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Incomplete reproductive isolation and strong transcriptomic response to hybridization between sympatric sister species of salmon

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Global change is altering ecosystems at an unprecedented rate. The resulting shifts in species ranges and reproductive timing are opening the potential for hybridization between closely related species which could dramatically alter the genetic diversity, adaptive capacity and evolutionary trajectory of interbreeding taxa. Here, we used behavioural breeding experiments, *in vitro* fertilization experiments, and whole-transcriptome gene expression data to assess the potential for and consequences of hybridization between Chinook and Coho salmon. We show that behavioural and gametic prezygotic barriers between socio-economically valuable Chinook and Coho salmon are incomplete. Postzygotically, we demonstrate a clear transcriptomic response to hybridization among F_1 Chinook-Coho offspring. Genes transgressively expressed within hybrids were significantly enriched with genes encoded in the nucleus but localized to the mitochondrion, suggesting a potential role for mito-nuclear incompatibilities as a postzygotic mechanism of hybrid breakdown. Chinook and Coho salmon are expected to continue to respond to climate change with shifts in migration timing and habitat use, potentiating hybridization between these species. The downstream consequences of hybridization on the future of these threatened salmon, and the ecosystems they inhabit, is unknown.

1. Background

Extinction via hybridization is a conservation concern in the current context of global climate change [1]. Sympatric sister species with strong ecologically based premating isolation but comparatively weaker levels of postzygotic isolation may be particularly susceptible to disturbances that tend to blend previously divergent habitats [2]. Environmental disturbances that disrupt prezygotic barriers have the potential to cause collapse of sympatric species pairs into hybrid swarms [3]. Such hybridization may be exacerbated by human-driven environmental alterations that threaten global biodiversity, including habitat fragmentation and degradation, climate change and overharvesting.

Among vertebrates, an estimated timeframe during which species boundaries remain vulnerable to collapse via hybridization ranges from 2 to 5 Myr since speciation [2]. Within the salmonids, a globally socioeconomically valuable group of teleosts, Chinook (*Oncorhynchus tshawytscha*) and Coho (*Oncorhynchus kisutch*) salmon have an approximate divergence time of 3.5 Myr and are thus deemed moderately susceptible to hybridization [2,4,5]. Environmental heterogeneity may have played a role in the adaptive divergence of

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these sister species: smaller Coho females prefer to construct redds in narrow tributaries with smaller gravel size compared to larger Chinook females that show preference for larger gravel sizes found in more open areas of the waterscape [6,7]. Such subtle differences in spawning habitat preference could factor significantly in the prezygotic isolation of Chinook and Coho salmon.

Reproductive isolation between these sister species does not appear to be complete. Viable F_1 hybrid offspring have been successfully produced in the laboratory and can survive at least a year [8]. F_1s are also fertile, capable of producing viable F_2 progeny, although these first- and second-generation hybrids typically have morphological asymmetries (i.e. unequal fin ray and/or gill raker numbers) thought to reduce fitness and ultimately survival [9]. However, findings of Chinook-Coho hybrids in nature were rare [10] until recently, when researchers genetically confirmed F_1 Chinook-Coho hybrids originating from the Cowichan River, Vancouver Island, Canada (H. A. Araújo 2019, unpublished data). These hybrids are thought to be the product of natural interspecific matings. These recent observations suggested that the appearance of hybrids is a consequence of climatic and habitat changes reducing water availability and leading to prolonged low water levels in the autumn. These low-water conditions subsequently delayed the peak of the spawning run of the area's Chinook (which usually spawn earlier than Coho), increasing temporal overlap of their breeding seasons, facilitating the two species to co-spawn.

Recently, several populations of both Chinook and Coho salmon have been assigned 'threatened' status by the Committee on the Status of Endangered Wildlife in Canada [11] because of demographic decline and rapid human-driven change in their spawning habitat [12]. In the Pacific northwest, river systems are vulnerable to environmental instability which could have unpredictable consequences on the timing of migrations and habitat use of Chinook and Coho salmon during the breeding season [13–15]. While hybridization in declining populations is an alternative outcome to extinction and would be expected to conserve some level of genetic biodiversity, it is difficult to predict the impact that the resulting hybrids may have on the ecosystem. Very little information exists on the mechanisms and strength of reproductive isolation between these sister species, but it is reasonable to expect that Chinook-Coho hybrids would dramatically alter the river community as a whole as seen in an example of reverse speciation in threespine stickleback [16].

To determine the strength of prezygotic reproductive isolation between these species we conducted mating experiments under scenarios of non-competitive mating (involving heterospecific pairs) and competitive mating (single female given the choice of two males, one Coho and one Chinook). We also performed fertilization experiments to test for the presence of reproductive barriers at the gametic level. Lastly, we used patterns of gene expression in F_1 hybrid juveniles as an indication of intrinsic postzygotic isolation acting at a molecular level, based on the previous observation that there is generally a negative relationship between hybrid fitness and degree of parental species' genetic divergence [17]. Such hybrid breakdown often manifests in the form of transgressive traits: those trait values that fall outside of the typical range of phenotypes between either parental species [17]. The frequency of occurrence of over- and under-expressed transcripts (collectively transgressively expressed genes) in hybrid offspring can be an indication of regulatory incompatibilities resulting from divergent genomes occurring in the same nucleus [18]. We used RNA-sequencing (RNAseq) to generate gene expression data and compared gene expression levels between laboratory-reared interspecific hybrids and progeny from pure crosses of both species to assess the relationship between gene expression profiles and the postzygotic consequences that might be incurred on F_1 hybrids.

2. Methods

(a) Fish and gamete collection

Sexually mature Chinook and Coho salmon used in the non-competitive and competitive breeding experiments, as well as in the fertilization experiments, were collected from the Chilliwack River Hatchery located in the Fraser Valley, approximately 120 km east of Vancouver, British Columbia, Canada. All behavioural breeding and gamete fertilization trials were conducted at Fisheries and Oceans Canada's Centre for Aquaculture and Environmental Research (CAER), West Vancouver, British Columbia, Canada. Fish were reared under approved Animal Use Permits no. 13-010 and no. 14-026 in compliance with Canadian Council on Animal Care guidelines.

(b) Breeding experiment: non-competitive

In November 2014, non-competitive breeding trials were conducted in a 2.1×30.5 m spawning channel divided into eight separate spawning arenas. The day before the start of the trials, mature fish were randomly assigned to one of eight spawning arenas. Arenas 1, 3, 5 and 7 each held one male Chinook and one female Coho, while arenas 2, 4, 6 and 8 each contained one female Chinook and one male Coho. Four 5 min observations on each arena were conducted daily (9.00, 11.00, 13.00 and 15.00) during which behaviours were recorded. These behaviours were: (i) digging by females, (ii) chasing, (iii) males attending females, (iv) quivering by males and (v) spawning (egg and milt release) [17]. The male behaviours of chasing, attending and quivering were summed across all days to give an overall count of observed male behaviour. A trial was considered complete and then concluded when the above behaviours ceased (normally by the death of one of the fish). Any remaining live fish were euthanized using a lethal dose (200 mg l^{-1}) of tricaine methanesulfonate (MS-222) buffered with 400 mg l^{-1} sodium bicarbonate. The arenas were then left undisturbed for three weeks to allow for development of any resulting fertilized eggs. After the three-week period, the arenas were sampled for fertilized eggs.

(c) Breeding experiment: competitive

During November 2015, competitive breeding trials were performed in the same spawning channel used for the non-competitive trials described above. The design of the competitive breeding experiment was similar to that of the non-competitive experiment, except a conspecific male was also present in each arena to provide a given female with a choice between males of the two species. Four 5 min observations on each arena were conducted daily as before (9.00, 11.00, 13.00 and 15.00) and the observed behaviours listed above were recorded, as was any evidence for male-male interactions. Again, the conspecific and heterospecific male behaviours were summed across all days to give a whole count of observed male behaviour. Aggressive acts between males were also counted. One arena (arena 3) was excluded owing to death of the female very early on in the trial. As previously described, the arenas were left undisturbed for three weeks following the end of the experiment. Redds (identified by depressions in the gravel) were subsequently

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searched for the presence of fertilized eggs. Developing embryos were discovered in three arenas and were subsequently preserved in 95% ethanol (EtOH) in preparation for genotyping. As hundreds of eggs may be present in a particular redd, we opted to genotype a random subset of all possible eggs, with the number of eggs depending on the number of redds found in each arena. Thus, 70, 167 and 140 embryos were genotyped from arenas 4, 6 (each containing a female Coho and heterospecific and conspecific males) and 7 (containing a female Chinook and heterospecific and conspecific males), respectively. It should be noted that eggs were also recovered from arena 5; however, these eggs were either not fertilized or died very shortly after fertilization as there was no evidence of an embryo inside and subsequently no DNA could be isolated for genotyping.

(d) Fertilization experiment: non-competitive (control)

Gametes were stripped from 20 Chinook (10 females and 10 males) and 20 Coho salmon (10 females and 10 males) at the Chilliwack River Hatchery, British Columbia, Canada (see the electronic supplementary material, Extended Methods for details). Approximately 100 eggs were used in each cross. The diluted sperm and eggs were then simultaneously added to a container of 100 ml of water. Five trials, each including four females (two Chinook females and two Coho females) and four males (two Chinook males and two Coho males), were conducted. Each trial involved mixing eggs and sperm of all possible male and female pairs, such that each trial resulted in 16 possible crosses (electronic supplementary material, figure S1a). Fertilized eggs were held in Heath trays in a flow-through incubator stack for three weeks until embryos reached the eyed stage and successful fertilizations could be scored on the basis of embryonic development. Several Coho females exhibited substantially lower fertilization success than other females, with some achieving a fertilization rate of zero in all crosses they were involved in. These females were removed from all subsequent analyses. Results were analysed with the statistical software JMP® Pro 9.2 (SAS Institute Inc.) using a mixed effects model that employed the restricted maximum-likelihood method (REML), in which individual females and males were assigned as random effects and female and male species were assigned as fixed effects.

(e) Fertilization experiment: competitive

Gametes originating from the same fish in the non-competitive fertilization trials were also used in a competitive fertilization experiment. Before application to the eggs, the sperm of each male was diluted to a concentration of 5^9 cells ml^{-1} using Ginsburg's ringer solution. As previously described, approximately 100 eggs were used in each cross and the diluted sperm and eggs were simultaneously added to a container of 100 ml of water. As before, four females (two Chinook and two Coho) and four males (two Chinook and two Coho) were used in each of five trials. Within each trial, a cross consisted of eggs from a single female being combined with a mixture containing equal concentrations of sperm from a single Chinook male and a single Coho male. A second batch of eggs from the same female was then combined with a second mixture of Chinook and Coho sperm derived from two different males, resulting in eight total crosses per trial (electronic supplementary material, figure S1b). Fertilized eggs were then left in a flow-through stacked incubator and allowed to develop for three weeks until embryos reached the eyed stage. At this point, successful fertilizations were counted, and embryos were sacrificed and preserved in 95% EtOH in preparation for subsequent genotyping. Results were analysed using a mixed effects model (REML) in JMP® Pro 9.2 (SAS Institute Inc.). Female identification and male sperm mixture were set as random effects with egg species (Chinook or Coho) being the fixed effects.

(f) High resolution melting analysis for embryo genotyping

A high-resolution melt assay (HRMA) targeting a single nucleotide polymorphism distinguishing pure Chinook from pure Coho was designed and validated using the pure species that parented the offspring that resulted in both the competitive breeding and the competitive fertilization experiments. Subsequently, embryos resulting from both the competitive breeding and the competitive fertilization experiments were genotyped to determine if the embryos were the product of inter- or intraspecific crosses. Embryos were dissected from their eggs, DNA was extracted (see the electronic supplementary material, Extended methods for details) and assay reactions were prepared using MeltDoctor™ HRM Master Mix according to the manufacturers' recommendations. Reaction conditions were set per the recommendations of the manufacturer on a 7500 Fast Real-Time PCR System. The resulting normalized melt curves for each individual genotyped were visualized and scored as either pure Chinook, pure Coho, or hybrid using the software High Resolution Melt v. 2.0 (all reagents and software from Applied Biosystems™, Foster City, CA, USA).

(g) RNA-sequencing: transcriptomic response to hybridization

All offspring used in the RNAseq experiment were produced, reared and sampled at the Fisheries and Oceans Canada Rosewall Creek Research Hatchery in Qualicum Beach, British Columbia, Canada. Two series of crosses resulting in four families each were made. In each series, pure species and reciprocal hybrid half-siblings were generated by crossing four individuals: one female Chinook and one male Chinook, and one female Coho and one male Coho in all combinations. Juveniles were grown in 10°C freshwater (fed to satiation with commercial salmon feed) to a weight of 5 g at which point they were euthanized, liver tissue was removed, preserved in RNA*later*® (Sigma-Aldrich, St. Louis, MO, USA) and stored at −20°C. Ten individuals from each family in both series were subsequently randomly selected for RNAseq (for a total of 80 individuals, 40 per series). RNA was extracted from liver tissue using a standard TRIzol® Reagent (Invitrogen, Carlsbad, California)-RNeasy Mini Kit (QIAGEN, Hilden, Germany) protocol. Total RNA was then sent to the Génome Québec Innovation Centre (Montréal, Québec) for library preparation and 100 bp paired-end sequencing (see the electronic supplementary material, Extended methods for details).

Preliminary data analyses were also performed by personnel at the Génome Québec Innovation Centre (Montréal, Québec) and included the assembly of a de novo project-specific transcriptome using Trinity [19] (see the electronic supplementary material, Chinook/Coho.fasta file). The transcriptome was annotated using Trinotate, reads were aligned to the *de novo* transcriptome using BWA [20], and a table of raw read counts per transcript per sample was generated using RSEM [21] (see the electronic supplementary material). The raw reads were then filtered to exclude any transcript for which an individual(s) showed expression counts of less than 25, eliminating lowly expressed transcripts which are often indistinguishable from sampling noise [22]. This filtering resulted in 6474 transcripts remaining in series 1 and series 2 for further analyses.

We used the Bioconductor software package edgeR [23,24] to produce a multidimensional scaling plot using the biological coefficient of variation option as a means of computing the distance between pairs of samples. We then grouped pure Chinook and pure Coho offspring from like crosses between our two families and tested for significant differences in gene expression. We used the VOOM transformation in LIMMA as implemented in edgeR to convert the raw transcript read counts to log-counts per million, determine differential expression of each reciprocal hybrid cross

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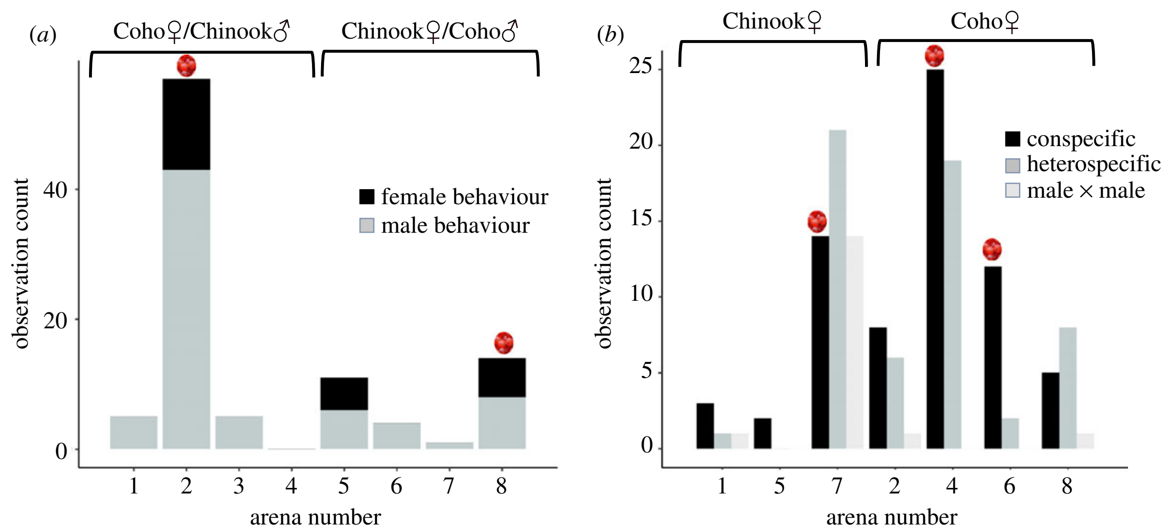

Figure 1. (*a*) Non-competitive spawning trials: summary of observed behaviours between female Coho and male Chinook (odd numbered arenas) and female Chinook and male Coho (even numbered arenas). Bars represent the sum of all behaviours (chasing, approaching, attending, quivering) exhibited by the males, and all behaviour exhibited by females (digging) over the course of the observation period. (*b*) Competitive spawning trial: summary of observed behaviours between female Chinook and heterospecific and conspecific males (odd numbered arenas) as well as female Coho and heterospecific and conspecific males (even numbered arenas). Bars represent the sum of all behaviours (chasing, approaching, attending, quivering) exhibited by the males over the course of the observation period. A large dot over the bar indicates arenas where fertilized eggs were recovered. (Online version in colour.)

relative to each group of pure offspring, and to estimate additive and dominance effects on the expression data. For each transcript, the additive effect (a) was determined as the distance between each mean pure species expression value (for either pure Chinook or Coho offspring) and the average of the two pure species expression values (M) (electronic supplementary material, figure S3). The dominance effects ($d1$ and $d2$, one value for each reciprocal hybrid cross), represent the difference between M and the mean expression value for each hybrid cross (electronic supplementary material, figure S3). A dominance coefficient is then calculated as $d/|a|$ [25]. Dominance coefficient categories were subsequently determined based on their value of $d/|a|$ as follows: underdominant < −1.25; recessive = −1.25 to −0.75; partially recessive = −0.75 to −0.25; additive = −0.25 to 0.25; partially dominant = 0.25 to 0.75; dominant = 0.75 to 1.25; overdominant > 1.25, with over- and underdominant, representing the two transgressive cases. Subsequent gene ontology (GO) term enrichment analyses were conducted using the web-based software GORILLA [26]. All figures were rendered using RSTUDIO (RStudio: Integrated Development Environment for R, 2015).

3. Results

(a) Behavioural and gametic reproductive isolation is incomplete

We observed imperfect prezygotic isolation in the no-choice mating trials, with interspecific mating occurring in two of the eight heterospecific pairs (figure 1*a*). Fish displayed characteristic salmon mating behaviour in all but one of the eight arenas in this study (fish in arena 7 displayed virtually none of the mating behaviours). We observed higher occurrences of male and female mating behaviour among heterospecific pairs in arenas 3 (Coho female with Chinook male) and 8 (Chinook female and Coho male) relative to the other arenas, and developing eggs were recovered from redds in both arenas. These results indicate that both male Chinook and female Coho as well as the reciprocal pairing will mate interspecifically in a scenario in which conspecific mates are unavailable, and these matings produce developing F_1 eggs.

We observed strong premating isolation in those breeding experiments in which females were given a choice between a conspecific and heterospecific mate. The absence of interspecific matings may have been owing more to the persistence of conspecific males in physically preventing the heterospecific male from approaching the female (indicated by the occurrence of male × male interactions; figure 1*b*) than to female preference, as all females in the seven arenas were partly receptive to the characteristic mating behaviours displayed by males of both species (figure 1*b*). Most notably, the frequency of encounters observed between the Chinook male and Coho female in arena 4 rivalled those observed between the female and the conspecific male (19 versus 25, respectively). In arena 7, the frequency of the heterospecific male's encounters with the Chinook female exceeded that observed between the female and the conspecific male (21 versus 14, respectively). Fertilized eggs were recovered from redds in three of these arenas (arenas 4, 6 and 7; figure 1*b*). A random sample of eggs from each nest was selected and embryos from these eggs were subsequently genotyped using our HRMA (described in Methods) to determine if any interspecific hybridization had occurred. Despite the interspecific mating behaviour observed in these three arenas, all 377 embryos genotyped were conspecific.

Fertilization rates in the non-competitive fertilization experiments were similar among conspecific and interspecific crosses involving Chinook eggs (0.68 and 0.69, respectively; electronic supplementary material, table S1). Fertilization rates were also similar among conspecific and interspecific crosses involving Coho eggs (0.44 and 0.48, respectively; electronic supplementary material, table S1). The results of the mixed effects model showed that Coho eggs had significantly lower fertilization rates than Chinook ($F_{1,19} = 4.38$, $p = 0.05$). Male species had no detectable effect on fertilization frequency either in conspecific (Chinook males mean rate = 0.68 versus Coho males mean rate = 0.48) or interspecific

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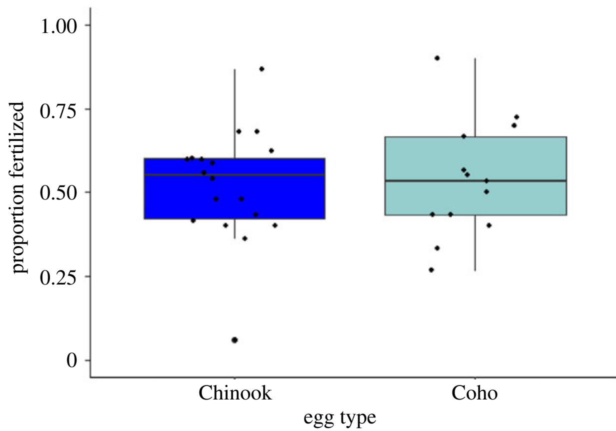

Figure 2. Conspecific fertilization rates for Chinook and Coho eggs when simultaneously mixed with conspecific and heterospecific sperm. Mean fertilization rates were not significantly different between sperm types when conspecific sperm was in competition with heterospecific sperm. (Online version in colour.)

crosses (Chinook males mean rate = 0.44 versus Coho males mean rate = 0.69; $F_{1,19} = 0.24$, $p = 0.63$; electronic supplementary material, table S1). We thus did not detect a significant interaction between male species type and female species type ($F_{19,19} = 0.29$, $p = 0.59$).

In the competitive fertilization trials, eggs from Chinook females showed only a slightly higher mean fertilization rate (±s.e.) by conspecific sperm than heterospecific sperm (0.52 ± 0.04 s.e. versus 0.48 ± 0.04 s.e., respectively; figure 2). Coho eggs showed a similar pattern with the mean fertilization rate from conspecific sperm only slightly exceeding that of heterospecific sperm by approximately 8% (0.54 ± 0.05 s.e. and 0.46 ± 0.05 s.e., respectively). Our test of the interaction term in a mixed effects model was unable to detect significant conspecific sperm precedence for either Coho or Chinook sperm ($F_{1,9} = 1.9208$, $p = 0.19$).

(b) Transcriptomic response to hybridization

RNA isolated from 80 individuals from two replicate families consisting of pure Coho ($n = 10 \times 2$), pure Chinook ($n = 10 \times 2$), and reciprocal hybrid offspring (offspring from Chinook female mated with Coho male, hereafter abbreviated Chinook♀/Coho♂, $n = 10 \times 2$; offspring from Coho female mated with Chinook male, hereafter abbreviated Coho♀/Chinook♂, $n = 10 \times 2$) underwent RNAseq. The *de novo* transcriptome generated from these specific individuals yielded 395 083 total transcripts with 66 607 unique components (electronic supplementary material, Data S1 Chinook_Coho.fasta). After filtering, 6474 transcripts, of which 3998 were annotated to gene name, remained for further analysis (electronic supplementary material, Data S2 Chinook_Coho_Raw_Read_Counts_Filtered_6474.csv). The whole-transcriptomic effect of hybridization clearly distinguishes hybrids from both parental species (figure 3).

Analysis of differential expression between pure Chinook and pure Coho offspring determined that, in this study, these species showed differential expression at 4616 of the 6474 transcripts (71.3%) that survived filtering (figure 4*a*; electronic supplementary material, Data S3 Chinook_versus_coho_edgeR_results.csv). A model calculating additive and dominance coefficients was fitted to gene expression in the hybrids relative to that in the pure species offspring, controlling for the effect of family (electronic supplementary material, figure S3). The estimated coefficients were then used to classify transcripts as additive, recessive, dominant, partially recessive, partially dominant, and over- or underdominant (electronic supplementary material, Data S4 Dominance_analysis.csv). The majority of transcripts (42.8% of genes in Coho♀/Chinook♂ offspring and 32.1% in Chinook♀/Coho♂ offspring) showed additive expression within the hybrids, having values approximately intermediate between those observed among offspring of the pure species. We focussed on those genes whose mean expression in the hybrids was more extreme than the mean expression observed in offspring from pure crosses (overdominance and underdominance), because such transgressive traits are often implicated in post-zygotic hybrid breakdown [17]. Among Coho♀/Chinook♂ offspring, 1630 of 6474 transcripts were transgressively expressed; 7.5% of these transcripts were overdominant in their expression and 8.7% were underdominant relative to their expression in pure Chinook and pure Coho offspring. A small fraction of these transgressively expressed genes were also differentially expressed between pure Chinook and pure Coho offspring (66/4616 = 1.4% underdominant; 42/4616 = 0.9% overdominant; figure 4*a*). Among Chinook♀/Coho♂ offspring, 1050 of 6474 transcripts were transgressively expressed; 12.4% of these genes exhibited overdominance and 12.7% underdominance when compared to expression in pure species offspring. As with the previous comparison, only a small fraction of these transgressively expressed genes were differentially expressed between pure type offspring (173/4616 = 3.7% underdominant; 192/4616 = 4.1% overdominant; figure 4*b*). Thus, differential expression between the pure type offspring does not appear to be predictive of genes that will be subsequently transgressively expressed in the reciprocal hybrids.

When the list of overdominant genes was compared to the filtered set of genes in our *de novo* transcriptome, GO analysis revealed significant enrichment of genes forming cellular components comprised of nuclear-encoded proteins primarily involved in the formation of ribosomal subunits including the small-subunit processome and the preribosome (figure 4*c*; electronic supplementary material, Data S5 Enriched_overdominant_GO_terms.csv). GO terms describing cellular components enriched in the underdominant genes of the hybrids include the ribonucleoprotein complex and cytosolic large ribosomal subunit which again comprise many nuclear-encoded proteins (figure 4*d*; electronic supplementary material, Data S6 Enriched_underdominant_GO_terms.csv). Most interestingly, gene enrichment tests indicated that those transcripts having underdominant expression in the hybrids were also significantly enriched with genes encoded in the nucleus but localized to the mitochondrion, cooperating with mitochondrially encoded genes strictly of the maternal species. Enriched cellular components of this nature included the respiratory chain complex, the oxidoreductase complex, NADH dehydrogenase complex, and inner mitochondrial membrane protein complex.

4. Discussion and conclusion

(a) Potential for hybridization

Previous *in vitro* fertilization experiments have found that Chinook-Coho hybrid zygotes form easily when gametes from the two species are mixed [8] but the possibility of natural hybridization was unknown. As an extension of these

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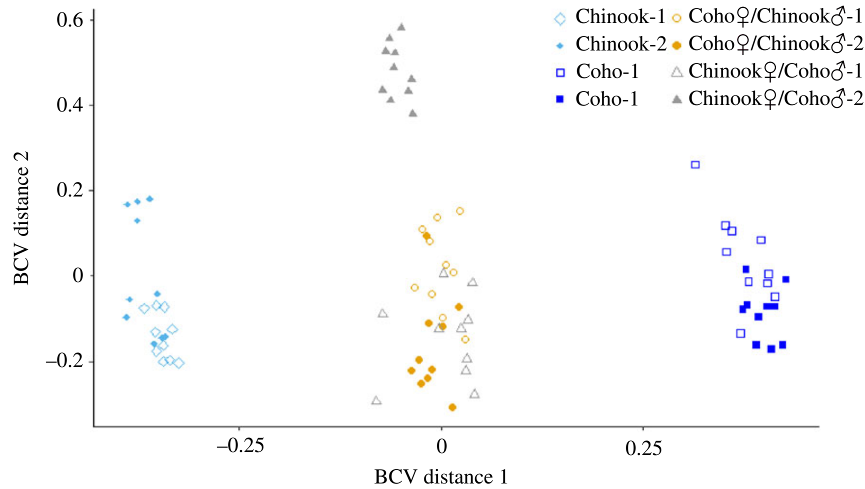

Figure 3. (*a*) Multidimensional scaling plot produced from 6474 transcripts having expression counts greater than 25 as a function of the biological coefficient of variation (BCV). Unfilled and filled shapes represent individuals from family 1 and family 2, respectively. (Online version in colour.)

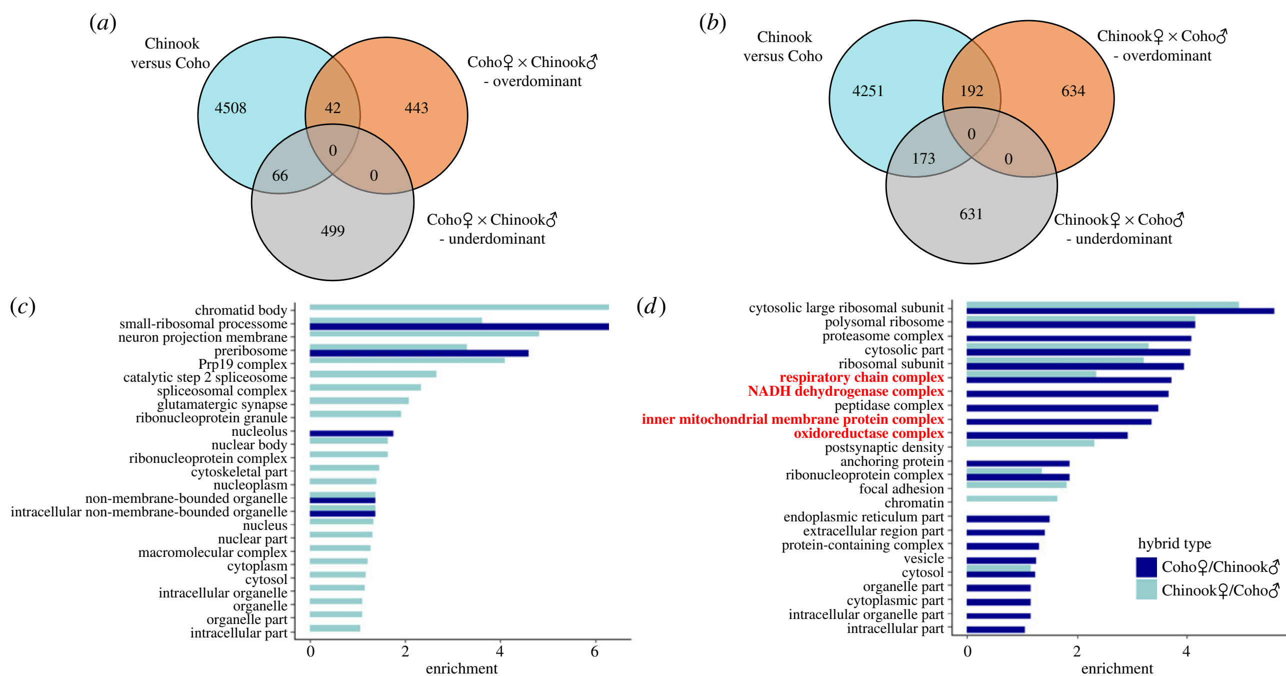

Figure 4. Venn diagram highlighting the congruence of genes differentially expressed between pure Chinook and pure Coho offspring, and those transgressively expressed in (*a*) Coho♀ × Chinook♂ and (*b*) Chinook♀ × Coho♂ hybrid offspring. (*c*) Summary of GO categories represented by genes significantly overdominant in hybrids relative to offspring from pure crosses. (*d*) Summary of GO categories represented by genes significantly underdominant in hybrids relative to offspring from pure crosses (false discovery rate corrected $q < 0.0003$). GO terms listed on the *y*-axis describe significantly enriched cellular components. Nuclear-encoded mitochondrial genes are emphasized with bold font. (Online version in colour.)

initial trials, the present study directly assesses the degree of prezygotic reproductive isolation between Chinook and Coho salmon at behavioural and gametic levels through a series of non-competitive and competitive breeding trials and *in vitro* fertilization experiments, respectively. Assortative mating was strong in competitive breeding and appeared, most obviously in the case of arena 7, to be owing to aggressive acts from the conspecific male which prevented the heterospecific male from accessing the female (figure 1*b*). However, premating reproductive isolation between these species was found not to be absolute, because no-choice spawning trials revealed interspecific mating behaviour, fertilization and viable F_1 hybrid progeny in one-quarter of the pairs. This observation was most unexpected, as the expectation was that there would be no interaction between the sister species, never mind interspecific hybridization! Thus, when conspecifics are absent, Chinook and Coho salmon will interbreed in both directions. In nature, changes in environmental cues (i.e. temperature, salinity) that initiate the return of Chinook to the river, for example, could generate steep asymmetries in the species' densities during their time of overlap on the breeding grounds and thus increase the chances of interspecific matings and the production of hybrid offspring. Shifts in migration timing in response to environmental changes associated with climate change, with some species returning earlier and others later, have already been reported among several species of anadromous Pacific salmonids [13,14]. Recent research has found evidence of shifts in the peak spawning migration time for Chinook salmon in the Cowichan River, British Columbia, trending

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towards later arrivals in years of prolonged summer droughts (H. A. Araújo 2019, unpublished data). This shift may be an important contributor in the observed Chinook-Coho salmon hybridization occurring in that watershed. Small local populations of either species are vulnerable to the impacts of climate change which threaten to increase asymmetries in abundance on the breeding grounds, thus increasing the chance for hybridization, resulting in local depression in numbers of the rarer species [15,27].

Reproductive isolation between Chinook and Coho salmon was also found to be incomplete at the gametic level. In the non-competitive fertilization experiments, when eggs were combined with either conspecific or heterospecific sperm, we observed little difference in fertilization rates among eggs derived either from Chinook or Coho. Similar studies involving closely related members of the sympatric, broadcast-spawning urchin species complex *Echinometra* also failed to observe higher fertilization rates among conspecific gametes when compared to unions formed by heterospecific gametes in non-competitive fertilization trials [28]. Sympatrically occurring species of hamlet (*Hypoplectrus nigricans* and *Hupoplectrus puella*) showed similar results [29]. Surprisingly, unlike the previous two examples, we saw little difference in the proportion of eggs fertilized by conspecific versus heterospecific sperm in the competitive fertilization trials. In a broadcast spawning species with little or no ability to actively select a mate (such as the members of the urchin complex), rapid evolution of gametic incompatibilities may be reinforced during speciation [30]. Gametic incompatibilities may be evolutionarily redundant among sympatric congenerics such as salmon that not only form a restricted number of polygamous unions (as opposed to broadcast spawners for example) but also depend heavily on behavioural mechanisms of species recognition when selecting a mate [29].

(b) Transcriptomic effect of hybridization

As allopatric populations of taxa diverge, genetic variants distinguishing the members of these groups accumulate. Some of the fixed variants in individuals from one population are sometimes not compatible with those alternate forms that have become fixed in members of the other population. Such Bateson–Dobzhansky–Mueller genetic incompatibilities (BDMIs) can then result in postzygotic hybrid breakdown should these divergent forms interbreed [30–32]. From a gene expression perspective, the merging of two divergent genomes can lead to 'genomic shock' elicited by this unexpected challenge (in this case hybridization) and which manifests as compensatory gene regulation, or more specifically transgressive gene expression [32,33]. Such transgressive expression has been subsequently associated with fitness declines in hybrid offspring [34,35]. We detected a strong whole-transcriptomic effect, as well as hundreds of genes transgressively expressed, within hybrid offspring relative to pure offspring. These transgressively expressed genes were enriched not only for genes comprising nuclear-nuclear protein complexes but also for those encoded in the nucleus but having function in the mitochondria. This finding is significant because mito-nuclear interactions are frequently implicated in the breakdown of advanced generation hybrids and backcrosses in a number of species from a range of taxa [36]. While the mitochondrial genome encodes 13 of the subunits of the electron transport chain required for oxidative phosphorylation, more than 1000 additional genes, encoded in the nuclear genome, are required for proper mitochondrial function [37]. At the crux of this specific subtype of BDMI is the general observation that F_1 hybrids, containing 50% of their nuclear genome matched to their mitochondrial type, exhibit more or less routine metabolic function as previously shown in the case of *Rana* leopard frogs [38] as well as *Tigriopus* copepods [39]. However, F_2 and more advanced generation hybrids between divergent animal taxa typically show a decline in fitness as recombination and independent assortment decrease the occurrence of allele and gene complexes that have had thousands of generations to coevolve [39–41]. In the case of Chinook-Coho hybrids, the observed transgressive expression is possibly the result of the separation of mito-nuclear gene complexes that have had the last 3.5 Myr to co-adapt. The fitness consequences of these mito-nuclear mismatches caused by hybridization between Chinook and Coho salmon are difficult to extrapolate. The complicated and lengthy life histories of salmonids render them a difficult group in which to conduct experiments on F_2s and more advanced generation hybrids in the laboratory, leaving the potential success of the hybrids in the natural environment open to additional research. The downstream consequences of extensive hybridization and introgression between Chinook and Coho salmon requires further investigation to determine the impact of such interbreeding on the future of the pure species as well as the ecosystems they inhabit.

Ethics. All trials were conducted at Fisheries and Oceans Canada's Centre for Aquaculture and Environmental Research (CAER), West Vancouver, BC, Canada. Fish were reared under approved Animal Use Permits no. 13-010 and no. 14-026 in compliance with Canadian Council on Animal Care guidelines.

Data accessibility. Raw sequencing data generated for this study have been uploaded to NCBI's SRA: PRJNA703990. The following datasets and code are available on the Dryad Digital Repository, with an associated Read_Me.txt file: https://doi.org/10.5061/dryad.dncjsxkzs [42]: Electronic supplementary material, Data S1. Chinook_coho.fasta, table_S2.RNAseq_sequencing_coverage_summary.csv, Data_S2._Chinook_coho_Raw_Read_Counts_Filtered_6474.csv, edgeR_code_pure_comparison. txt, Data_S3.Chinook_versus_coho_edgeR_results.csv, Full_Model_Dom_Terms_control_for_family.txt, Data_S4._Dominance_analysis.csv, Data_S5._Enriched_overdominant_GO_terms.csv, Data_S6._Enriched_underdominant_GO_terms.csv, Figure_1a_Data.csv, Figure_1b_data.csv, Figure_2_data.csv, Figure4c.Both_crosstypes_Overdom.csv, Figure4d.Both_crosstypes_Underdom.csv, R_code_Figures_revised. txt, 2014_Non_Competitive_Breeding_Experiment_Behavioural_Observations.docx, 2015_Competitive_Breeding_Experiment_Behavioural_Observations.pptx.

Authors' contributions. J.L.M.: conceptualization, data curation, formal analysis, investigation, writing-original draft, writing-review and editing; A.A.H.: conceptualization, investigation, writing-review and editing; J.L.S.: conceptualization, data curation, methodology; R.H.D.: conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, writing-original draft, writing-review and editing; D.S.: conceptualization, formal analysis, investigation, methodology, resources, supervision, writing-original draft, writing-review and editing. All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Competing interests. We declare we have no competing interests.

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