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Behavioral and Reproductive Effects of Environmental Enrichment and *Pseudoloma neurophilia* infection on Adult Zebrafish (*Danio rerio*)

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Recent studies have shown beneficial effects of environmental enrichment (EE) for zebrafish, while infection of zebrafish with the common pathogen *Pseudoloma neurophilia* has negative effects. This study investigates the effects of *P. neurophilia* infection and EE in housing and breeding tanks on measures of behavior, growth, and reproduction. Zebrafish were socially housed and were either infected, *P. neurophilia*-infected (PNI) (n = 12 tanks), or SPF for *P. neurophilia* (SPF) (n = 24 tanks). Fish were housed with or without EE, which consisted of placing plastic plants in the tanks; sprigs from plants were placed in half of the breeding tanks for half of breedings, alternating breeding tanks without EE weekly. Behavioral testing included the Novel Tank Diving Test (NTT) and Light/Dark Preference Test (LDT) conducted prior to breeding. At the end of the study, biometric data were collected. Histopathology and molecular analysis for common diseases in fish confirmed that SPF fish remained SPF and that fish from all PNI tanks were infected. PNI fish produced significantly fewer eggs and had lower body weights and lengths than did SPF fish. Fish with EE had longer body lengths, than did fish without EE, and male fish had longer body lengths than female fish. The biometric results and reproductive measures show that SPF fish exhibited better growth and suggest that EE in housing tanks could improve fish growth. The behavioral test results were inconclusive regarding whether infection status or EE altered anxiety-like behavior. Our results support other recent studies showing negative effects of *P. neurophilia* infection on zebrafish.

Abbreviations: BCS, body condition score; dpf, days post fertilization; EE, Environmental enrichment; H&E, hematoxylin and eosin; hpf, hours post fertilization; ISKNV, Infectious spleen and kidney necrosis virus; LDT, light/dark preference test; NTT, novel tank diving test; PNI, *Pseudoloma neurophilia*-Infected Group; SARL, Sinnhuber Aquatic Research Laboratory; SPF, SPF-*P. neurophilia* group; ZIRC, Zebrafish International Resource Center

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The use of laboratory zebrafish (*Danio rerio*) has expanded dramatically in recent decades from an animal model for developmental genetics into one that is employed in a wide variety of research areas, including: neuroscience, animal behavior, cancer, tissue regeneration, immunology, infection, aging, and toxicology research.[9,20] Many of these research areas use adult zebrafish, which are more likely to be affected by spontaneous disease than are embryos and larvae.[8] Coinciding with the expanding popularity of zebrafish in research is the prevalence of microsporidiosis, caused by the microsporidian parasite *Pseudoloma neurophilia*.[23,36] *P. neurophilia* is one of the most common pathogens detected in laboratory zebrafish,[23,27] causing both subclinical infections and progressive clinical disease in heavy infections.[7,11] Clinical signs of *P. neurophilia* infection include emaciation, spinal deformities, reduced growth, altered behavior, decreased fecundity, and increased mortality.[7,11,28,29] Zebrafish with clinical signs of *P. neurophilia* can often be visually identified by their low body condition score,[6] scoliosis, and resultant swimming abnormalities and should be removed from experiments and breeding stock.

P. neurophilia is an obligate intracellular parasite with a tropism for neural tissue. *P. neurophilia* is transmitted both vertically and horizontally.[26] Some evidence indicates that surface disinfection of embryos is not completely effective at preventing transmission of the parasite,[10,11] making its exclusion from laboratory animal facilities challenging. In addition, no treatment is currently available for microsporidiosis, which makes elimination of established infections difficult. However, rigorous pathogen screening protocols have helped establish lines of laboratory zebrafish that are SPF for *P. neurophilia*.[14]

Naturally occurring clinical and subclinical infections in animals are well established to cause confounds and high variability that can lead to invalid or misinterpreted experiments and an accumulating body of experimental evidence has begun to characterize the adverse effects of *P. neurophilia* and other pathogens on zebrafish research.[2,13,15,21,22,24,28,29] However, subclinical PNI zebrafish often go undetected, and thus may be enrolled in, and potentially compromise, biomedical research projects. In addition, uninfected zebrafish may acquire the infection during the course of an experiment, potentially creating undesired effects on research outcomes. In one study, PNI zebrafish were found to have a significantly reduced habituation

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to a startle response as compared with both exposed negative tankmates and unexposed control zebrafish.[29] In other studies, PNI zebrafish displayed more freezing behavior or altered shoaling behavior relative to uninfected control zebrafish.[21,28] These findings suggest that microsporidiosis could initiate a stressed or anxiety-like behavioral phenotype, which could confound behavioral research findings.[28] Previous reports have suggested that the effects of *P. neurophilia* infection may vary with sex and genetic background.[5,24] A retrospective study evaluating histopathologic specimen from the Zebrafish International Resource Center (ZIRC) found a 12.4% higher prevalence of *P. neurophilia* infected males in routine health cases and a 7.5% higher prevalence in males with clinical cases.[5] Another study found that when zebrafish from the AB line that had an endemic *P. neurophilia* infection were subjected to stress or treated with cortisol, their mortality rate significantly increased over a 7 wk period, while no mortality was observed in the TL line of zebrafish.[24] However, experimentally-infected TL zebrafish weighed 27% less than control TL zebrafish, indicating that although mortality rate was not affected in the TL line, the growth rate was affected.[24] Larval zebrafish appear to be particularly susceptible to *P. neurophilia* infections, leading to the suggestion that infection could compromise developmental neurotoxicity testing and other development studies.[15] Thus, the exclusion and elimination of *P. neurophilia* from laboratory zebrafish colonies would be beneficial to research.

To date, methods for providing environmental enrichment (EE) are not well-established for many aquatic species,[12] and zebrafish may often not be routinely provided with EE. However, a growing number of studies demonstrate that EE influences zebrafish behavior in certain situations.[8,16,18,19,32,33] For example, for zebrafish exposed to unpredictable chronic stress, access to EE for 21 or 28 d attenuated both behavioral and chemical responses to stress.[19] While socially housing zebrafish is the default at most research facilities, zebrafish occasionally require single housing for experimental or other husbandry or clinically-related purposes. In cases where zebrafish are individually housed, the addition of an artificial plant as EE has been shown to decrease anxiety in behavioral measures, compared with single-housed fish in tanks without EE.[8] In breeding tanks, zebrafish appear to prefer spawning in a grass-enriched environment, as evidenced by a higher number of viable embryos produced in breeding tanks containing EE.[16] In a separate study, EE did not affect fry survivability.[33] The authors of that study concluded that further studies are warranted to identify optimal EE strategies that promote species-typical behaviors and reduce stress and anxiety-like behaviors in laboratory zebrafish.

Multiple behavioral assays have been developed to assess behavior in zebrafish, including two tests of anxiety-like behaviors - the novel tank diving test (NTT) and the light:dark preference test (LDT). Based on geotaxis, the NTT uses the zebrafish's instinctual escape response to seek protection in an unfamiliar environment by diving, freezing, and reducing exploration in the vertical water column space.[3] As zebrafish gradually acclimate to a novel environment, increased exploration into the test tank's top half normally occurs.[35] Anxiolytic drugs, such as fluoxetine and diazepam, increase the time zebrafish spend at the top of the NTT tank.[31] In contrast, anxiogenic stimuli increase time spent at the bottom of the tank, immobility, and erratic movements.[31] The LDT is based on the zebrafish's overt scototaxis, or innate preference for dark rather than brightly lit environments.[16] Zebrafish exposed to anxiogenic substances or stressors spend more time in dark areas of a test tank than do control zebrafish.[16] While both the NTT and the LDT test for anxiety-like behaviors in zebrafish, one study evaluating the use of these 2 tests suggested that for a comprehensive assessment, the best approach is to use both NTT and LDT tests.[1]

Our goal in this study was to identify the effects of *P. neurophilia* infection and EE on anxiety-like behaviors and reproductive performance in adult AB line zebrafish. Juvenile PNI and SPF zebrafish were socially housed with or without EE in their tanks. After 5 wk, zebrafish were behaviorally tested in the NTT and LDT and then bred for 6 wk, alternating whether EE was provided in the breeding tank or not each week. At the end of the breeding experiment, all adult zebrafish were euthanized, measured, and weighed, and samples were submitted for analysis by histopathology and real-time PCR to confirm PNI or SPF status. We hypothesized that *P. neurophilia* infection would increase anxiety-like behaviors in NTT and LDT behavioral assays, would not be significantly impacted by EE, and would have a negative impact on fecundity. We hypothesized that no significant difference in anxiety-like behaviors would be present between fish that were group-housed with EE or without EE and that a significant difference in reproductive performance would be found between breeding tanks that contained EE and those that did not.

Materials and Methods

Animals and husbandry. All experimental procedures performed in this study were reviewed and approved by the University of North Carolina Chapel Hill Animal Care and Use Committee. Animals were housed in an AAALAC-accredited facility in compliance with the *Guide for the Care and Use of Laboratory Animals,*[12] as detailed on protocols.io (dx.doi.org/10.17504/protocols.io.xusfnwe). Six hundred SPF- *P. neurophilia* AB line zebrafish (*Danio rerio*) embryos were received at 1-d after fertilization (dpf) from Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. Embryos were surface disinfected with bleach by the sending institution at 6 h after fertilization (hpf). In addition to *P. neurophilia*, the production colony at SARL was also free from *Edwardsiella ictaluri*, *Myxidium streisingeri*, *Pseudocapillaria tomentosa*, *Mycobacterium abscessus*, *Mycobacterium fortuitum*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, and *Mycobacterium peregrinum*, based on annual PCR and histopathology health monitoring at SARL. After receipt, 30 fry per culture dish were kept in E3 solution (http://cshprotocols.cshlp.org/content/2011/10/pdb.rec66449) at room temperature in a room with a 14-h light, 10-h dark photoperiod. At 5 dpf each plate was transferred to a stationary 1.1 L tank with 100 mL of system water and 100 mL of L-type saltwater rotifers (*Brachionus plicatilis)* and remained in polyculture for 3 d. By 8 dpf the tanks were placed on the flow-through Tecniplast stand-alone system (pH 6 to 8, conductivity 300 to 600 µSiemens/cm, temperature 27 to 29 °C) with a flow rate of one drip per second. Each tank received approximately 30,000 rotifers daily. At 21 dpf, the flow rate was increased to a fast drip, and pelletized feed GemmaMicro 150 was provided once daily. Juvenile fish were large enough for Gemma Micro 300 after 65 dpf. The fish remained on the rack system during their growth phase and were transferred to stationary 10-gallon glass aquariums at 90 dpf for the actual study.

Monoinfected *P. neurophilia*-positive adult zebrafish ($n = 24$) of unknown age and genotype were obtained from the University of Southern California. Two fish were euthanized immediately upon receipt and submitted to IDEXX BioAnalytics (Columbia, MO) for testing to confirm health status. Real-time PCR results confirmed the presence of *P. neurophilia*, and were uniformly negative for *Edwardsiella ictaluri*, *Flavobacterium columnare*,

Ichthyophthirius multifiliis, Infectious spleen and kidney necrosis virus (ISKNV), *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium haemophilum*, *Mycobacterium marinum*, *Mycobacterium peregrinum*, *Myxidium streisingeri*, *Piscinoodinium pillulare*, *Pleistophora hyphessobryoconis*, and *Pseudocapillaria tomentosa*. Adult monoinfected zebrafish were randomly divided into 2 equal groups, with each half housed in one of 2 10-gallon rectangular glass aquariums (Aqua Culture, Ontario, Canada) on one side of a 0.30 mm nylon mesh divider (McMaster-Carr, Cleveland, OH). Water temperature was maintained within the range of 19.5 to 27.2 °C by submersible glass aquarium heaters (Hydor, Sacramento, CA), and sponge filters (Aquarium Technology Decatur, GA) were used to maintain water quality parameters (as detailed on protocols.io (dx.doi.org/10.17504/protocols.io.xusfnwe)) along with weekly water changes (90%). Separate supplies were used for each tank of fish to avoid cross-contamination.

***Pseudoloma neurophilia* infection.** At 3 mo of age, zebrafish (n = 300) were sexed; males (n = 120) and females (n = 180) were randomly placed into one of four 10-gallon rectangular glass aquariums (Aqua Culture, Ontario, Canada) where they were exposed to 1 of 2 experimental housing conditions for 8 wk: cohoused in the same aquarium with confirmed PNI adult zebrafish (n = 15) on the other side of a nylon mesh divider (PNI Group) or cohoused without zebrafish on the other side of the mesh divider (SPF group). SPF fish that were not cohoused with infected fish had a mesh divider placed in the aquarium so that the available tank size was identical for both groups. Mesh dividers were used to physically separate the 2 cohorts of fish that were of different ages and size to avoid injury, aggression, or cannibalism between the 2 cohorts of fish in the infection tanks.

Transmission of *P. neurophilia* occurs via ingestion of infected tissues from live or dead fish.[11,13,23,27] Any infected adult zebrafish that displayed clinical symptoms of *P. neurophilia* (including low body condition score (BCS ≤ 2/5), abnormal swimming behaviors, or lethargy) were euthanized, and their spinal tissue extracted, minced, and fed to juvenile fish to improve chances of disease transmission. Feeding infected tissues to the PNI group may itself have affected behavior or breeding. Feeding these tissues to the SPF group would have exposed the SPF group to *P. neurophilia*, which would have compromised the study. Feeding fish products to SPF fish is not necessary to maintain their SPF status. This study was not designed to address nutritional effects on behavior and breeding, so euthanizing adult SPF fish in order to feed them to SPF fish to control for the difference in nutrition is not consistent with 3Rs.

Gentle manual expression was used to express unfertilized eggs that were then fed to juveniles in the 2 infected groups. At the end of 8 wk of exposure by cohousing, all remaining infected adults were euthanized, and their spinal cords and brains collected and fed to juveniles in the *P. neurophilia*-exposed groups.

Experimental housing. At 5 mo of age, fish were moved from their static tanks to 1.1L tanks on a flow-through aquatic housing system (Tecniplast, Buguggiate, VA, Italy) where they were socially housed at 2 to 5 fish per tank. Fish were housed in mixed sex groups. PNI (n = 12) and SPF (n = 24) tanks were placed on opposite sides of the zebrafish housing rack to avoid the potential for splashes and infection of the SPF. Half of all tanks were provided with EE consisting of plastic plants (3 boxwood sprigs attached to mats and 3 floating boxwood sprigs; BioServ, Flemington, NJ). This EE was chosen due to plants being arranged on a sinking mat for use in home tanks with removable sprigs that float as EE for use in breeding cages. EE remained in the housing tanks for the remainder of the experiment. The other half of the tanks received no EE. Figure 1.

Behavior testing. After 5 wk of exposure in all 4 groups [PNI with EE, (n = 6); PNI with no EE (n = 6); SPF with EE (n = 12) and SPF with no EE (n = 12)] each fish was tested once in each behavioral test. Fish from the same tank were all tested individually in the same test session. Behavior testing occurred in the animal housing room. All behavior testing occurred between 1000 and 1500 h; this time interval is similar to that used in another study with behavior trials, but the test period was longer for this study due to sample size and pathogen containment.[8] Test tanks were filled with water from the flow-through system, and clean tanks and freshwater were used for each trial. All behavioral trials were recorded on videotape (Amcrest 720P, Houston, TX) at 43.5 cm from the test tank. All videotaped behaviors were digitally scored using behavior tracking software (EthoVision XT 14, Noldus, Leesburg, VA).

Novel tank test (NTT). Fish were transferred with a net into a clear 25.5 cm × 16 cm × 24.5 cm 2.5 L plastic tank (Pentair, Minneapolis, MN) with no acclimation period. The tank was marked at 10 cm from the bottom of the tank by placing 2 black permanent marker lines on each side of the tank. These marks were later used as a landmark to adjust arena settings in behavior tracking software. Behaviors measured included total distance moved, velocity of movement, frequency of transitions, and cumulative duration above and below the horizontal line. Behaviors were scored over 6 min.

Light-dark test (LDT). Clear 25.5 cm × 16 cm × 24.5 cm 2.5 L plastic tanks (Pentair, Minneapolis, MN) were divided in half vertically and one half of the tank exterior was painted with opaque nontoxic black paint and the other half was externally painted with nontoxic white paint (Plaid, Atlanta, GA). A 6 cm viewing window. which was slightly smaller than viewing window used in another study,[8] was left unpainted in the center of the tank for video recording. A custom-designed plastic divider was placed in the tank's center to confine the fish to the clear center area. Fish were transferred with a net into an acclimation area located in the center of the tank at the level of the viewing window for an acclimation period of 2 min. After 2 min, the plastic divider was removed and fish were allowed access to all areas of the tank. Behavioral parameters measured included velocity of movement and cumulative durations in the white-painted half of the tank and the tank's black-painted half. Behaviors were scored over a 15 min period.

Breeding. At 244 dpf, single pair, weekly spawning trials began for all groups. Every week for 6 wk, 1 male and 1 female were randomly chosen from each of the 36 tanks and kept physically separated from one another overnight by a divider placed in standard breeding tanks (Pentair, Minneapolis, MN). The following morning at 0900, the water was changed in each tank before removing the divider and plants, if present, and natural mating was permitted for 30 min. Embryos were collected in E3 medium (Cold Spring Harbor Laboratory Press, 2011) and immediately sorted into culture dishes of 50 or fewer embryos. Embryos were stored in an incubator at 28.5 °C with a 12:12 h light: dark cycle and sorted again 24 dpf. A final count of all hatched embryos was performed at day 6 after mating (Figure 2). During the first week of breeding, half of the tanks were chosen at random to receive one plastic plant sprig on each side of the divider. The next week, the other half received the plant, alternating in the same way in subsequent weeks.

Euthanasia, biometric data and sample collection. All fish were euthanized via rapid chilling in accordance with the *AVMA Guidelines for Euthanasia of Animals*.[1] Immediately after

Figure 1. Housing tanks, EE (top) and no EE (bottom).

euthanasia, fish were weighed and fork length (the length of the fish from mouth to center caudal fin) was measured. A longitudinal incision was made in the body wall of 12 euthanized PNI zebrafish and 6 SPF fish before fixation in 10% buffered formalin for histopathology. One euthanized fish from each of 12 PNI and 24 SPF tanks was placed in a sterile microcentrifuge tube and frozen to be submitted for real-time PCR.

Histopathology. Formalin-fixed zebrafish were submitted to IDEXX BioAnalytics (Columbia, MO) for slide preparation and interpretation. Fixed zebrafish were parasagittally sectioned, placed in tissue cassettes (Tissue-Tek Uni-Casette, Sakura Finetek, Nagano, Japan), and decalcified for 4 h in a 25% formic acid/10% sodium citrate solution. Tissues were subsequently embedded in paraffin using routine methods, sectioned at 5 µm, and stained using hematoxylin and eosin (H and E) and Ziehl-Neelsen acid-fast stains (StatLab Medical Products, McKinney, TX) prior to evaluation.

Molecular analysis. Frozen zebrafish were submitted to IDEXX BioAnalytics (Columbia, MO) to be tested for zebrafish pathogens by real-time PCR, including *Edwardsiella ictaluri*, *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacteritum haemophilum*, *Mycobacterium marinum*, *Mycobacterium peregrinum*, *Myxidium streisingeri*, *Pseudocapillaria tomentosa*, and *P. neurophilia*. Individual whole fish were homogenized, and total nucleic acids were extracted using a commercially available platform (NucleoMag VET Kit, Macherey-Nagel GmbH and KG, Düren, Germany). Fluorogenic real-time PCR assays were based on the IDEXX proprietary service platform. Real-time PCR assay primers and hydrolysis probes were designed using PrimerExpress version 3.0 software (Applied BioAnalytics, Waltham, MA). Real-time PCR analysis was performed at IDEXX BioAnalytics (Columbia, MO) using standard primer and probe concentrations (Applied BioSystems) and LightCycler 480 Probes Master (Roche Applied Science, Indianapolis, IN) in a 384-well plate configuration in a commercially available instrument (LightCycler 480, Roche Applied Science). All IDEXX BioAnalytics real-time PCR assays have been validated to detect fewer than 10 template copies of target DNA per reaction. In addition to positive and negative controls for each real-time PCR assay, a hydrolysis probe-based real-time PCR assay targeting a universal bacterial reference gene (*16s rRNA*) was amplified for all samples to determine the amount of DNA present in the test sample, to verify DNA integrity, and to confirm the absence of PCR inhibition.

Statistics. Statistical analyses were conducted using SAS (version 9.4, SAS Institute, Cary, NC). For biometric data (n = 46 SPF EE; n = 48 SPF no EE; n = 21 PNI EE; n = 22 PNI no EE), general linear models were fit to weight and separately to length. These models included fixed effects for sex (male, female), enrichment (EE, no EE), disease status (PNI, SPF), and all their interactions. The models also included a random effect for tank to control for any tank differences. Type III sums of squares were used for analysis and all post hoc mean comparisons were run using least squares means. For the NTT and LDT, each response variable in trials (n = 145 and n = 156, respectively), a mixed ANOVA was run allowing for fixed effects of fish disease status (PNI, SPF) and enrichment group (EE, no EE), and their interaction. A random effect was included for tank to control for any differences. For each model, residual diagnostics were examined to ensure model fit and to verify that the assumptions were satisfied.

Figure 2. Breeding tanks, no EE (top) and EE (bottom).

Due to heteroscedasticity in the residuals, some response variables (LDT and embryo counts) were natural-log transformed. Summary data are presented as mean ± SEM. The significance level was set at $\alpha = 0.05$. The Tukey–Kramer adjustment was performed for all pairwise comparisons within each model to control the Type I error rate.

Results

Novel Tank Diving Test. SPF and PNI fish showed no significant difference in swim distance ($P = 0.2593$) (Table 1). EE ($P = 0.1838$), and disease status by enrichment type ($P = 0.699$) also did not significantly affect mean distance traveled. Fish with EE did not swim significantly faster than did fish without EE ($P = 0.0992$). Disease status did not have a statistically significant effect on mean velocity of movement ($P = 0.2801$) or on frequency of entries into the top half of the tank ($P = 0.1936$), but a significant difference was found between enrichment groups ($P = 0.0374$), with EE fish approaching the top half of the tank more frequently, on average as compared with non-EE fish. PNI fish occupied the bottom of the tank significantly more than did SPF fish ($P = 0.0032$), but EE did not have a significant effect on the mean frequency of entries into bottom half of tank ($P = 0.6296$). Neither disease status nor enrichment had a statistically significant effect on duration spent at top of tank ($P = 0.9636$ and $P = 0.1583$, respectively). Disease status had a significant effect on mean duration spent in bottom half of tank, with SPF fish spending more time at the bottom half of the tank than PNI fish ($P = 0.0345$). However, enrichment did not have a statistically significant effect ($P = 0.2100$).

Light:Dark Preference Test. Disease status ($P = 0.0991$), EE ($P = 0.3883$), and disease status by enrichment ($P = 0.2752$) did not have statistically significant effects on frequency of entries into the light half of the tank (Table 2). Disease status ($P = 0.0167$) and EE ($P = 0.0337$) both had statistically significant effects on frequency of entries into the dark half of the tank, but disease status by enrichment type ($P = 0.1774$) did not. Disease status ($P = 0.6503$), EE ($P = 0.2893$), and disease status by enrichment type ($P = 0.2643$) did not significantly affect the mean amount of time spent in the light half of the tank. Disease status ($P = 0.4185$) and environmental enrichment ($P = 0.2667$) did not significantly affect the mean amount of time spent in the dark half of the tank. Disease status by enrichment type had a statistically significant effect on the time spent in the dark half of the tank ($P = 0.0298$). For SPF fish, those with enrichment spent significantly more time in the dark half of the tank ($P = 0.0177$).

Breeding Data. Infection status significantly affected breeding, with SPF fish producing more eggs (SPF EE, 21 ± 5; SPF no EE, 14 ± 4) than PNI fish (PNI EE, 2 ± 2; PNI no EE, 3 ± 2) ($P = 0.0009$) (Table 3). Infection status had a statistically significant effect on embryos surviving at 6 dpf, with SPF fish having more viable embryos at 6 dpf (SPF EE, 15 ± 4; SPF no EE, 11 ± 3) than PNI fish (PNI EE, 0; PNI no EE, 2 ± 1) ($P = 0.0008$). Enrichment in the housing tank or breeding tanks had no significant effects.

Biometric Data. Measurements were collected from all fish that survived to the end of this study ($n = 137$ from a total of 170). The average body weights for females were 0.41 ± 0.06 g (SPF EE), 0.26 ± 0.03 g (SPF no EE), 0.13 ± 0.02 g (PNI EE), and 0.12 ± 0.02 g (PNI no EE). The average body weights for males

Table 1. Novel tank diving test

Infection status	Housing EE	Distanced traveled (cm)	Velocity of movement (cm/s)	Frequency of entries into top half of tank	Duration spent in top half of tank (s)	Frequency of entries into bottom half of tank	Duration spent in bottom half of tank (s)
SPF	EE	1676 ± 172	5.5 ± 0.7	13.6 ± 4.2*	48 ± 8	13.2 ± 2.7	302 ±10**
	No EE	1535 ± 124	4.6 ± 0.3	6.4 ± 1.1	9 ± 1	9.5 ± 1.1	323 ± 7**
PNI	EE	1461 ± 200	5.7 ± 1.3	14.3 ± 4.4*	16 ± 3	16.4 ± 2.7***	284 ± 16
	No EE	1172 ± 191	3.7 ± 0.6	9.7 ± 2.8	17 ± 4	16.8 ± 3.78***	286 ± 20

Values denote means ± SEM. *P = 0.037; Fish with EE in housing tanks approached the top half of the tank more frequently than those without EE. **P = 0.035; SPF fish spent more time at the bottom half of the tank than PNI fish. ***P = 0.003; PNI fish frequented the bottom of the tank, on average, more than SPF fish.

Table 2. Light:Dark preference test

Infection status	Housing EE	Frequency of entries into light half of tank	Frequency of entries into dark half of tank	Duration spent in light half of tank (s)	Duration spent in dark half of tank (s)
SPF	EE	44 ± 4	56 ± 5*/**	194 ± 23	414 ± 30***
	No EE	42 ± 4	33 ± 4*	283 ± 28	278 ± 34
PNI	EE	28 ± 0	31 ± 6**	262 ± 45	291 ± 44
	No EE	39 ± 8	43 ± 22	239 ± 45	337 ± 51

Values denote means ± SEM; *P = 0.0167; **P = 0.0337; SPF fish and fish with EE approached the dark half of the tank more frequently, on average. ***P = 0.0177; SPF EE fish spent significantly more time in the dark half of the tank.

Table 3. Breeding performance over 6 wk

Infection status	Housing EE	Total no. eggs	Mean no. eggs	Mean no. hatched	Survival to hatch %	Spawning success %
SPF	EE	754[a]	21* ± 5	15** ± 4	62%	35%
	No EE	505[a]	14* ± 4	11**± 3	73%	33%
PNI	EE	89	2 ± 0	0	0	0%
	No EE	82	3 ± 2	2 ± 1	24%	14%

Values denote means ± SEM. Total # of SPF eggs has been divided by 2 because there were twice as many SPF tanks as PNI tanks. *P = 0.001 comparing values for SPF and PNI, **P = 0.001 comparing values for SPF and PNI.

were 0.36 ± 0.02 g (SPF EE), 0.34 ± 0.02 g (SPF no EE), 0.18 ± 0.03 g (PNI EE), and 0.16 ± 0.02 g (PNI no EE). No significant differences were detected between sexes and enrichment, but infection status had a statistically significant effect, with SPF fish having significantly higher body weights ($P < 0.0001$) than PNI fish did (Figure 3). Mean body lengths for females were 3.0 ± 0.1 cm (SPF EE), 2.6 ± 0.2 cm (SPF no EE), 2.1 ± 0.1 cm (PNI EE), 2.1 ± 0.01 cm (PNI no EE). Mean body lengths for males were 3.0 ± 0.0 cm (SPF EE), 2.9 ± 0.0 cm (SPF no EE), 2.4 ± 0.1 cm (PNI EE), and 2.3 ± 0.1 cm (PNI no EE) (Figure 4 and Figure 5). Enrichment type, sex, and infection status were all significantly affected body length. SPF fish were significantly longer than PNI fish ($P < 0.0001$), fish housed with EE were significantly longer than fish housed without EE ($P = 0.016$), and male fish were significantly longer than female fish ($P < 0.0001$).

Histopathology. H and E and Ziehl–Neelsen-stained slides were evaluated from 12 PNI zebrafish and 6 SPF zebrafish euthanized at the end of the study. Aggregates of *P. neurophilia* spores were observed in 12/12 (100%) of PNI zebrafish (See Figure 6). The severity of *P. neurophilia* infection varied among individual fish, ranging from a focal aggregate of *P. neurophilia* in the hindbrain to multifocal infection of multiple tissues, including hindbrain, spinal cord, vertebral column, and skeletal muscle, accompanied by mild to marked lymphohistiocytic infiltrates. In contrast, *P. neurophilia* organisms were not observed in any SPF zebrafish evaluated. No evidence of mycobacteriosis or infection with any agent other than *P. neurophilia* was observed in any zebrafish evaluated by histopathology.

Molecular Analysis. At the conclusion of the study, 12 PNI zebrafish and 24 SPF zebrafish were tested for multiple zebrafish pathogens by real time PCR. *P. neurophilia* was identified by real-time PCR in 12/12 (100%) of PNI zebrafish and 0/24 (0%) of SPF zebrafish at the end of the study. One PNI zebrafish and one SPF zebrafish tested weakly positive for *Mycobacterium chelonae* (both near the lower limit of detection of the assay) at the end of the study. Test results for all other pathogens were uniformly negative.

Discussion

This study compared biometrics, anxiety-like behaviors, and fecundity between PNI and SPF zebrafish that were group-housed, with or without artificial plants as EE. PNI zebrafish displayed adverse outcomes, including smaller body size and poor reproduction as compared with SPF fish. The disparity in numbers of tanks between SPF ($n = 24$) and PNI ($n = 12$) fish was due to more SPF fish surviving to adulthood than did PNI fish. Furthermore, fish housing density was impacted by *P. neurophilia* infection and subsequent loss of fish. This difference may have introduced an unplanned variable into our results because housing density was reduced by fish mortality due to *P. neurophilia* infection. EE increased fish growth but did not affect fecundity. Body length varied by sex, with male fish exhibiting longer body lengths than female fish. Zebrafish normally show very little sexual dimorphism, other than coloration and shape.[30]

In the NTT, the PNI fish had higher frequencies of entry to the bottom of the tank than did the SPF fish, but the SPF fish

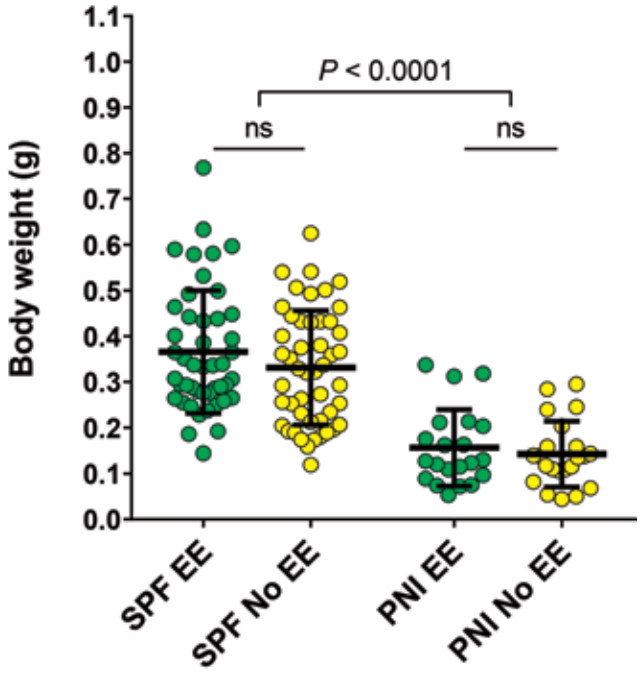

Figure 3. Body weights at end of study. Dots= individual fish, mean = dark line ± SD (lighter error bars). $P < 0.0001$ between weights for SPF compared with PNI.

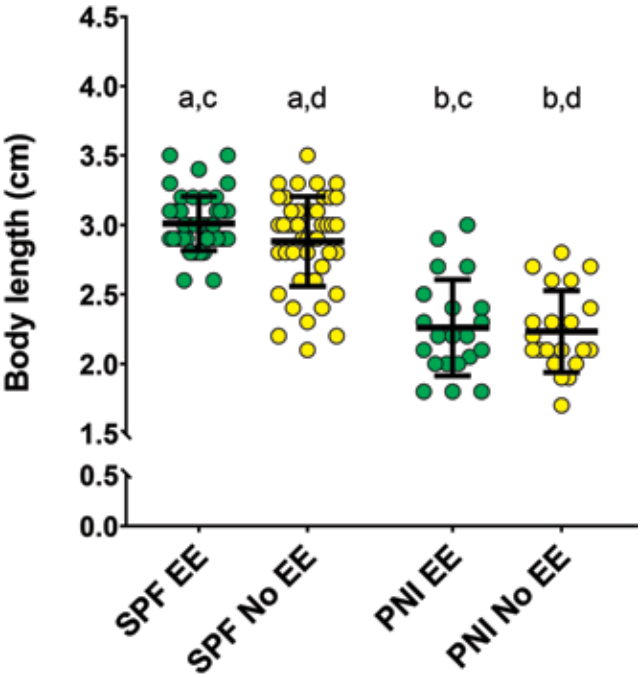

Figure 4. Body lengths at end of study. Groups = SPF EE, SPF no EE, PNI EE, PNI no EE. Mean ± SEM a, b = $P < 0.0001$ between lengths for SPF compared with PNI. c, d = $P = 0.016$ for EE fish compared with no EE. $P < 0.0001$ males compared with females (not shown).

spent more time at the bottom of the tank than PNI fish. So, we found that for NTT, both PNI and SPF fish displayed anxiety-like behaviors in terms of frequency of entries into the bottom zone of the tank and duration of time spent at the bottom of the tank, respectively.

In the LDT, SPF fish had higher frequencies of entry into the dark side of the tank and the SPF EE fish spent more time in the dark side of the tank, suggesting greater anxiety-like behavior for both groups. This finding contrasts with a recent report that infection status did not affect the time spent in the tank's light or dark side.[21] However, that study also found that infected fish had a significantly reduced number of crossings between compartments, which they attributed to overall decreased locomotion by infected fish.[21] In our study, infection status had some effect on the behavioral measures, but we cannot confirm that PNI fish showed more anxiety-like behavior based on more than one measure in one of the 2 tests.

The differences in anxiety-like behavior were varied between fish housed in tanks with EE or with no EE. In the NTT, fish with EE had a higher frequency of entry to the top of the tank than did fish housed without EE, regardless of infection status. This behavior suggests reduced anxiety-like behavior in fish housed with EE and is similar to a study that found fish with EE spent more time in the top part of the tank than did fish with no EE.[17] However, in the LDT, fish housed with EE had more frequent entries into the dark side of the tank, consistent with greater anxiety-like behavior in the EE groups. While behavioral assays revealed mixed results with respect to anxiety-like behaviors in fish in the EE groups, they indicate that the presence of EE in housing tanks can affect outcomes in commonly used zebrafish behavioral assessments. However, the type of behavior that was affected differed between the groups in that fish with EE appeared less exploratory. When fish housed with EE were moved to behavioral testing tanks without EE, they showed behavior that could be interpreted as alarm-like. Although the present study did not evaluate the effect of EE in individually housed fish, our findings support a previous study that found increased anxiety-like behavior in singly housed fish without EE, but not in group housed fish, whether or not the group housed fish had EE in their tanks.[8] A potential explanation for these findings is that social enrichment may have greater effects on anxiety-like behaviors than does physical enrichment. Because all fish in our study were socially housed, the social enrichment may have masked the effect of the physical enrichment provided in the form of the plastic plants.

In the present study, SPF fish produced significantly more eggs than did PNI fish. This finding is in contrast to a previous study that did not find a significant effect on mean number of eggs laid by infected and uninfected fish.[24] The fecundity seen in the present study is overall lower than previously published studies of AB line reproduction.[33] Another study surveyed the data from multiple labs and found that the range from the 5 labs using the AB line was an average clutch size of 63 to 282,[4] compared with our average of 14 to 21 eggs for the SPF fish. However, our spawning success of 62% to 73% in the SPF fish is comparable to that found in a study conducted by others.[4] The older age of the fish in our study could have been a contributing factor for the lower reproductive values. Because the study design included experimental infection and the assessment of behavior in adults prior to breeding, we started breeding our fish at 244 dpf and continued breeding for 6 wk, which is much older than breeding zebrafish in another study.[3] Another possible contributing factor is the amount of time the male and female were kept together in their breeding tanks before egg collection. For our study, breeding fish were housed in breeding tanks for a shorter time (30 min) rather than the 2 h periods used in another study.[3] This shorter time was used based on our experience collecting embryos from other breeding zebrafish at our institution's animal facility.

EE had no significant effect on breeding performance, regardless of whether that EE was in the housing tanks or in the breeding tanks. In contrast, another study found that EE in

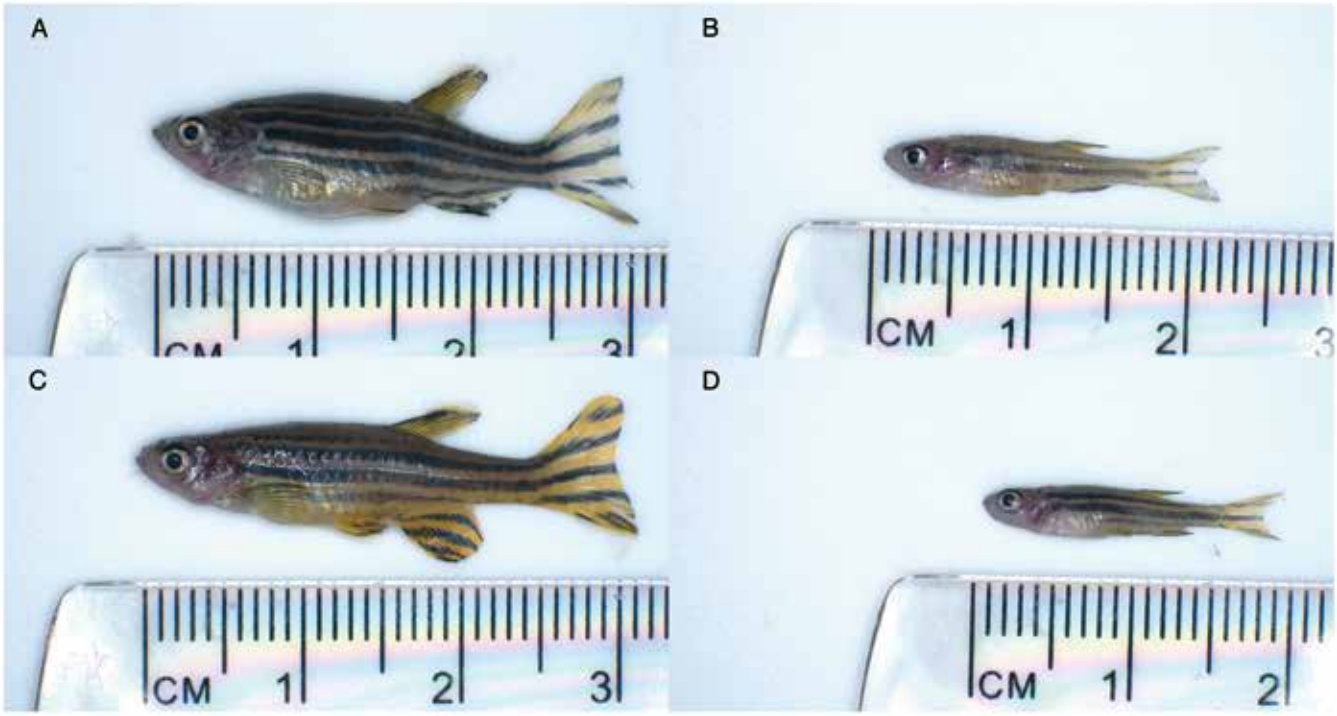

Figure 5. Examples of fish at end of study. (A) female SPF EE. (B) female PNI EE. (C) male SPF EE. (D) male PNI EE. (The no EE groups are not shown.) SPF length > PNI ($P < 0.0001$), EE length > no EE ($P < 0.016$), male length > female ($P < 0.0001$).

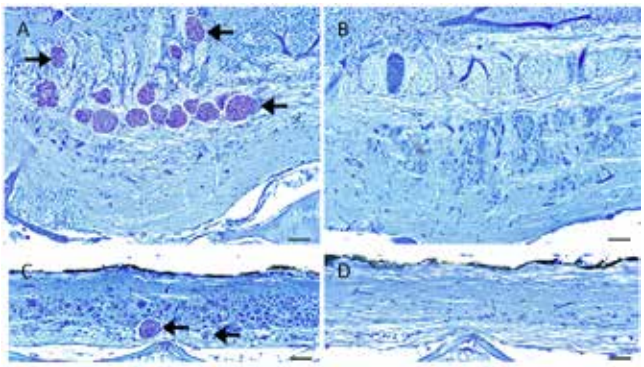

Figure 6. Histologic sections of zebrafish central nervous system tissue demonstrating *Pseudoloma neurophilia* infection status. (A) Sagittal section of brain (rhombencephalon) displaying multifocal aggregates of *P. neurophilia* organisms including spores (arrows). (B) Sagittal section of uninfected brain (rhombencephalon). (C) Sagittal section of spinal cord displaying multifocal aggregates of *P. neurophilia* organisms (arrows) including spores. (D) Sagittal section of uninfected spinal cord. Ziehl–Neelsen acid-fast stain; scale bar, 50 mm.

breeding tanks improved egg count.[33] That study also found that grass enrichment in breeding tanks resulted in a higher egg count than either leaf enrichment or no enrichment in the breeding tanks; our study used a plastic leaf-like enrichment. A grass-type of enrichment might have improved reproductive measures. More studies examining different types of enrichment and their various effects on zebrafish are needed.

Infection status had a significant effect on biometric data collected at the end of the experiment. SPF fish weighed more and were longer than PNI fish, indicating a detrimental effect on growth in fish that are experimentally infected with *P. neurophilia.* Similar results were found in a previous study in which fish of the TL line experimentally infected with *P. neurophilia* weighed significantly less than control TL line fish, and that fish of the AB line were generally smaller in the infected group than in the uninfected group.[24] Another publication described a decrease in body mass and body length in infected fish,[21] similar to our results. A more recent study examining the body condition of AB line fish after exposure to *P. neurophilia* reported a significant decrease in body condition in females only.[25] Our study also found a sex difference, as males had longer body lengths than females, regardless of infection status or housing condition. An additional biometric outcome in our study was that EE groups had significantly longer body lengths than groups without EE. A previous study found that both sex and EE affect body condition and that female fish with EE had a higher body condition score than did females without EE, but no difference in body condition was observed in male zebrafish.[17] Similarly, our results suggest that EE may have a positive effect on fish growth.

Histopathologic and molecular analyses confirmed the infection status of all PNI fish examined and showed an absence of infection with *P. neurophilia* or other pathogens in the SPF fish, except for 2 samples that were weakly positive on real-time PCR for *Mycobacterium chelonae* (one SPF sample and one PNI sample). *Mycobacterium chelonae* is a common facultative pathogen that readily establishes itself in biofilms on surfaces in aquatic systems, but typically causes chronic subclinical infections in zebrafish and is thus not considered one of the more pathogenic species of mycobacteria in zebrafish.[34] Although the histopathology results did not show any evidence of mycobacteriosis in other fish, the presence of subclinical infection cannot be completely excluded. However, because *M. chelonae* was detected in both SPF and PNI samples, its low prevalence could be presumed across all groups in this study and as a result it is considered unlikely to have significantly affected results between groups in this study.

In summary, histopathologic and molecular analyses confirm that SPF fish remained free of infection with *P. neurophilia* and all infected tanks (PNI) were positive for the pathogen. For the NTT and LDT, both infection status and EE affected the

behavioral measures of anxiety, but the results were mixed and we cannot conclude that either infection or housing without EE increased anxiety-like behavior in the tests we performed. Fish infected with *P. neurophilia* had reduced fecundity, body weight and body length. EE in the housing tank or in the breeding tank did not significantly affect egg count, but zebrafish with EE in their housing tanks had longer body lengths. Male fish had longer body lengths than female fish, and SPF fish had greater body weights and longer body lengths than did PNI fish. The biometric results and reproductive measures show that fish not infected with *P. neurophilia* had better growth and development and suggest that EE in housing tanks could increase fish growth. PNI fish appeared to show effects on processes that are energetically costly, such as growth, locomotion, and fecundity, and another study recommended further research on the effect of infection on other costly biologic processes, such as immune response.[21] Our results support other recent studies outlining the adverse effects of *P. neurophilia* infection on zebr afish.[2,13,15,21,22,24,28,29] The many potential unintended effects in infected fish argue for efforts toward the eradication of *P. neurophilia* in laboratory zebrafish.

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Competing interests

Marcus J Crim is an employee of IDEXX BioAnalytics, a division of IDEXX Laboratories Inc., a company that provides veterinary diagnostics.

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