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ORIGINAL ARTICLE

Genes, Brain and Behavior

Behavioral transcriptomic effects of triploidy and probiotic therapy (*Bifidobacterium*, *Lactobacillus*, and *Lactococcus* mixture) on juvenile Chinook salmon (*Oncorhynchus tshawytscha*)

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Abstract

Aquaculturists use polyploid fish to maximize production albeit with some unintended consequences including compromised behaviors and physiological function. Given benefits of probiotic therapies (e.g., improved immune response, growth, and metabolism), we explored probiotic supplementation (mixture of *Bifidobacterium*, *Lactobacillus*, and *Lactococcus*), to overcome drawbacks. We first examined fish gut bacterial community composition using 16S metabarcoding (via principal coordinate analyses and PERMANOVA) and determined probiotics significantly impacted gut bacteria composition ($p = 0.001$). Secondly, we examined how a genomic disruptor (triploidy) and diet supplements (probiotics) impact gene transcription and behavioral profiles of hatchery-reared Chinook salmon (*Oncorhynchus tshawytscha*). Juveniles from four treatment groups (diploid-regular feed, diploid-probiotic feed, triploid-regular feed, and triploid-probiotic feed; $n = 360$) underwent behavioral assays to test activity, exploration, neophobia, predator evasion, aggression/sociality, behavioral sensitivity, and flexibility. In these fish, transcriptional profiles for genes associated with neural functions (neurogenesis/synaptic plasticity) and biomarkers for stress response and development (growth/appetite) were (i) examined across treatments and (ii) used to describe behavioral phenotypes via principal component analyses and general linear mixed models. Triploids exhibited a more active behavioral profile ($p = 0.002$), and those on a regular diet had greater Neuropeptide Y transcription ($p = 0.02$). A growth gene (early growth response protein 1, $p = 0.02$) and long-term neural development genes (neurogenic differentiation factor, $p = 0.003$ and synaptysomal-associated protein 25-a, $p = 0.005$) impacted activity and reactionary profiles, respectively. Overall, our probiotic treatment did not compensate for triploidy. Our research highlights

novel applications of behavioral transcriptomics for identifying candidate genes and dynamic, mechanistic associations with complex behavioral repertoires.

KEYWORDS
behavioral transcriptomics, Chinook salmon, gut microbiota, gut-brain axis, probiotic, triploidy

1 | INTRODUCTION

Due to growth in the human population,[1] an increase in production of fish for human consumption[2] has been necessary to meet increasing demands,[3,4] surpassing the production levels of capture fishing practices.[2,5] To ensure sustainable food production while meeting animal welfare needs,[6] advances in technologies, including producing polyploid individuals, have been employed.[7] In particular, triploidization is a widespread aquaculture technique used to induce sterility to increase the harvestable mass of individuals[8,9] and reduce the environmental impacts of escapees from farms.[10,11] During the triploidization process, organisms (e.g., teleost fish), undergo heat or pressure shock at the egg fertilization stage[12,13] which results in genome disruption and functionally sterile individuals with three sets of chromosomes.[9,12]

One result of this genome disruption is differential gene expression in a host of tissues (e.g., intestinal epithelium), resulting in altered gut microbiota diversity/structure.[14] Due to a reduction in energetic requirements for gametogenesis (as a result of sterility) triploid individuals have more energy available for somatic growth and are expected to have enhanced growth compared with diploid counterparts,[15,16] however results in the literature have been mixed.[15] In addition to enhanced growth,[9] triploid organisms also exhibit increases in expression of genes related to metabolism and growth, resulting in faster growth via increased feeding behaviors.[17–20] Despite the potential for increased growth, there have been well-supported negative impacts of triploidy via altered gene expression on the organism's immune system,[21–23] survival,[21,23] stress response,[17] neuroendocrine function and neurogenesis effects.[24]

To our knowledge, little research focuses on how altered gene expression in triploid fish drives behavioral changes. In certain instances, triploids have been found to be less aggressive (e.g., Chinook salmon[25] and Atlantic salmon[10]), exhibit poorer foraging ability (e.g., Atlantic salmon[26–28] and brook trout (*Salvelinus fontinalis*)[28]), and have lowered feeding activity relative to their diploid counterparts (e.g., Chinook salmon[25] and Atlantic salmon[26]). Furthermore, triploids have been shown to have lowered levels of sensitivity to environmental factors including light and sound (e.g., sweetfish (*Plecoglossus altivelis*)[29]), and reduced responsiveness (e.g., zebrafish larvae (*Danio rerio*)[30]). Behavioral lateralization, a measure of asymmetric expression of cognitive function,[31–34] has yet to be investigated in triploid fish; however, triploidy has been found to impact the size of structures within the brain,[27] which could affect the asymmetries of bilateral neural structures driving laterality.[34] Despite these diverse differences between diploid and triploid fish, their underlying genomic mechanisms remain unclear, but perhaps arise from the complex interactions between multiple genes within the genome and the environment.[35] Nevertheless, given that behaviors serve as the gateway between neurophenotypes and responses to environmental stimuli, the mechanisms driving these relationships remain uncharacterized, thus necessitating a behavioral genomics approach to elucidate why triploidy may not always address issues in food security and animal welfare.

An individual's diet can serve as a mediator between the gut, brain, and behavior of animals.[36] Probiotics are live microorganisms which confer a health benefit to the host.[37,38] Probiotics can serve as food additives designed to create a healthy gut environment,[39] and can aid in modulating brain activity,[40] gene expression[41–44] and subsequent behavioral responses.[45] Probiotics have been widely used in aquaculture,[11] including the commercial probiotic mix used in our study,[46] and can possibly overcome some of the drawbacks of triploidy. Probiotics work through a variety of mechanisms involving altered gene expression: enhancing the host's intestinal metabolites (e.g., short-chain fatty acids) to modulate gene transcription[47–49]; coordinating multiple signaling pathways[47]; and suppression of virulence-related gene expression while upregulating genes associated with commensalism between host and gut microbiota.[49] Positive changes in neural gene expression have also been noted with probiotic use. These effects include increased growth-related gene transcription (i.e., *GH* and insulin-like growth factor 1 receptor) (e.g., Senegalese sole larvae (*Solea senegalensis*)[42] and rainbow trout (*Oncorhynchus mykiss*)[43]); and expression of heat shock proteins (i.e., HSP70 and HSP90AA) related to stress response and possibly indicative of increased resistance to stressful situations.[42] Downregulation of genes involved in meiosis, intracellular protein transport, cell adhesion, and so on, as well as upregulation of genes related to post translation modifications (e.g., Chinook salmon[44]) have also been observed, possibly indicative of microbial alterations to host signaling pathways.

Microbes added to the host gut via probiotic treatments can affect behaviors through releasing/producing common chemical messengers (e.g., neurotransmitters) or hormones, which have been shown to impact the function of neuro-endocrine circuitry of the host.[50,51] Downstream effects include modulation of the central nervous system and stress response,[52] which ultimately influences behavioral responses.[53–55] In fish given probiotics, alterations to anxiety-related behaviors (e.g., Nile tilapia (*Oreochromis niloticus*)[56]), reduced behavioral stress responses (through increased surface swimming patterns and lowered swim speeds, for example, zebrafish (*Danio rerio*)[57]), reduced stress coping ability (e.g., Arctic char (*Salvelinus*

alpinus)[58]), increased exploratory behaviors (e.g., zebrafish[57]), and greater foraging performance (rainbow trout[59]) have all been reported. Additionally, probiotics have been found to promote behavioral flexibility (i.e., an animal's ability to adjust its behavior according to changes in environmental stimuli[60]) via a positive feedback loop between higher microbiome diversity promoting greater foraging success and increased diet breadth.[61]

Behavioral genomic approaches have shown probiotics (containing strains of *Bifidiobacterium* and *Lactobacillus*) to modulate a variety of neural pathways to influence downstream behaviors,[62] including mood, anxiety, and cognition.[63] Probiotic treatments have also been found to increase gene expression involved in serotonin signaling and metabolism,[45,52] crucial for regulation of a fish's behavioral response[64–66]; and increased gene expression of brain derived neurotrophic factor (*BDNF*), which aids in regulating exploratory and social behaviors.[45] While a growing body of research exists on how probiotics impact the gut-brain-behavior axis, there remains limited information on the use of probiotics as therapy to redress the negative integrated behavioral and physiological functions of triploidy.

The objective of our research was to explore the behavioral and neural transcriptomic effects of triploidy (genomic disruptor) on juvenile Chinook salmon and test whether probiotic therapies affect these traits using a behavioral transcriptomics approach. We first began by testing the effects of our probiotic treatment (a commercially available mixture of multiple *Bifidobacterium* and *Lactobacillus* probiotic strains and a single *Lactococcus* strain) on the bacterial communities (hereafter microbiota) within the gut of fish fed a probiotic diet versus a control. The expectation was that our Jamieson Essentials Probiotic: 10 billion Active Cells (Jamieson Laboratories, Toronto, Ontario, Canada; see Table 1 for details) would alter the gut microbiota. Next, in a second set of experiments, we quantified gene transcription in genes related to neural function (i.e., synaptic plasticity and neurogenesis), behavioral responses (i.e., neuronal plasticity and stress-induced responses), and performance (i.e., growth and metabolism) in fish belonging to four treatment groups (i.e., diploid vs. triploid and regular vs. probiotic feeds). By also investigating the behavioral phenotypes of these fish, (i.e., responsiveness, activity, boldness, and aggression), we extended our transcriptional study to explain behavioral variation through a novel behavioral transcriptomics perspective. Overall, our goal was to determine whether genome disruption and altered diet influence the integrated behavioral transcriptomic responses of juvenile Chinook salmon to recommend whether probiotic therapies should be utilized by culturists.

TABLE 1 List of the strains of bacteria (with number of colony-forming units (CFU)) in the Jamieson brand probiotic mixture used as the probiotic feed treatment for juvenile Chinook salmon.

Bacterial strain	Probiotic complex in 10 Billi on colony-forming units (CFU)
Bifidobacterium animalis subsp. *lactis* (UABla-12)	2.0
Lactobacillus paracasei (UALpc-04)	1.5
Bifidobacterium breve (UABbr-11)	1.0
Lactobacillus gasseri (UALg-05)	1.0
Lactobacillus rhamnosus (UALr-06)	1.0
Lactobacillus rhamnosus (UALr-18)	1.0
Lactobacillus acidophilus (DDS®-1)	0.5
Lactobacillus plantarum (UALp-05)	0.5
Bifidobacterium longum subsp. *longum* (UABl-14)	0.39
Bifidobacterium bifidum (UABb-10)	0.3
Lactobacillus casei (UALc-03)	0.3
Lactobacillus reuteri (UALre-16)	0.3
Lactococcus lactis (UALl-08)	0.2
Bifidobacterium longum subsp. *infantis* (UABi-13)	0.01

2 | MATERIALS AND METHODS

2.1 | Study location and species

Our study was performed at a Chinook salmon farm on Quadra Island, British Columbia (Yellow Island Aquaculture Ltd. (YIAL): 50°07′59.124″ N, 125°19′36.4392″ W). The breeding design included captively bred XX females and sex-reversed XX males. The original brood stock at YIAL was started in 1985, where individuals were sourced from two hatcheries on Vancouver Island, British Columbia (Big Qualicum [49°23′37.954″ N, 124°37′0.512″ E] and Robertson Creek [49°20′6.835″ N, 124°58′52.043″ W]).[67] All offspring produced were female from a monosex stock.

2.2 | Probiotic effects on the gut microbiota experiment

2.2.1 | Breeding design

The probiotic impacts on the gut microbiota portion of the study used a nested breeding design (2×1) replicated six times where we assessed 132 fish from multiple crosses (October 2019), with eggs incubated in replicate cells of vertically stacked incubation trays. Full experimental flowchart can be found in Figure 1. After eggs hatched and reached the stage of first feeding in their incubation stacks, fish were moved to replicate 200 L tanks (March 2020). Rearing tanks were supplied with fresh running water from a groundwater source at a consistent flow rate (average temperature of 8.5°C, dissolved oxygen levels of 6.23 mg/L), continuous aeration, and a 16:8 h light–dark cycle. Fish were fed an extruded sinking pellet EWOS® Micro Crumble feed (1.2 mm in diameter) which was administered daily at 9 am, 1 pm, and 5 pm at approximately 3% of their body weight until the experimental challenge (October 24, 2020), and tanks were cleaned

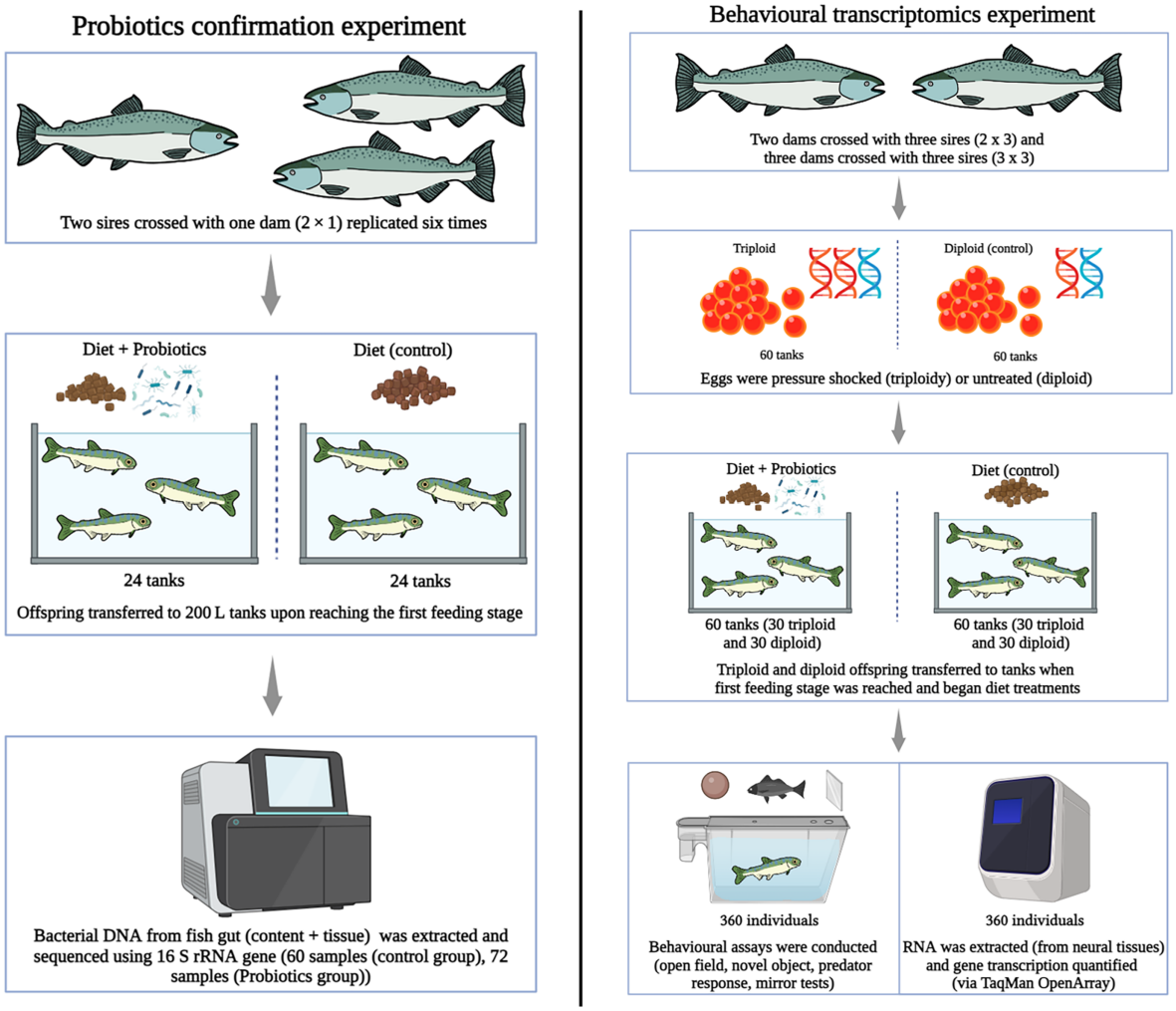

FIGURE 1 Experimental flowchart for the probiotic conformation and behavioral transcriptomic portion of the study. Different individuals were used for these experiments.

weekly. At the time of sampling, tank density ranged from 30 to 40 fish.

2.2.2 | Probiotic experiment

Of the 48 tanks used in this study, 24 tanks received regular food while 24 tanks received the same amount of probiotic treated food (3 probiotic capsules per 100 gm of fish feed). The probiotic was a commercially available formulation developed for human use (60 billion colony forming units (CFU) (Jamieson Laboratories, Toronto, Ontario, Canada; Table 1)). The probiotic feed consisted of EWOS® Micro Crumble feed combined with powder from the Jamieson probiotic mixture, 10 mL of sodium alginate binder (1.0 g Alginate powder mixed with 100 mL of water until dissolved), and 10 mL of 0.5% calcium chloride at a dosage of 10^9 cfu per kg of feed. Feed was prepared daily at the time of feeding. After 10 days three fish per tank were humanly euthanized in accordance with the ethical practices established by the Canadian Council on Animal Care (CCAC) and the University of Windsor's Animal Care Committee (ACC) in an overdose solution of clove oil.[68] Fish were not fed the day of sampling and had a mean mass of 23.3 g (±7.2 SE) with no treatment effect on body weight. Subsequently the fish were dissected and preserved in a highly concentrated salt solution (ammonium sulfate, 1 M sodium citrate, 0.5 M EDTA, H_2SO_4 to bring the pH to 5.2) for subsequence gut DNA extraction. Of the 48 tanks, four control tanks from two families (replicated) had 100% mortality and were excluded from the study, bringing the total number of fish to 60 and 72 for control and probiotics treatment, respectively. All the preserved fish were stored at −20°C.

2.2.3 | Bacterial DNA extraction and 16S library preparation

Bacterial DNA was extracted from fish gut (content and tissue) using a sucrose lysis buffer, as described previously.[69] Bacterial

communities were 16S metabarcoded using universal V5-F (787 F-acctgcctgccg-ATTAGATACCCNGGTAG) and V6 (1046 R-acgcca ccgagc-CGACAGCCATGCANCACCT) polymerase chain reaction (PCR) primers to target the V5-V6 variable 16S regions. Our 16S metabarcoding methodologies were as previously described.[70] Briefly, two rounds of PCRs were used, where the first round consisted of denaturing at 95°C for 3 min, followed by 28 1-min cycles of 94, 55, and 72°C. PCR products from the first round were then purified using Sera-Mag Magnetic Beads (GE, Healthcare Life Science, UK), these products were used for the second round PCR to ligate the adaptor and barcode sequences for sample identification and sequencing. The second round PCR consisted of 3 min at 95°C, eight 1-min cycles of 94, 60, and 72°C, and a final extension at 72°C for 7 min. Concentration of purified PCR product mix (library) was measured on a Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada). The resulting sequencing library was sequenced on a PGM ION Torrent sequencing platform.

2.2.4 | Bioinformatics analysis

The resulting FASTQ file was imported into the Quantitative Insights Into Microbial Ecology (QIIME2-2023.5) platform.[71] DADA2 pipeline[72] was used to denoise single-end sequences, dereplicate, filter chimeras and Amplicon Sequence Variant (ASV) pick using the removeBimeraDenovo function with the "consensus" method. Furthermore, samples with low sequence depth (less than 3000 reads; selected based on alpha rarefaction plot), low abundance taxa (less than 10 ASVs) and ASVs that showed up in only one sample were removed. This decreased the total number of samples to 52 (out of 60) fish for the control group and 67 (out of 72) for the probiotic group. Bacterial taxonomic identification was completed using the SILVA 138-99 reference database.[73] The *Qiime diversity core-metrics-phylogenetic* command in QIIME2 was used to calculate the beta diversity indices. Phylogeny constructed by fasttree plugin[74] was used for calculating the Unweighted UniFrac (to estimate β-diversity). Bray–Curtis dissimilarity matrix (β-diversity) was calculated using the Vegan package in R.[75] Those metrics were used to test for the specific effects of our probiotic treatment on the gut bacterial community composition.

2.3 | Behavioral-transcriptomics experiment

2.3.1 | Breeding design

This portion of the study employed a subset of six 3×3 (not replicated) crosses where 18 females were artificially fertilized with milt from 18 male Chinook salmon (Figure 1). Triploidization occurred the same day, where one-half of the dam's eggs were hydrostatically pressure shocked to serve as a genomic modulation for this study, while the other half was left untreated (i.e., remained diploid). Fertilization and the triploidization process for these fish occurred between October 19 and 22, 2018.

2.3.2 | Rearing design

Of the crosses outlined above, only 15 families (from a 3×3 and a 3×2 cross) were included in this study due to family-egg survival. Fish were moved from incubations stacks to replicate rearing tanks when fish reached the exogenous feeding stage (February 16–29, 2019). Here, family ID and ploidy treatment were identified, with half the tanks assigned to their feed treatments, for a total of 120 tanks (2 ploidies × 2 feed treatments × 15 families × 2 replicates). Rearing tanks were set up in the same manner as those used in the probiotic confirmation trial (i.e., 200 L rearing tanks supplied with fresh running water at a consistent flow rate, continuous aeration, and a 16:8 h light–dark cycle). Average temperature of 9.05°C ± 0.485 SD, dissolved oxygen levels of 9.07 mg/L ± 0.167 SD. At the time of sampling, tank density ranged from 11 to 76 fish.

Individuals from both triploid and diploid replicate families were assigned to either a regular-feed diet or a probiotic additive-feed diet, to serve as a gut-microbiota manipulation for this study. All fish began diet treatments when they could exogenously feed (April 5, 2019). The regular diet consisted of EWOS® Micro Crumble feed, while the probiotic diet consisted of the regular feed combined with powder from the Jamieson probiotics used in the probiotic confirmation trial prepared as described above.

2.3.3 | Behavioral-transcriptomics experiment

When fish reached 8 months post-fertilization, a subset ($n = 360$) underwent behavioral experiments (from June 5 to June 10, 2019) to determine their behavioral profiles (Figure 2). These same individuals were then sacrificed for neural tissues, which were used in transcriptional analyses. Specifically, at the end of the behavioral trial, fish were immediately netted from the behavioral arena and euthanized in 3 L of clove oil anesthetic. The clove oil anesthetic was composed of 1.5 L of water, 5–6 drops of clove oil (active components: eugenol, eugenyl acetate, ß-carophyllene methyl eugenol, Sigma-Aldrich, St. Louis, MO, USA), and 3 drops of ethanol to improve solubility of clove oil. Fish were effectively anesthetized when equilibrium was lost, and they could no longer maintain an upright position. Fish were weighed (mean mass of 4.70 g ± 1.05 SE), spinal cords were pithed, and immediately following, neural tissues (i.e., whole brains) were sampled and placed in RNAlater within 10 minutes post-euthanization. Samples were allowed to become fully saturated with RNAlater via cooling at 5°C for 24 h before being stored at −20°C until RNA extraction for transcriptomic analyses. Whole brains were used to ensure sufficient tissue was available for RNA extraction.

2.3.4 | Tissue sample processing

A total of 357 individuals (of the 360 individuals from the behavioral trials) were included in the transcriptional analyses (three samples

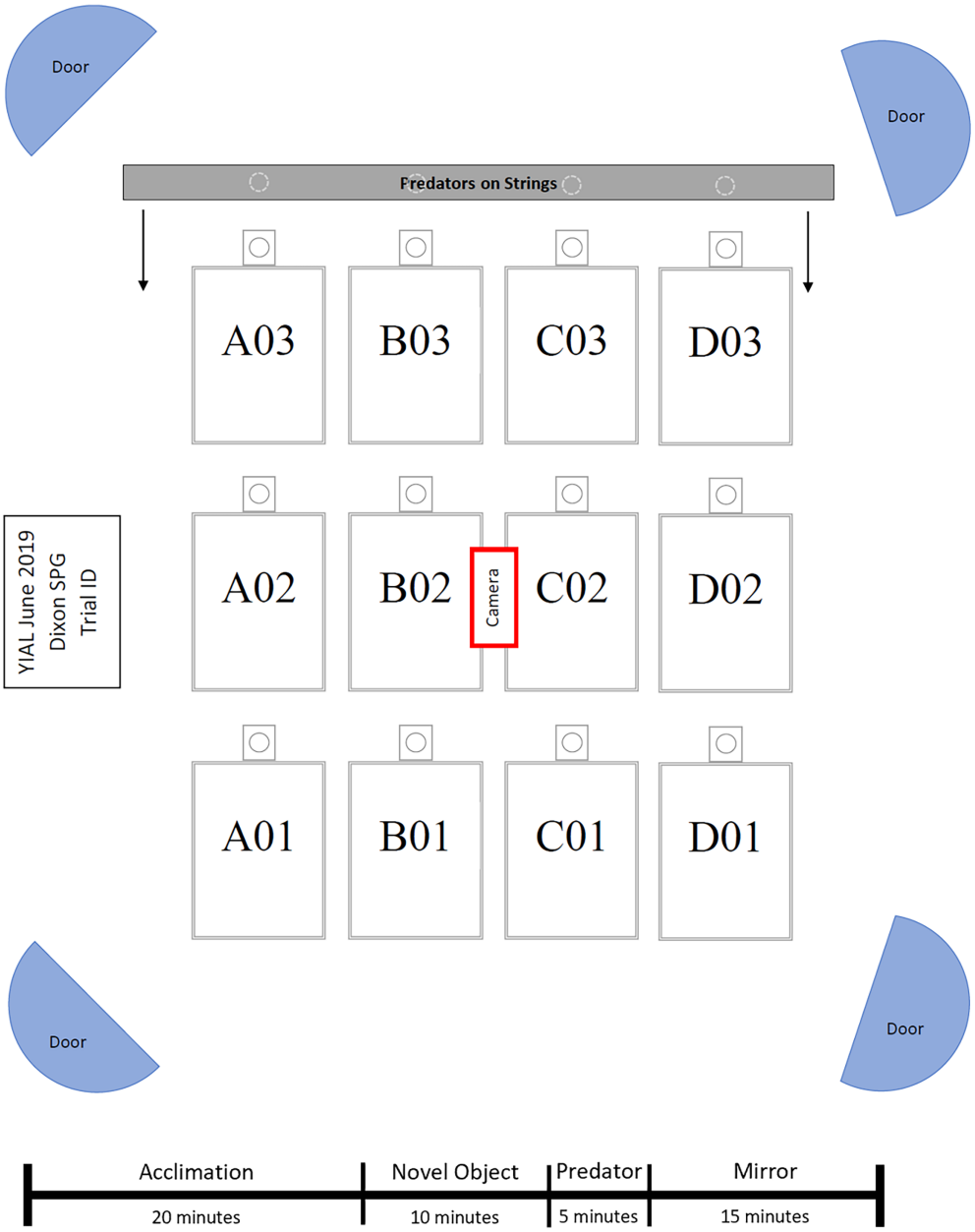

FIGURE 2 Schematic diagram of the behavioral arena set up. A total of 30 trials were run, four-tank numbers tested (individuals assigned A-D based on tank number), with three individuals from a given tank tested at a given time (assigned a number of 01-03). Each behavioral trial consisted of four assays: an open field test (acclimation period), novel object test, predator response test, and a mirror test. Predator silhouettes were moved across arenas at a steady pace, and mirrors were placed on the opposite side of where predator movement was initiated.

failed to provide sufficient quality/quantity RNA for transcriptional analyses). Whole brain RNA extractions were completed with TRIzol™ (ThermoFisher Scientific, Waltham MA, USA), following a protocol adapted from Wellband & Heath.[76] RNA was then treated with DNase to remove possible genomic DNA contamination from the extracted RNA. A subset of samples was run on a Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada) to ensure quality of extracted RNA, RNA integrity numbers of samples ranged from 8.80 to 10. RNA was converted to cDNA using the high-capacity cDNA reverse transcription kit (ThermoFisher Scientific, Waltham, MA, USA), following the manufacturer's protocol altered for half-reactions. Samples were normalized prior to Taqman® OpenArray® qPCR analyses by ensuring all samples contained a cDNA concentration between 100 and 200 ng/μL.

2.3.5 | Candidate genes

Twenty-six genes were included in this study due to their role in one of the following functions: synaptic plasticity (i.e., sonic hedgehog (*Shh*), FOS proto-oncogene (*C-Fos*), autism susceptibility candidate 2 (*Auts2*), *Htr1aa*, and *TPH2*), neurogenesis (i.e., doublecortin (*DCX*), neurogenic differentiation factor (*NeuroD1*), and cellular retinoic acid binding protein 1a (*Crabp1a*)), synaptic plasticity/neurogenesis (i.e., dihydropryimidinase-related protein 2 (*Dpsyl2*), synaptysomal-associated protein 25-a (*SNAP-25a*), *BDNF*, and double cortin like kinase 1 (*Dclk1*)), metabolism/appetite (i.e., cholecystokinin (*CCK*), orexin/hyoicretin receptor 2 (*hcrtr2*), proopiomelanocortin (*POMC*), and neuropeptide Y receptor type 1-like (*NPY*)), stress response (i.e., mineralcorticoid receptor (*MR*), glucocorticoid receptor 1 (*GR1*),

glucocorticoid receptor 2 (*GR2*), cortcotropin-releasing factor (*CRF*), and arginine vasotocin (*AVT*)), and growth (i.e., *GH*, early growth response protein 1 (*EGR-1*), *IGF-1*, insulin-like growth factor binding protein (*IGFBP2b*), and tumor suppressor p53 (*p53*)). These specific gene groups were selected as they relate to behavioral responses and growth; in particular, synaptic plasticity, neurogenesis, and stress-response gene groups are anticipated to relate to behavioral flexibility, sensitivity, and responses to stressors.[77–81] Other genes were selected due to their role in growth and hence production in aquaculture (as metabolism/appetite genes relate to food consumption, and growth genes can indicate a growth benefit).[82–84] Two endogenous control genes (i.e., elongation factor 1-a (*EF-1a*) and acidic ribosomal phosphoprotein (*ARP*)) were selected for normalization[85] using the geometric mean of both endogenous control genes. Transcription of endogenous control genes was examined across treatments to ensure *EF-1a* and *ARP* levels were unchanged.

2.3.6 | Primer and probe analyses

The majority of Taqman® qPCR primers and probes relating to the candidate genes were taken from previous work.[86,87] A subset of primer/probe sequences were developed for this study using cDNA sequences from the National Center for Biotechnology Information (NCBI) for Chinook salmon (i.e., *Auts2*, *DCX*, *NeuroD1*, *hcrtr2*, and *NPY*) or the related species rainbow trout (i.e., *Htr1aa*, *TPH2*, *Dpysl2*, *CCK*, and *POMC*) and Primer Express® version 3.0 (ThermoFisher Scientific, Waltham, MA, USA). Primers were tested with Chinook salmon cDNA, using a gradient PCR (55–65°C), to ensure that the primers amplified at the required final qPCR temperature of 61°C. Sequences of primers for all the genes used in this study can be found in Supplementary Table 1, while putative gene functions are listed in Table 2.

2.3.7 | Transcriptional analyses

To quantify gene transcription, Taqman® OpenArray® chips (Applied Biosystems) (Applied Biosystems) were used. Chips were designed with 48 subarrays, each composed of 56 wells. The wells for each chip were loaded with 48 samples, and the 56 wells were preloaded with the primers and probes for the 28 candidate genes in duplicate. Gene transcription was analyzed using ExpressionSuite Version 1.3 (Applied Biosystems) and all assays with relative cycle threshold (*Ct*) values exceeding 35 were omitted from further analyses. Primer efficiencies were calculated using LinRegPCR.[88] Estimated starting concentrations were log-transformed to have a normal distribution so that parametric statistics could be used in subsequent statistical analyses.

2.3.8 | Behavioral experiment

For a given behavioral trial, four rearing tanks were randomly selected and three fish (to serve as replicates) were haphazardly netted from each tank (for a total of 12 fish per trial) and placed into 12 individual arenas for the duration of the behavioral experiment. This process was repeated with different rearing tanks five times each day for the study duration, resulting in a total of 30 trials over the six-day period and 120 families tested ($n = 360$ individuals). Individual fish were placed in arenas to examine behavioral responses to a novel environment, novel object, predator stimulus, and a conspecific stimulus to collectively determine distinct behavioral profiles emerging across treatments. Behavioral trials were conducted in an enclosed, tarped-off area to prevent potential disturbances from the surrounding environment. Fish were transferred from rearing tanks in a water-filled 3 L pitcher to individual 9 L (35×23×18 cm) opaque arenas (Aquatic Habitats, FL) which were arranged in a 3×4 layout (Figure 2). Arenas contained a blue tile (9.5×4.5×0.5 cm) to serve as a shelter area for the fish as it presents a darker area within the arena that matches their rearing tanks while still allowing researchers to record usage of this space from above. Once an individual was placed in the arena, netting was secured over the arena to prevent escape. Arenas were equipped with a low-pressure source of inflow groundwater (the same source supplying water to the rearing tanks), and water was fully exchanged between trials to prevent any possible effects of hormones released (e.g., stress hormones) from fish from the previous trial. Dissolved oxygen levels and temperature were monitored before the start of behavioral trials with a Labquest (Vernier Software & Technology, USA) to ensure consistency. Average dissolved oxygen level was 9.07 ± 0.167 while average temperature was 9.05°C ± 0.485.

Behavioral assays began after all fish were in their behavioral arenas, and researchers had left the enclosed behavioral arena. The entire assay lasted for 50 min and consisted of four components: an acclimation test, a novel object test, a predator test, and a mirror test. Videos of the behavioral trials were recorded using a GoPro Hero 4 (Woodman Labs, USA) mounted above the arenas and re-positioned as needed for each trial to minimize visually obstructed areas within the arenas and glare from the water's surface. During these trials, the camera was set to record at 1080p, 30fps, and on a linear setting, to record all 12 of the arenas simultaneously in high quality. Each fish was given a unique alphanumeric identifier code to allow for unbiased analyses during the video-analysis phase.

2.3.9 | Behavioral assays

Fish were left undisturbed for 20 min to acclimate to their new environments. This time period served as an open field test to record baseline activity level and explorative tendencies (i.e., use of arena zones (center, peripheral, and shelter) exhibited by an individual, and activity—duration of time spend highly mobile, mobile, and immobile).[89] At the 20-minute mark, a small (3 mm diameter) brown glass bead was added to each arena. This particular bead was selected since it was similar in coloring to the feed pellets given to the fish; however, the bead was of larger size which the fish had no previous experience of, and hence responses to the bead would be indicative of the fish's degree of neophobia.[90] Interaction with the bead (i.e., time to

TABLE 2 Table of candidate genes and their functions. Functional group indicated above genes in italics.

Gene	Main function
Synaptic plasticity	
Sonic hedgehog (*Shh*)	Embryonic development, cell growth, specialization and normal patterning of the forming body
C-FOS	Immediate early response gene involved in cell proliferation and differentiation
Autism susceptibility candidate 2 (*Auts2*)	Neurodevelopment, regulates neuronal migration
5-hydroxytryptamine (serotonin) receptor 1A a (*Htr1aa*)	Serotonin receptor involved in neuromodulation and behavioral plasticity
Tryptophan 5-monooxygenase (*TPH2*)	Catalyzes the synthesis of serotonin
Neurogenesis/synaptogenesis	
Doublecortin (*DCX*)	Neural migration and neurite outgrowth
Neurogenic differentiation factor (*NeuroD1*)	Neural cell cycle regulation
Cellular retinoic acid binding protein 1a (*Crabp1a*)	Neurogenesis/regulate transport/metabolism in the developing embryo and throughout life
Synaptic plasticity & neurogenesis/synaptogenesis	
Dihydropryimidinase-related protein 2 (*Dpsyl2*)	Neural development of axonal growth and cell migration
Synaptysomal-associated protein 25-a (*SNAP-25a*)	Release of neurotransmitters at the end of synaptic terminals, post-synaptic receptors, and synaptic plasticity
Brain-derived neurotrophic factor (*BDNF*)	Neuronal survival and synaptic plasticity
Double cortin like kinase 1 (*Dclk1*)	Involved in the calcium-signaling pathway controlling neuronal migration in the developing brain and may participate in functions of the mature nervous system
Metabolism & appetite	
Cholecystokinin (*CCK*)	Satiation signals and influences digestion/feeding processes and suppresses food intake
Orexin/hyoicretin receptor 2 (*hcrtr2*)	Regulates feeding behavior (predicted) by enabling orexin receptor activity
Proopiomelanocortin (*POMC*)	POMC neurons may exert a tonic inhibitory effect on feeding and energy storage
Neuropeptide Y Receptor Type 1-like (*NPY*)	Involved in regulation of food intake (stimulates)
Growth	
Growth hormone (*GH*)	Necessary for normal growth of the body's bones/tissues
Early growth response protein 1 (*EGR-1*)	Regulates response of growth factors, DNA damage and ischemia
Insulin-like growth factor 1 (*IGF-1*)	Stimulating the start of the cell cycle for other growth factors
Insulin-like growth factor binding protein (*IGFBP2b*)	Receptor to bind and regulate IGF actions in controlling growth, development, reproduction, and aging
Tumor suppressor p53 (*p53*)	Tumor suppressor which regulates cell growth/division
Stress response	
Mineralcorticoid receptor (*MR*)	Ion/water transport regulation during stress
Glucocorticoid receptor 1 (*GR1*)	Bind glucocorticoids to initiate a stress response in the central nervous system to stressful situations
Glucocorticoid receptor 2 (*GR2*)	Bind glucocorticoids to initiate a stress response in the central nervous system to stressful situations
Cortcotropin-releasing factor (*CRF*)	Regulation of stress response/maintaining homeostasis during stress, and can suppress food intake
Arginine vasotocin (*AVT*)	Important for coping with changing environmental salinity

approach and number of approaches), behavioral changes (i.e., change in mobility to the novel object), and activity (i.e., duration of time spent highly mobile, mobile, and immobile) were examined for 10 min before the next assay was carried out. After 10 min, a row of four plush fish made from dark-colored material was suspended from a wooden dowel and moved across the row of arenas approximately 15 cm above each one. These "predators" were designed to resemble a silhouette of a larger fish (i.e., *Micropterus* spp.) to serve as a visual predator stimulus and thus elicit anti-predator responses. The overhead predator served as a true predator evasion test, as individuals exhibited an immediate startle response indicated by a C- or S-start response.[91] Behaviors post-predator were examined for a duration of 5 min, to determine predator-induced responses (i.e., change in mobility) and activity levels (i.e., duration of time spent highly mobile, mobile, and immobile). Following the predator stimulus (after 5 min), mirrors were placed in one end of the arena (opposite of the in-flow

water tube and shelter) to assess sociality or aggression to a 'conspecific'. However, previous research has questioned the effectiveness of the mirror test, with the standard mirror test serving as a less reliable prediction of individual sociability than when using a live conspecific.[92] The standard mirror test was still used in the present study to control for size differences between individuals and the variability in behaviors exhibited by live stimuli. Fish remained in arenas for 15 min, with the final 10 min of this time analyzed for the fish's activity and time spent in the mirror zone. Fish were considered in the mirror zone (4.3 cm × 20.5 cm) when the majority of their body (i.e., more than half) was located within this zone. Laterality, measured as body position to the mirror was also analyzed, where eight different body positions were considered from left/right parallel, forward/backward perpendicular, and 45° intervals were noted. Laterality was measured as a metric for sociality, as fish have shown an eye preference during social interactions,[93] where preference for the left is indicative of sociality,[33] while right is indicative of aggression.[94,95] Shelter use (i.e., number of visits and time spent) was recorded throughout each assay. Presentation of behavioral tests remained consistent across trials to minimize the variation in disturbances to fish by the researchers, particularly for the mirror test, which researchers manually placed in arenas. Novel object test and predator test also remained in this order as the predator stimuli was considered a more stressful disturbance than the bead (neutral), as individuals may exhibited a greater fear response when presented with the bead if following the predator test.

The responses to the introduction of the novel object, predator stimulus, and mirror-insertion were collectively considered as disturbances to the fish (i.e., to the bead, predator silhouette, and the researcher placing the mirror into the arenas) and analyzed independently from the other variables in the behavioral assays. We determined behavioral sensitivity and flexibility based on a fish's response to these three disturbances, where sensitivity indicates whether there was a response to each stimulus, similar to Fraser et al.[96]

2.3.10 | Video analyses

The open field test assay was examined with EthoVision XT14 (Noldus Inc.), a behavioral tracking software, while Solomon Coder,[97] an open-source semi-automatic behavioral coding software, was used to analyze behaviors for the novel-object, predator-simulation, and mirror trials. For the open field test, distance moved (cm), velocities (cm/s) (maximum and average), mobility (mobile and immobile in seconds), latency (s) to enter the center of the arena, and duration of time (s) spent in zones (i.e., periphery, center, and shelter) were measured (Supplementary Table 2). Trials examined with Solomon Coder used behavioral coding sheets where (i) durations of behaviors (i.e., mobilities (immobile, mobile, and highly mobile), zone locations (center, peripheral, and shelter), and laterality (direction of fish towards the mirror)), (ii) count data for behavioral responses (i.e., changes in mobility status and number of bead approaches), and (iii) latencies (i.e., time of first approach to the novel object and to resume pre-disturbance behaviors) were examined (Supplementary Table 2). All behaviors analyzed in Solomon Coder were coded every 0.2 s.

Behavioral sensitivity was determined by examining the fish's behavior (i.e., activity level) 30 s prior to the disturbance, and comparing this mobility immediately following the disturbance, and measured both as a binomial response (i.e., a change in response or no change, totaled across assays) and as a graded response (i.e., assigned a value based on change). For the graded response, a "0" value refers to least sensitive—that is, no change in behavior across the three disturbances, while a value of six refers to the most sensitive—that is, fish either went immobile to highly mobile or the reverse. Flexibility was assessed through examining the latency (in seconds) to resume the fish's initial mobility-based behavior (i.e., mobility level) across all three disturbances.[60]

2.4 | Statistical analyses

2.4.1 | Probiotic confirmation experiment

Differences in the bacterial community composition between control and probiotic diet treatments was visualized using a Principal-coordinate analysis (PCoA) using both the Bray–Curtis dissimilarity and Unweighted UniFrac distance matrices to visualize clustering of the samples based on their treatment groups. The significance of those clusters was assessed using permutational multivariate analysis of variance (PERMANOVA) analysis in R using Vegan package. To identify ASVs that are differentially abundant between the treatment groups, Analysis of Compositions of Microbiomes with Bias Correction (ANCOM-BC)[98] was used in the QIIME2 plugin (qiime composition ancombc; https://docs.qiime2.org/2023.5/plugins/available/composition/ancombc/).

2.5 | Behavioral-transcriptomic experiment

2.5.1 | Transcriptomics

Principal component analyses (PCAs) were used for gene transcription within each functional gene group to explore their within-group associations. This ensured that the genes within groups could be used in our subsequent statistical models as being representative of related functions. PCAs were conducted in JMP version 14 (SAS Institute Inc., Cary, NC, USA) with a Varimax rotation to ensure each variable was meaningfully included in only one factor when multiple factors emerged. Variables with loading values <0.55 were excluded as they were not considered relevant contributors to the components. Factor eigenvalues ≥ 1 were retained and used in subsequent analyses if they explained >10% of the variance seen across individuals. Only the neural function group of genes (both synaptic plasticity and neurogenesis) separated out into two groups, with early-development neural functions as one group, comprising *Shh*, *Auts2*, *Htr1aa*, and *DCX*; and a long-term neural development group that included *NeuroD1*,

Crabp1a, *Dpysl2*, *SNAP-25a*, *BDNF*, and *Dclk1* (Supplementary Table 3.1—Supplementary Table 3.4). As a result, we tested genes within 5 different functional groups instead of four.

General linear mixed models (GLMM) were run using the lme4 package in R[99] with a Gaussian family and identity link function to explore the effects of treatment (ploidy (categorical: diploid or triploid), feed (categorical: regular or probiotic), and their interactions), mass (g) (continuous range: 1.86–7.64), rearing tank densities (continuous, range: 13–79) and time of day (continuous, range: 9.4–15.53) on our response variable: the corrected concentration (continuous range: 3.35–12.1) values from candidate genes. Additionally, dam ID, sire ID, family ID, and replicate (nested in treatment group and family ID) were included as random effects for all GLMMs. We used sequential Bonferroni (Bonferroni-Holm)[100] to adjust our *p*-values when testing individual gene transcription models within a functional group to correct for multiple hypothesis testing.[101]

2.5.2 | Behavioral Transcriptomics

Behavioral measurements were transformed (i.e., *log*, *1/log*, or box cox) as needed and then standardized prior to PCA analyses[102] (Supplementary Table 4.1—Supplementary Table 4.5). Separate PCAs were run for traits extracted from each of the different behavioral assays to reduce redundancy and find meaningful behavioral associations within each assay. PCAs were also run on the combined disturbance sensitivity and flexibility variables. Principal components from each behavioral assay plus disturbance attributes were then collectively combined into an overall PCA (Table 3) to determine if any behavioral types (consistent behaviors) emerged across the entire behavioral experiment.

Four behavioral profiles emerged across assays (see Results). Two individuals were statistically confirmed as outliers for the "activity" PCA and were removed from this component for subsequent analyses. Where necessary, these profiles underwent data transformations to satisfy assumptions of normality before being used in subsequent statistical models. Behavioral genomics GLMMs were then run with a Gaussian-specified family and an identity link function to explore the main effects of treatments (ploidy, feed, and their interactions), mass (g), rearing tank densities and time of day on each behavioral type. Fixed effects also included the concentrations of all candidate genes per functional group (range 2–6 genes per statistical model per functional group). Dam ID, Sire ID, family ID, and tank number (nested within family ID) were included in GLMMs as random effects but were removed as they contributed non-significantly to the model, thus reducing model complexity. We used sequential Bonferroni corrections across the functional groups per behavioral profile, as each behavioral profile was tested five times with the different functional gene groups.

3 | RESULTS

3.1 | Probiotic confirmation experiment

Our PCoA plot showed that the microbiota in gut samples were separated based on their treatment group (probiotic or control) using both Unweighted UniFrac and Bray–Curtis dissimilarity (β-diversity; Figure 3A,B). Moreover, the PERMANOVA analysis (using Bray–Curtis dissimilarity matrix) showed that the community structures were significantly different between the treatment groups ($F_{1,117} = 7.32$, $p = 0.001$). In addition, our ANCOM-BC analysis showed that ASVs

TABLE 3 Results from the principal component analysis loadings for factors from individual principal component tests (open field, novel object, predator, mirror and behavioral sensitivity/responsiveness tests).

Behavioral measurement	PCA component reactionary	PCA component activity	PCA component boldness and aggression	PCA component laterality and extremes
Exploration (Novel Object)	**0.700**	−0.036	−0.216	−0.277
Predator Response (Predator)	**0.752**	−0.019	0.258	0.153
Sensitivity to Novelty (Behavioral Flexibility)	**0.935**	−0.068	−0.026	−0.007
Activity (Open Field Test)	0.218	**0.626**	−0.054	0.281
Activity (Novel Object)	−0.079	**0.730**	−0.069	−0.182
Activity (Predator)	−0.021	**0.695**	−0.083	0.013
Activity (Mirror)	−0.149	**0.584**	0.123	−0.054
Boldness (Open Field Test)	0.006	0.193	**0.645**	−0.164
Aggression (Mirror)	−0.019	−0.011	**0.506**	0.098
Response to Habituated Stimuli (Behavioral Flexibility)	−0.041	0.289	**−0.618**	0.010
Extremes (Open Field Test)	0.096	0.153	−0.109	**−0.657**
Laterality (Mirror)	0.032	0.112	−0.125	**0.720**

Note: Bold values were loadings that were considered significant (>0.55). Components from this test revealed four different behavioral profiles: reactionary, activity, boldness/aggression, and laterality and extremes.

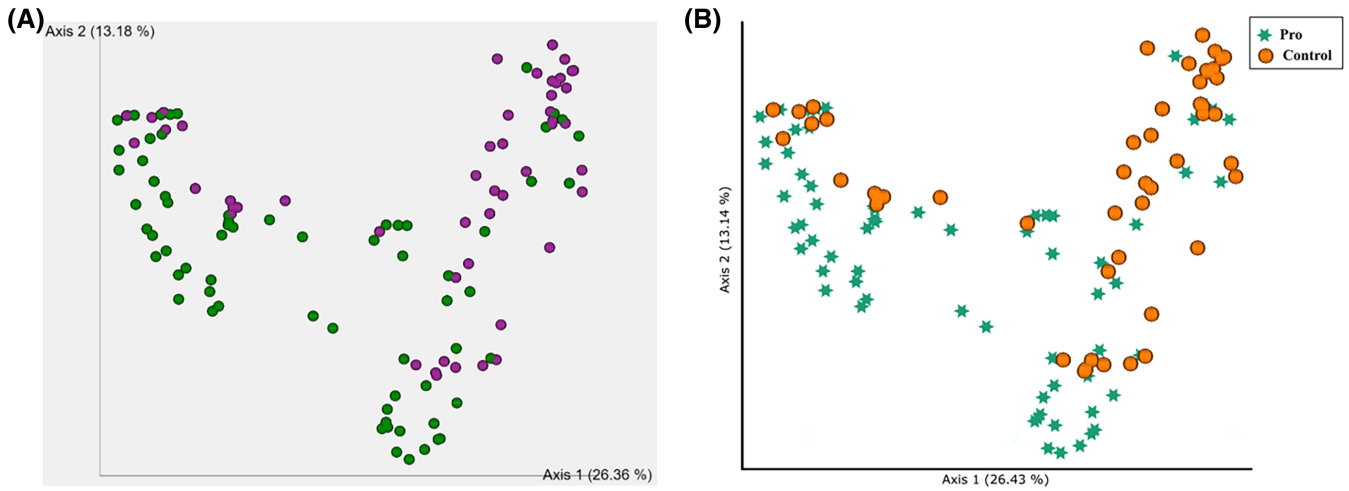

FIGURE 3 PCoA of the fish gut microbiome colored based on treatments using the Bray–Curtis dissimilarity matrix (A) and the Unweighted UniFrac matrix (B).

related to feed probiotic strains were the main taxa associated with the observed differences in gut microbiota (Supplementary Table 5).

3.2 | Behavioral transcriptomics experiment

3.2.1 | Gene transcription

From the TaqMan OpenArray, six of the 26 genes candidate genes did not provide enough gene transcription data (possibly due to low levels of transcription for individual samples) to be included in the remainder of the statistical analyses. Of these, two were genes related to synaptic plasticity (*C-Fos* and *TPH2*), two from the metabolism/appetite functional group (*CCK* and *hcrtr2*), one involved in growth (*IGFBP2b*), and one stress response gene (*GR1*). PCA results for gene functional groups indicated there were a total of four different functional gene groups: early-neural development (i.e., *Shh*, *Auts2*, *Htr1aa*, and *DCX*), long-term neural development (i.e., *NeuroD1*, *Crabp1a*, *Dpysl2*, *SNAP-25a*, *BDNF*, and *Dclk1*), metabolism/appetite (i.e., *POMC* and *NPY*), growth (i.e., *EGR-1*, *IGF-1*, *p53*, and *GH*), and stress response (i.e., *MR*, *GR*, *CRF*, and *AVT*). All loadings from the PCA can be found in Supplementary Tables 3.1–3.4.

3.2.2 | Transcriptional variation across treatments

Variation in the transcriptional profiles of the selected genes was examined to determine the impacts of treatment on the levels of gene transcription. Ploidy did not have a significant effect on transcription for genes within the five functional groups after within group Bonferroni-Holm corrections (summary of relationships between treatments, genes, and behaviors can be found in Figure 4). However, probiotic treatment had a significant effect on gene transcription for the metabolism and appetite gene *NPY* ($t(128) = 2.48$, $p = 0.02$) (Figure 5), where those on a regular diet had higher neural gene transcription of *NPY* (Figure 5). Full results for gene transcription can be found in Table 4. Time of day had a significant impact on gene transcription in two functional gene groups: long-term neural development (*BDNF*: $t(103) = -2.40$, $p = 0.02$) and growth (p53: $t(226) = -2.56$, $p = 0.01$). Mass and tank density had no significant impact on gene transcription (corrected *p*-values non-significant (n.s.)); nor did the random effects of sire ID, dam ID, family ID, or replicate (all corrected *p*-values n.s.).

3.2.3 | Within-assay behavioral associations

Within each behavioral assay, behaviors were examined for redundancy and to identify meaningful associations. The PCA for the open field test resulted in three factors that explained a total variance of 81.1% (Supplementary Table 4.1). These were Activity (32.7%), Extremes of Locomotion (25.9%), and Boldness/Exploration (22.5%). Individuals with high scores for activity spent the majority of their time mobile, moved the most distance in the arena, and spent less time in the center of the arena. Individuals with high scores for the extremes of locomotion reached the greatest maximum velocities and moved the greatest distance in one bout of movement within the behavioral arenas. Lastly, individuals with high scores for boldness and exploration spent less time overall in the periphery, and more of this time was spent moving in this zone; these individuals also transitioned between the center and periphery the most.

The PCA for the novel object test resulted in two factors, accounting for 83.9% of the variance (Supplementary Table 4.2): Novel Object (NO) Responsiveness (61.9%), and Activity (22.0%). Individuals with a high score in NO responsiveness exhibited a higher

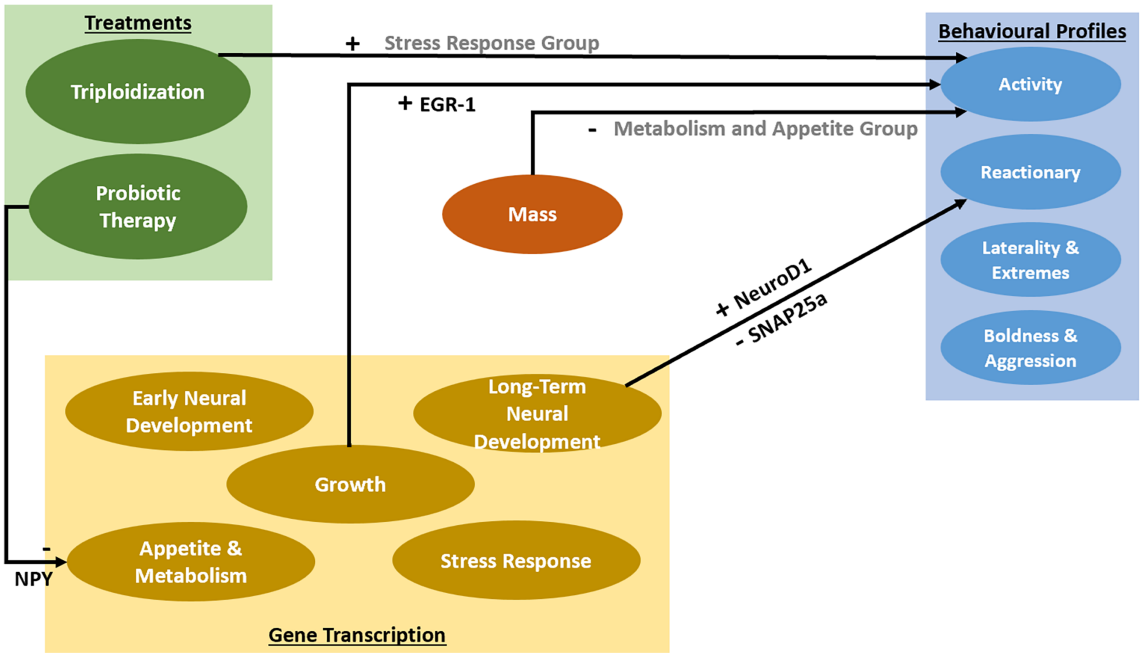

FIGURE 4 Summary diagram of findings (significant interactions), incorporating treatments, mass, gene transcriptional profiles, and behavioral profiles. Time of day and rearing tank density not included in this summary. Significant interactions of direct effects indicated with directionality indicated. Where genes are directly involved, gene is indicated in black text, while gene groups included in the behavioral models, but not directly driving the impact, are indicated in gray.

FIGURE 5 Significant model-predicted results of regression analysis of food treatment effects on gene transcription. Probiotic therapies had a significant effect on gene expression for the NPY ($n = 135$) gene, where those on probiotic diets had decreased gene expression compared with regularly fed counterparts. Model-predicted relationships are plotted; data points show predicted-model data.

mobility (both active and highly active), and subsequently approached the bead more often while taking less time to initially approach the bead. Individuals that scored high in the activity variable spent the least amount time immobile throughout this assay.

The PCA for the predator response test resulted in two factors accounting for 81.2% of variance (Supplementary Table 4.3): Predator Responsiveness (44.7%), and Activity (36.5%). An individual with a high score for Predator Responsiveness initially exhibited a large spike in locomotion with the predator stimulus but spent less time immobile during the remainder of the trial. An individual that scored high in the activity component remained mobile during this assay, regardless of the stimulus presentation.

The PCA from the mirror test resulted in three factors, which accounted for 69.4% (Supplementary Table 4.4): Aggression (26.1%), Activity (22.2%), and Laterality Towards the Mirror (21.1%). An individual with a high score in aggression spent more time facing towards and being active in the mirror. An individual with a high activity score spent more time being both mobile and highly mobile when in the mirror zone. Those that scored high in laterality spent more time with their left side facing the mirror, and less time with the right side facing the mirror.

The behavioral flexibility/sensitivity PCA resulted in two factors, accounting for 76.7% (Supplementary Table 4.5): Sensitivity to Novelty (51.6%), and Responses to Habituated Stimuli (25.1%). Individuals that scored high on sensitivity to novelty responded the most across all three behavioral assays, exhibiting the greatest degree of responses (e.g., going from immobile to highly mobile for all three stimuli), and taking longer to resume their pre-disturbance behavior to novel stimuli (i.e., bead and the predator silhouette). Fish that scored high on responses to a habituated stimulus took longer to resume their behavior to the disturbance.

TABLE 4 Gene transcription GLMM results by functional group, with *t*-values and significance values (indicated in parentheses) for each variable included in the model indicated.

Gene model	Ploidy	Feed	Ploidy * feed	Mass	Tank-rearing densities	Time of day	Model sample size
Early neural development							
Shh ($p < 0.0125$)	0.853 (0.395)	1.902 (0.059)	−0.802 (0.424)	−0.651 (0.516)	−0.661 (0.509)	−1.371 (0.172)	196
Auts2 ($p < 0.0167$)	0.326 (0.745)	0.376 (0.707)	0.251 (0.802)	−0.213 (0.831)	0.135 (0.893)	−0.842 (0.401)	240
Htr1aa ($p < 0.05$)	0.834 (0.405)	0.797 (0.426)	0.092 (0.927)	−0.015 (0.988)	−0.683 (0.496)	−0.273 (0.785)	208
DCX ($p < 0.025$)	1.359 (0.176)	0.766 (0.445)	0.282 (0.778)	0.611 (0.542)	−1.300 (0.196)	−0.867 (0.387)	171
Long-term neural development							
NeuroD1 ($p < 0.01$)	0.025 (0.980)	0.728 (0.467)	−0.076 (0.939)	0.724 (0.470)	−1.186 (0.237)	−1.316 (0.189)	316
Crabp1a ($p < 0.0125$)	−0.303 (0.762)	0.200 (0.842)	0.563 (0.574)	0.920 (0.358)	−1.100 (0.274)	−0.103 (0.918)	332
Dpysl2 ($p < 0.008$)	0.121 (0.904)	1.486 (0.141)	0.178 (0.859)	−0.450 (0.652)	−0.871 (0.386)	−0.023 (0.982)	341
SNAP-25a ($p < 0.05$)	0.418 (0.677)	1.237 (0.219)	−0.044 (0.965)	−0.887 (0.376)	−1.520 (0.131)	−1.494 (0.138)	350
BDNF ($p < 0.025$)	0.165 (0.869)	1.521 (0.132)	−0.033 (0.973)	1.079 (0.282)	−0.833 (0.407)	**−2.399 (0.018)**	244
Dclk1 ($p < 0.0167$)	0.309 (0.757)	0.692 (0.490)	0.435 (0.664)	−0.592 (0.554)	−1.676 (0.102)	−2.220 (0.027)	308
Metabolism and appetite							
POMC ($p < 0.05$)	−0.023 (0.982)	0.655 (0.515)	0.140 (0.889)	0.776 (0.439)	1.691 (0.095)	−1.171 (0.245)	137
NPY ($p < 0.025$)	0.985 (0.326)	**2.476 (0.015)**	−0.914 (0.362)	−1.108 (0.270)	0.755 (0.452)	−1.453 (0.149)	135
Growth							
IGF-1 ($p < 0.0125$)	0.671 (0.503)	0.206 (0.837)	0.646 (0.519)	0.362 (0.718)	0.387 (0.700)	−2.228 (0.027)	263
p53 ($p < 0.0167$)	1.589 (0.113)	1.367 (0.173)	−0.953 (0.342)	0.717 (0.474)	−0.449 (0.655)	**−2.563 (0.011)**	235
GH ($p < 0.025$)	−0.316 (0.753)	0.740 (0.461)	0.984 (0.328)	0.583 (0.562)	0.359 (0.720)	−1.464 (0.147)	88
EGR-1 ($p < 0.05$)	−1.270 (0.210)	−1.356 (0.182)	1.276 (0.208)	−0.539 (0.592)	0.010 (0.992)	0.185 (0.854)	82
Stress response							
MR ($p < 0.0167$)	0.283 (0.777)	0.455 (0.649)	0.252 (0.801)	1.023 (0.307)	−0.125 (0.900)	−0.586 (0.558)	318
GR2 ($p < 0.0125$)	−0.495 (0.621)	0.396 (0.693)	0.439 (0.661)	−0.548 (0.584)	−0.511 (0.611)	−1.017 (0.310)	258
CRF ($p < 0.025$)	1.559 (0.120)	2.417 (0.016)	−0.713 (0.477)	−0.215 (0.830)	−1.664 (0.098)	−1.030 (0.304)	235
AVT ($p < 0.05$)	1.063 (0.289)	1.695 (0.092)	−0.192 (0.848)	0.274 (0.784)	−0.130 (0.897)	−1.272 (0.205)	176

Note: Sequential Bonferroni corrections were done within each functional gene group. Accepted significance values indicated next to gene model and significant result *p*-values after corrections are indicated in bold.

3.2.4 | Across-assay behavioral phenotypes

Behaviors were examined to determine the presence of behavioral types across behavioral assays. The cohesive behavioral PCA model, an aggregate of the open-field, novel-object, predator, mirror, and behavioral sensitivity/flexibility PCA factors, resulted in four behavioral types, or profiles, which accounted for 52.0% of the variance (Table 3). These profiles were: Reactionary (16.8%), Activity (15.9%), Boldness/Aggression (10.2%), and Laterality and Extremes of Locomotion (10.0%). Individuals with high scores for Reactionary spent more time being highly mobile during the novel-object assay and interacting with the bead, had a more drastic change in behavior to the predator stimulus and spent less time immobile after it was removed, responded more overall across all the disturbance stimuli, and took longer to resume behaviors post-disturbance. Individuals that scored high in the Activity component were more active in the open-field, novel-object and mirror tests (specifically in the mirror zone). Individuals with a high Bold/Aggressive score were less immobile and spent less time in the periphery and explored the arena more often during the open-field test. They also spent more time displaying behaviors suggestive of aggression (i.e., forward-facing the mirror and not stationary), and resumed behaviors induced by the researcher disturbance much quicker. Fish that scored high on the Laterality/Extremes of locomotion profile spent more time with the left side of their body facing the mirror and had the lowest scores for velocity and total distance traveled.

3.2.5 | Behavioral variation across treatment and functional genes

Reactionary

Reactionary (box cox transformed) (Summary of results found in Table 5, full results can be found in Supplementary Table 6): Ploidy, probiotic feed, and their interaction had no significant effect on the reactionary behavioral type (corrected *p*-values not significant across

TABLE 5 Summary of behavioral profile GLMMS, with gene groups as explanatory variables and specific gene group incorporated into the model as indicated.

Gene group model	Behavioral Profile	Accepted *p*-value	Variable	*t*-Value (significance level)
Long-term neural development	Reactionary	0.0125	*NeuroD1*	3.04 (0.003)
			SNAP-25a	−2.86 (0.005)
Metabolism and appetite	Activity	0.025	Mass (g)	−3.02 (0.005)
Growth	Activity	0.05	*EGR-1*	3.34 (0.021)
			Time of Day	−3.88 (0.02)
Stress response	Activity	0.01	Ploidy	3.23 ($1.74e^{-3}$)

Note: Only variables with a significant impact on behavioral profiles are included here, full comprehensive models can be found in Table 6 of the supplementary materials.

all gene function groups). However, transcription at two genes from the long-term neural functional group significantly impacted reactionary fish: *NeuroD1* ($t(158) = 3.04$, $p = 0.003$; Figure 7A) and *SNAP-25a* ($t(157) = -2.86$, $p = 0.005$; Figure 7B). Specifically, increased transcription of *NeuroD1* led to an increasingly reactionary behavioral profile, whereas increased levels of the *SNAP-25a* transcript were related to lower reaction profile scores. Mass, tank density, and time of day had no significant impact on this behavioral profile (corrected p-values non-significant across all gene function groups). Dam ID, Sire ID, and Family ID did not significantly contribute to the reactionary models and were removed; nor was there a significant random effect of replicate (corrected p-values n.s.).

Activity

Activity (log-transformed) (Summary of results found in Table 5, full results can be found in Supplementary Table 6): Ploidy had a significant effect on fish displaying an active behavioral type when genes from the stress response functional group were included ($t(92) = 3.23$, $p = 0.002$), with triploid individuals being more active than diploids (Figure 6). Food treated with probiotics did not impact activity (corrected p-values n.s.), nor did the interactions between ploidy and feed type for any of the gene groups in these activity behavioral models (corrected p-values n.s.). *EGR-1* transcription impacted the active behavioral profile ($t(5) = 3.34$, $p = 0.02$), with individuals with a higher level of *EGR-1* transcription displaying a more active behavioral profile (Figure 7C). Tank density had no impact on activity levels (corrected p-values n.s.), while mass (metabolism and appetite: $t(34) = -3.02$, $p = 0.005$) and time of day (growth genes: $t(4) = -3.88$, $p = 0.02$) significantly impacted activity levels. Heavier individuals were less active than lighter counterparts, and individuals were less active when sampled later in the day. Dam ID significantly contributed to activity profiles when immediate neural, long-term neural, and metabolism and appetite functional gene groups were included. Sire ID, Family ID and replicate did not significantly contribute to the activity models.

Boldness and aggression

Boldness and aggression (log-transformed) (Summary of results found in Table 5, full results can be found in Supplementary Table 6): Ploidy, probiotic feed, and their interaction had no significant effects on individual boldness-aggression profiles (corrected *p*-values n.s. across all gene function groups). Likewise, no genes in any of the functional groups had a significant impact on bold/aggressive behavioral profiles (corrected *p*-values n.s.). No significant effect was found for mass, tank density, or time of day (corrected *p*-values n.s.). Dam ID, Sire ID, and Family ID and replicate did not significantly contribute to the boldness and aggression models.

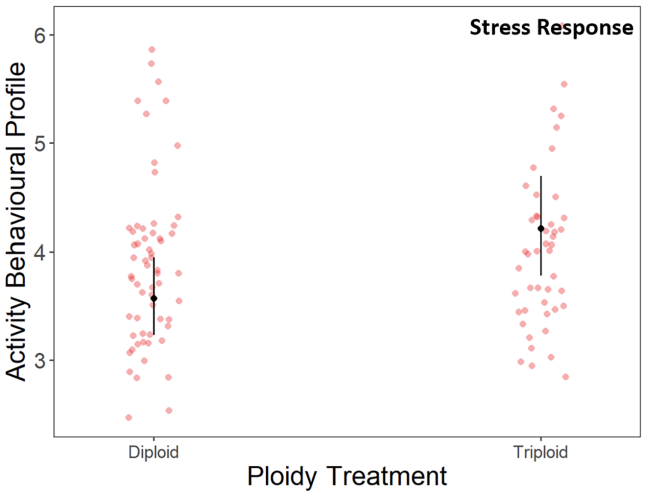

FIGURE 6 Significant model-predicted results of regression analysis of ploidy treatment effects on active behavioral profile. Triploidization had a significant effect on the activity levels of individuals, with triploids more active than diploid counter parts when the stress response gene functional group ($n = 106$) was incorporated into the behavioral profile models. Triploidization did not have a significant effect on reactionary, boldness/aggression or the laterality/extremes of locomotor behaviors. Model-predicted relationships are plotted, data points show the model-predicted data.

Laterality and extremes of locomotion

Laterality and extremes of locomotion (Summary of results found in Table 5, full results can be found in Supplementary Table 6): Ploidy, probiotic feed, and their interaction had no significant effects on the behavioral profile of laterality and extremes of locomotive behavior

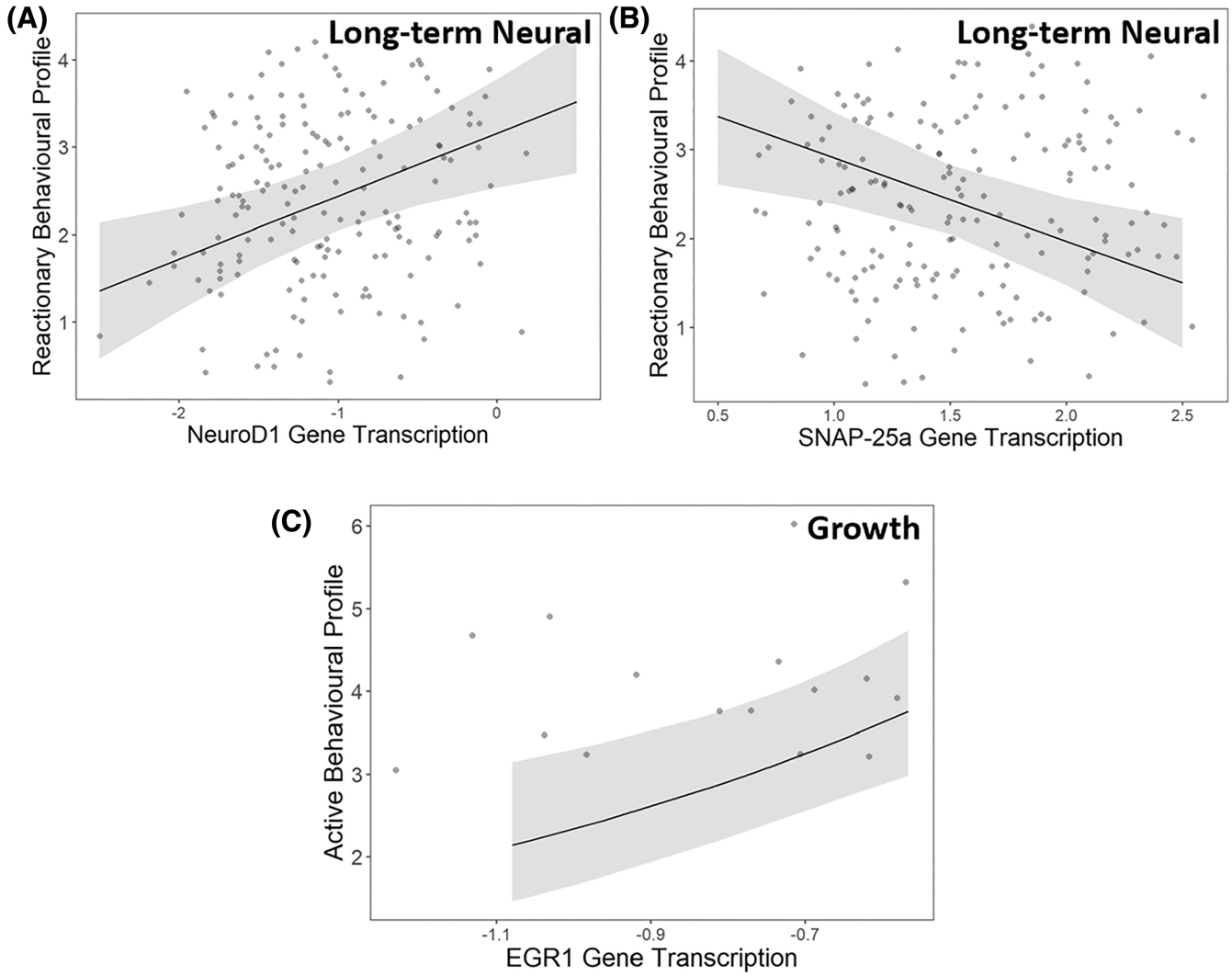

FIGURE 7 Significant model-predicted results of regression analysis of gene transcription effects on behavioral profiles. Significant gene effects on behavioral profiles. Reactionary profiles showed gene effects, particularly for NeuroD1(A) and SNAP-25a (B) transcription ($n = 174$). Individuals exhibiting greater NeuroD1 expression also showed an increased level of reactionary behaviors, while those with an increased level of SNAP-25a expression had a decrease in level of reactionary behaviors. Active profiles showed a significant gene effect of EGR-1 (C) transcription ($n = 16$), where individuals with higher EGR-1 transcription also had increased activity levels. Model-predicted relationships are plotted, data points show the predicted-model data.

(all corrected *p*-values n.s.). No genes nor functional groups had a significant impact (all corrected *p*-values n.s.). Mass, tank-rearing densities, and time of day had no significant effect in any of the functional-gene models (all corrected *p*-values n.s.). No random effects were significant, either. Significant effects of treatment and gene expression on behavioral profiles can be found in Figure 4.

4 | DISCUSSION

Our probiotic challenge study using a commercially available probiotic mix established that gut microbiota in the Chinook salmon gastrointestinal tract were different for individuals fed the probiotic supplement compared with those on a control diet. These findings contribute to the growing literature on probiotics in aquaculture and confirm the effect of an orally supplemented (in this case with a commercially available probiotic mixture) diet on gut microbiota in Chinook salmon. Despite this evidence for our probiotic treatment affecting the gut microbiome, we did not find strong evidence for genomic impact of triploidy or the effect of probiotic feed (i.e., environmental effect) on gene transcription or fish behaviors. Instead, we found triploid individuals to be more active, but only when taking into account stress-response genes, indicating that stress genes likely serve as an underlying mechanism acting on activity levels. Similarly, our probiotic therapy directly influenced neural gene transcription (i.e., *NPY*), which highlights a link between gut modifications and the brain, but again, we did not find evidence of it being translated into differential behaviors (at least for those we studied), suggesting

that the connections between the gut and fish behavior are complex and multifaceted. In addition, none of the variables impacted by triploidy were also affected by the probiotic treatment, indicative that our probiotics did not recover neural/behavioral changes associated with triploidization. Our more fruitful findings emerged from our behavioral transcriptomics analyses, identifying a subset of candidate genes that influenced behavioral profiles regardless of ploidy and diet, indicating selected salmon genes play a greater role in behavioral variation than previously thought.

4.1 | Variation in gene transcription

Variation in gene expression is a key driver in phenotypic differences among individuals.[103–105] Contrary to our predictions, ploidy did not have an impact on the levels of gene transcription within any of the selected functional gene groups. Albeit the function of the candidate genes chosen reflected behavioral and/or mass differences noted in triploids such as lowered levels of sensitivity,[29] mass gains[9] or deficits,[106] and responsiveness in response to environmental stimuli,[30] our findings were not surprising given that there was also little influence of ploidy on the behavioral profiles in our fish. One possible reason for our results may be due to dosage compensation (e.g., triploid cyprinid (*Squalius alburnoides*)[107]), where gene dosage effects (increased levels (1.5×) of gene transcription in triploids[108,109]) are compensated for through gene silencing, where one of the three alleles is silenced, resulting in gene transcription equivalent to diploid levels.[110] Given that this is the first study to investigate the connections between transcriptional and behavioral profiles it is also possible that Chinook salmon of differing ploidy do not express transcriptomic or behavioral differences at the juvenile life stage.

We did, however, find that probiotics decreased the transcription of *neuropeptide Y receptor type 1-like* (*NPY*) (metabolism and appetite gene group). This result is unexpected, given that *NPY* is involved in the regulation of food intake, where it stimulates the brain and initiates feeding behaviors.[83,111] We had predicted probiotics to have a positive impact on appetite, and result in a higher transcription of *NPY*, opposite to our findings. Despite this result, we found no association between the down regulation of *NPY* and changes in behavioral profiles. However, as *NPY* expression has been found to stimulate appetite and increase in instances of starvation or food deprivation,[112,113] probiotics may induce the opposite effect and down regulated *NPY* transcription due to fish on this diet being satiated. Our general lack of effects is not consistent with previous studies that have shown probiotics to alter gene expression of neural survival genes (e.g., *BDNF*[45]), serotonin signaling/metabolism genes (e.g., *TPH2* and Htr1aa[45]), growth genes (e.g., *IGF-1*[114]), and many immune genes (see review from Dawood et al.[115]). A possible explanation is the type of probiotics we used may not be composed of bacterial strains suited for salmon aquaculture. As there are a total of 14 bacterial strains in this probiotic, it is possible that the effects of some strains may interfere with others or the natural microflora within the gastrointestinal tract,[116] as each strain may have a different effect on gene expression levels. Lastly, it is important to note that salmoniformes experienced a whole genome duplication event,[117] and as a result, some of the selected genes may not have the expected functions, and instead may have functions not yet characterized.

Interestingly, our findings show that a subset of genes related to long-term neural development (i.e., *BDNF*), and growth (i.e., *p53*) exhibited a significant diel expression pattern. Daily rhythms occur in gene expression to benefit the organism by ensuring that biological functions occur at the optimal times of day.[118] *BDNF* is crucial for survival and growth of neurons,[119] where increases in *BDNF* aids in promoting synaptic plasticity, neurogenesis, cell survival, and results in the greater capacity for learning and memory.[120,121] Decreasing levels of *BDNF* transcription thus may be indicative of greater levels of synaptic plasticity and neural cell development/survival earlier in the day for Chinook salmon. In our study, transcription of *BDNF* significantly decreased as the day progressed, where earlier in the day also coincided with higher activity levels in our fish. *BDNF* has been found to be important for generating circadian rhythmicity in zebrafish, and aids in maintaining the rhythmicity of locomotor behavior.[122] *p53*, whose function is to initiate repair mechanisms of DNA-damaged cells or cellular apoptosis when repair is not possible[123] has been found to oscillate and modulate the expression of other circadian genes (e.g., Period 2, a circadian regulator).[124] In all, these findings have relevance for aquaculture production in that the first feeding of the morning may be crucial in terms of the greatest growth performance potential.

4.2 | Variation in behavioral profiles

Four distinct behavioral profiles emerged in our sample of juvenile Chinook salmon, as determined from our behavioral assays. Fish could be categorized either by their degree of reactivity, activity levels, boldness and aggression, and their behavioral lateralization (a proxy for sociality) combined with locomotor extremes. These behavioral profiles can serve as performance indicators for how they may respond to changes in their environment,[125] and furthermore have ecological consequences that affect an individual's growth and performance.[126]

Contrary to our predictions, we found triploid individuals were more active than their diploid counterparts with the inclusion of stress response genes as covariates. These findings are contrary to previous studies, where no differences in the locomotor activity profiles of fish of different ploidies were observed (e.g., Atlantic salmon,[127] and Coho salmon (*Oncorhynchus kisutch*)[128]). Furthermore, no other effects of ploidy were found to affect the behavioral profiles of juvenile fish in our study. Similarly, Benhaïm et al.[127] found no difference in boldness between diploid and triploid Atlantic salmon; however, aggression and sociality have been shown to either differ between ploidies (e.g., Kavumpurath and Pandian[129] and Carter et al.[10]) or be unaffected (e.g., O'Keefe & Benfey[28] and Garner et al.).[25] Lastly, we found no effects of ploidy or probiotics on sociality nor locomotor extremes; and to our knowledge, some studies have examined sociality

(e.g., Chinook salmon,[25] Atlantic salmon,[26] brown trout (*Salmo trutta*)[130]), while none have previously focused on the impacts of ploidy on anxiety-related behaviors (e.g., as more erratic movements relate to higher anxiety[131,132]) in fishes.

Probiotic effects on behaviors were not observed in this study, contrary to our predictions. Results from previous studies on feeding-related behaviors have been mixed, with some fish species fed different strains of probiotics having either increased (e.g., rainbow trout[59]) or decreased[133] levels of feed motivation, where the latter was further associated with an increased transcription of appetite-suppressing genes (*leptin* and *melanocortin-4-receptor*) and a decrease in transcription of appetite-stimulating genes (*cannabinoid receptor 1* and *NPY*).[133] A lack of probiotic effect on activity levels has also been reported in zebrafish,[134,135] similar to our study. Studies that have demonstrated locomotor differences have been conducted in germ-free organisms, which may not lead to the same results, since in our study, we modified the already existing gut microbiota in our fish.[136] However, the impacts of probiotic therapies influencing anxiety-like behaviors have been well studied. In zebrafish, both *Latobacillus plantarum*[134,136] and a mixture of *Latobacillus rhamnosus* and *Bifidobacterium longum*[57] were found to reduce anxiety-like behaviors, unlike in our study. However, the anxiety-like behaviors exhibited in those previous studies tend to refer to the usage of space in a novel tank, where bottom-dwelling behaviors are related to a lowered state of anxiety,[137] whereas our measures of anxiety-like behavior included reaching the maxima of locomotive behavior, and an inability to cope with disturbances by readily resuming normal behaviors post-stimulus.

4.3 | Genetic effects on behavioral profiles

Variation in gene expression can result in variation in behaviors exhibited by individuals.[138,139] In our study, we found that increased transcription of *NeuroD1* of the late response neural gene group increased instances of the reactionary behavioral profile. This transcription factor is responsible for eliciting neural development, neural survival, differentiation, and synaptic plasticity,[140,141] with increased expression triggered after chronic or mild stress.[142] Its association with the highly reactionary and disturbance-sensitivity profile is therefore not surprising. Next, we found increases in *SNAP-25a* (late response neural functional group) were related to dampened reactionary responses in fish. Indeed, this finding is plausible, as *SNAP-25a* plays a crucial role in the release of neurotransmitters at the end of synaptic terminals, post-synaptic receptors, and synaptic plasticity[143]; and decreases in *SNAP-25* have been associated with attention-deficit hyperactivity disorder (ADHD)-type behaviors (i.e., hyperactivity)[144,145] and cognitive functions.[146,147] This increased hyperactivity may be similar to high levels of reactive behaviors associated with low levels of *SNAP-25a* transcription found in our study, as the reactionary behavioral profile was based on startle-type responses and mobility when stimuli were presented. Taken together, a very reactive individual had increased *NeuroD1* (promoting increased neuronal plasticity) and decreased *SNAP-25a* (decreasing neurotransmitter exocytosis of neural synapses) transcription, which could be indicative of greater connections between neurons with lowered levels of effective communication related to the reactionary profile.

Additionally, our study found that individuals with greater transcription of *EGR-1* (growth functional group) exhibited a more active behavioral type. While *EGR-1* is a member of the immediate-early gene group involved in organismal growth through cell proliferation, differentiation, and development,[148] expression of *EGR-1* occurs widely throughout the brain especially within key areas for controlling cognition, emotional response, social behaviors and sensitivity to rewards.[149] Additionally, *EGR-1* is part of a group of transcriptional regulators that initiate transcriptional responses to environmental changes,[150] and is involved in neural processes of memory and learning, specifically for long-term memory consolidation.[151] In mice, *EGR-1* deficient mice have been found to have reduced levels of locomotor activity compared with wild type mice as a result of disrupted rhythmic regulation,[152] which is in line with our findings that higher levels of *EGR-1* transcription promoted higher activity levels. Furthermore, for stressors (both predictable and unpredictable), Cerqueira et al.[151] found that expression of *EGR-1* was increased. Their findings could aid in explaining increased activity levels with increasing *EGR-1* transcription, as the activity levels were measured after a novel environment and novel stimuli were introduced which could be perceived as unpredictable stressors.

4.4 | Conclusion

In this study, we used behavioral transcriptomics to investigate the interactions between the gut (via probiotic therapies), brain (via neural gene transcription), and behaviors in individuals experiencing a disruption to their genome (triploidy). Our study is not the first to explore the connections between gut, brain, and behavior in fish,[45,134,136] but rather adds to the growing body of literature, and is the first to include these interconnections in triploid fish on a probiotic diet. It is possible that our lack of effects is due to our analytical methods, given that we took a multi-gene and multi-behavioral, holistic approach, which necessitated both stringent statistical corrections and complex, multi-variable models, where weak effects may be swamped; however, while a simplified statistical approach may have detected more effects, they would have little biological relevance due to the lack of important confounding covariates. Nevertheless, our findings suggest that triploidization in Chinook-salmon aquaculture may not place triploid individuals at transcriptional- or behavioral disadvantages compared with their diploid counterparts at the juvenile stage, and triploidization should still be implemented as a method to maintain sustainable hatchery practices. Additionally, the probiotics we chose to use did not provide the anticipated benefits nor improve welfare for Chinook salmon, but rather may decrease appetite through genetic mechanisms, albeit the impact on feeding behaviors (and subsequent mass) has yet to be tested in this context.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ETHICS STATEMENT

All of the experiments were conducted in accordance with the University of Windsor's Animal Care Committee (AUPP 18-08) and the Canadian Council of Animal Care to ensure ethical research practices.

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