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In vitro immune function in laboratory-reared age-0 smallmouth bass (*Micropterus dolomieu*) relative to diet

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ABSTRACT

Smallmouth bass (*Micropterus dolomieu*) are used as an indicator species in environmental monitoring and assessment studies. However, laboratory-based studies for methods development and effector assessment are limited for this species. Nutrition, a known modulator of teleost physiological responses including immune function, is a critical knowledge-gap sometimes overlooked in the design of laboratory studies. We report the results from a study evaluating a commercially available artificial pelleted diet for bass and live feed (fathead minnows). Following a six-month diet-acclimation period, age-0 smallmouth bass were assessed using morphometric measures, histologic and immune-function end points using conventional methods, miniaturized cell isolation and assay methods as well as imaging flow cytometry. Fish on the two diets did not significantly differ in length, weight, or condition factor, indicating that growth was similar in the two groups. Histologic examination revealed relatively higher levels of macrophage aggregates and accumulation of ceroid/lipofuscin in the spleen as well as hepatocyte changes in the pellet-fed group. Leukocytes from the pellet-fed group exhibited significantly elevated bactericidal activity and significantly depressed mitogen response compared to fish fed live feed. Following exposure to a known inducer of inflammatory responses, bacterial lipopolysaccharide, responses including the transition of leukocytes to an apoptotic/necrotic condition differed significantly based on diet. Histologic findings were consistent with the occurrence of diet-related oxidative stress in the pellet-fed fish. Oxidative stress can be induced by multiple factors including environmental pollutants. For a diet to be useful in laboratory-based studies, it cannot elicit response that could also be induced by experimental treatments. To do so greatly complicates the detection of experimental effects. Until an artificial diet is developed for smallmouth bass that does not produce potentially confounding conditions for laboratory-based studies, use of a live feed appears to be the best option.

1. Introduction

Smallmouth bass (*Micropterus dolomieu*) are an economically important sportfish throughout their native range as well as within areas of the Chesapeake Bay where they were introduced in the 1800s [1,2]. Skin lesions and mortality of adult and young-of-year smallmouth bass have occurred throughout the Potomac and Susquehanna Rivers (Chesapeake Bay watershed, USA), raising concerns of the public and management agencies about both the fishery and ecosystem health of these watersheds [3–5]. Co-infections of bacterial, viral, as well as parasitic infections have been associated with these events [4,6,7] suggesting immunosuppression. Estrogenic endocrine disruption as evidenced by testicular oocytes (intersex), vitellogenin in male fish [8–10] and the occurrence of contaminants linked to intersex [4,7,11,12] has been confirmed within the areas of the Chesapeake watershed where disease events occur. The observation of a complex disease etiology that includes multiple obligate and opportunistic pathogens and parasites as well as endocrine disruption necessitates laboratory investigations into the potential changes in physiologic functions including the immune response.

Assessments of immune function using laboratory-held fish are valuable for both methods' development and effector analysis. When the intent, however, is to apply results from these studies to wild populations some consideration needs to be given to approximate natural conditions in the laboratory setting. Holding protocols that fail to provide such conditions risk altering or masking responses to such an extent as to make acquired data irrelevant. While developing the criteria for maintaining fish for this purpose, a major issue that needs to be

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addressed is nutrition. The relationship between teleost nutrition, general health, and immune function is well established [13–15].

Smallmouth bass, an apex predator, are not commonly cultured on artificial feeds and little research has been directed toward nutritional requirements. Here, we evaluate the impacts of feeding smallmouth bass either an artificial pelleted diet formulated for largemouth bass or live fathead minnows (*Pimephales promelas*). Diet effects are determined using morphometric measures, histology and multiple measures of immune function as endpoints.

2. Materials and methods

2.1. Laboratory smallmouth bass

In November 2016, 400 age-0 smallmouth bass were obtained from Schultz's Fish Hatchery (Lake Ariel, PA). Two hundred fish were trained to accept an artificial pelleted-diet, while the other 200 were reared on live feed. Fish were housed indoors at the U.S. Geological Survey (USGS) Leetown Science Center (Kearneysville, WV) in 1287 L circular tanks (n = 100 per tank initial density). Tanks were supplied with flow-through spring water heated to an in-tank temperature of 20 °C using a heat exchanger located outside the holding facility. Typical total tank-volume replacement occurred 5x daily. Tank illumination was provided by a combination of natural and fluorescent light with photoperiod matching natural light supply. Initial average weight of fish was 9.8 g (single measure divided by number of fish in measure). One tank of each feed type was used for this study.

The pellet-fed smallmouth bass were initially fed a 0.8 mm slow sink pelleted feed containing 50% protein and 15% fat formulated for bass (Melick Aquafeed; Catawissa, PA) and then transitioned to a 1.0 mm diet of the same formulation when fish sized increased. The feed was delivered using a Baby Belt Feeder (Pentair Aquatic Ecosystems, Inc.; Apopka, FL) with feed evenly dispensed throughout daylight hours. Target feed rates were approximately 4% body weight per day. The live-fed fish were initially fed small (~1 g) fathead minnows supplied by Schultz's Fish Hatchery and obtained at the same time as the smallmouth bass. Subsequent supplies of disease-free fathead minnows were obtained from Anderson Minnows (Lonoke, AR). As was the case with the feed rates with the pelleted diet, minnow feed rates were approximately 4% base body weight per day. Bass were fed minnows every day for the first two months and then switched to feeding every third day to provide a larger number of minnows during a single feeding. This provided an opportunity for all fish within this diet group to feed and was necessary to prevent the most aggressive feeders from consuming the bulk of the minnows. Actual feed rates varied because of size differences in individual bass as well as differences in individual feeding performance. Feed rates for individual smallmouth bass were not determined. Minnows were obtained from the supplier approximately every three weeks, fed the same 0.8 mm pellet diet fed to the smallmouth bass and held in the same room in a tank supplied by flow-through spring water at 13–15 °C. Dead as well as moribund smallmouth bass, as indicated by dark appearance and atypical swimming behavior, were euthanized, weighed and assessed by histologic examination. Smallmouth bass were acclimated to holding conditions and feeding regime for six months prior to sampling in May 2017.

2.2. Smallmouth bass sampling

Fish were euthanized with an overdose (250 mg L^{-1}) of tricaine methanesulfonate (Tricaine-S, Western Chemical Laboratories, Ferndale, WA) immediately upon removal from the tanks and processed as follows: the fish were measured for total length, weighed and bled from the caudal vessels; anterior kidney was collected using aseptic technique and processed for leukocyte isolations as described below. The remaining tissues were placed in Z-fix (Anatech Ltd; Battle Creek, MI) for fixation, processed, embedded into paraffin, sectioned at 5 μm and stained with hematoxylin and eosin [16]. Fulton's condition factors were determined for individual fish using the equation $K = W/L^3$, where K = condition factor, W = weight of the fish, and L = length of the fish [17]

2.3. Anterior kidney leukocytes

Anterior kidney leukocytes isolation was performed using modification of the methods previously described [18]. Methods were modified to increase the efficiency of leukocyte isolation from the small amount of anterior kidney available in the age-0 fish. Unless otherwise indicated, reagents and media components were obtained from Millipore Sigma (Burlington, MA). Immediately following excision, anterior kidney tissues were placed in 5 ml of cold L-15 media supplemented with 290 μg ml^{-1} L-glutamine, 10 units ml^{-1} sodium heparin, 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2% (vol/vol) fetal bovine serum (FBS, characterized U.S. origin, Hyclone (GE Life Sciences; Logan, UT); supplemented media designated L-15/2%). Media and tissues were transferred to a 7 ml manual Tenbroeck tissue grinder to produce a cell suspension and the tissue grinder was then rinsed with an additional 5 ml of cold L-15/2%. The resulting suspension was held overnight at 4 °C prior to leukocyte isolation (12–16 h hold times). This hold time was employed to simulate that used in on-going studies involving wild smallmouth bass captured in remote areas. In the case of the wild fish, the hold time was related to time required for transport. Such hold times have been previously reported in studies involving white perch (*Morone americana*) [19] and were shown to have no impact on smallmouth bass leukocyte yield or viability in pilot studies. Cell suspensions were pelleted by centrifugation at 500 RCF for 10 min at 4 °C. Samples were resuspended in 2 ml L-15/2% and centrifuged as above. After this wash step, cells were resuspended in 2 ml of L-15/2% and then layered on top of 2 ml of 32% percoll in Hanks balanced salt solution without phenol red. Cells on percoll were centrifuged for 15 min at 500 RCF at 4 °C. Purified leukocytes were harvested from the media/percoll interface and centrifuged as described above for 2 min. Isolated leukocytes were resuspended in L-15/2% for counting. Total viable leukocyte counts were performed using trypan blue exclusion and a Countess II automated cell counter (Thermo Fisher; Waltham, MA). Cell counts were performed in triplicate and the final count was the average of the three. Countess II viable-cell-counts were optimized for smallmouth bass leukocytes prior to use in this study. Final instrument settings were validated using parallel cell counts from a propidium iodide exclusion assay with data acquisition performed using a Flow Sight (Amnis/Luminex; Austin, TX) image-based flow cytometer (data not shown).

2.4. In vitro immunoassays

Anterior kidney leukocytes from smallmouth bass on the two diets were evaluated for mitogenesis and bactericidal activity. Individual cell yields from single groups of fish were insufficient to meet the requirements of both assays; therefore, leukocytes used in the bactericidal and mitogenesis assays came from two sets of fish sampled on separate days.

To reduce cell number requirements, all assays were performed in Falcon 384-well Tissue Culture Plates (Corning Cat. No. 353961; Thermo Fisher). Following viable leukocyte counts, cells used in the bactericidal assays were resuspended in L-15 media containing 290 μg ml^{-1} L-15 media containing 290 μg ml^{-1} L-glutamine and 0.1% (vol/vol) FBS (L-15/0.1%). The L-15/0.1% was replaced with L-15 media containing 290 μg ml^{-1} L-glutamine and 5% (vol/vol) FBS (L-15/5%) following 2 h of incubation. The incubation with L-15/0.1% was performed to select for highly adherent cells, such as macrophages, specifically targeted in the bactericidal assay [18,20]. Specific leukocyte selection was not desired for cells used in the mitogenesis assays thus these cells were directly resuspended in L-15/5%. Viable-leukocytes were resuspended at 1×10^7 viable leukocytes ml^{-1}. Initial

plating volumes were 25 µl well^{-1} (bactericidal assays) or 12.5 µl well^{-1} (mitogenesis assay). All plates were incubated at 20 °C in humidified containers opened to allow for air exchange.

2.4.1. Bactericidal activity

Adherent leukocytes were evaluated as previously described [20]. A brief description of this method is as follows: target bacteria were incubated in the presence or absence of adherent leukocytes and the number of bacteria remaining following incubation was enumerated; The ratio of colony forming units (leukocytes present: leukocytes absent) was subtracted from 1 and expressed as a percentage (percent bactericidal activity); Viable adherent cell counts, determined from wells that did not receive bacteria, were applied to the calculation of corrected bactericidal activity, which was determined as follows: (percent bactericidal activity x (1/viable leukocytes)). *Aeromonas sobria* (American Type Culture Collection #43979), a known pathogen of age-0 smallmouth bass [6], was used as the target bacteria in all assays. Final leukocyte counts used for the correction of bactericidal activity were performed using trypan blue exclusion and the Countess II automated cell counter as described above. Wells receiving bacteria were replicated in duplicate. Final cell counts were based on individual wells.

2.4.2. Mitogenesis assay

Leukocyte mitogenesis assays were performed based on modifications of the methods previously described [21] using mitogens obtained from Millipore Sigma. All mitogens were diluted in L-15/5%. The anterior kidney leukocytes were treated at 12.5 µl well^{-1} with either concanavalin A (CON A) at 10 µg ml^{-1} (0.25 µg well^{-1} final concentration), phytohemagglutinin – P (PHA-P) at 20 µg ml^{-1} (0.5 µg well^{-1} final concentration), lipopolysaccharide (LPS, from *E. coli* O111:B4) at 100 µg ml^{-1} (2.5 µg well^{-1} final concentration), or mitogen-free media (control wells). Total incubation time with mitogens was 48 h. Cell replication was detected using Click-iT® EdU (5-ethynyl-2′-deoxyuridine) Assay Kits (Molecular Probes) following a modification (correction for assay scale) of the manufacturer's protocol (MP 10419). Six- µl well^{-1} of EdU in L-15 (3.12 µM well^{-1}) was added to mitogen treated and control wells after 30 h of incubation. Following an additional 18 h of incubation, culture supernatants were removed from all wells and leukocytes were washed with 25 µl well^{-1} of solution consisting of 1% bovine serum albumin (BSA) in Dulbecco's phosphate buffered saline (DPBS/1% BSA). The DPBS/1% BSA was removed from the wells and replaced by 6 µl well^{-1} of Click-iT® fixative. Leukocytes were fixed for 15 min at room temperature. Cells were washed as described above and then 25 µl well^{-1} of Click-iT® saponin-based permeabilization and wash reagent was added. Permeabilization proceeded for 15 min and 25 µl well^{-1} of DPBS-based Click-iT® reaction cocktail containing Alexa Fluor® 647 azide was then added. Labeling of integrated EdU occurred at room temperature over 30 min. Leukocytes were washed with 25 µl well^{-1} of Click-iT® saponin-based permeabilization and wash reagent and then 30 µl well^{-1} of this same reagent was added in preparation for analysis using the Flow Sight imaging flow cytometer.

Flow Sight instrument settings were as follows: Illumination – 642 laser at 100.0 mV, 785 laser at 5.00 mV; Fluidics – minimum flow speed. Cell analysis was performed without compensations (single fluorescence channel employed) using IDEAS version 6.2.

(Amnis/Luminex) as shown in Fig. 1 and Fig. 2. Primary gating was performed on initial cell-image data to identify cells in focus (region 1) and to identify individual round cells (region 2). Region 2 was then divided further into three regions based on cell diameter and dark field (side scatter) intensity (regions 3, 4, and 5). Cell diameters ranged from 6 to 12 µm and were grouped relative to side scatter using cell density distributions. Replicating cells were identified based on fluorescence intensity associated with the incorporation and subsequent detection of EdU with detection threshold established based on histogram distribution and on the minimum intensity at which target-specific fluorescence could be observed in individual cells. Apoptotic/necrotic cells were defined using cell diameter and side scatter as previously described [22]. Specific markers for apoptotic and necrotic cells were not employed so no differentiation between the two processes was made. Raw mitogenesis data were expressed as the number of EdU-positive cells occurring in regions 3, 4 and 5. All mitogenesis data were evaluated using the same analysis template to provide consistency across samples and treatments.

Final analysis of the fish-specific stimulation indices was produced using the ratio of EdU-positive mitogen-treated leukocytes to EdU-positive negative control leukocytes from the same population. The number of EdU-positive cells was corrected to account for the total number of cells in the region in which they occurred. The region-specific calculation with correction was made using the following formula:

$$\frac{((\textit{Mitogen Treated Rx Rep Pos Cells})/(\textit{Mitogen Treated Rx Total Cells}))}{((\textit{Negative Control Rx Rep Pos Cells}/(\textit{Negative Rx Control Total Cells}))}$$

Where Mitogen Treated Rx Rep Pos Cells is the number of mitogen-treated (CON A, PHA-P or LPS) EdU-positive cells in regions 3, 4 or 5; Mitogen treated Rx Total Cells is total number of mitogen (CON A, PHA-P or LPS) treated cells in regions 3, 4 or 5; Negative Control Rx Rep Pos Cells is the number of EdU-positive negative control cells in region 3, 4 or 5; and Negative Control Rx Total Cells is the total number of negative control cells in region 3, 4 or 5. Observed cell proliferation in the negative control cells was expressed as the percent of EdU-positive cells relative to the total number of cells in the region. Total measures for stimulation indices and percent negative control cell proliferation were calculated using the same equation except that the values for regions 3, 4, and 5 were combined.

2.5. Statistical analysis

Preliminary analysis of all measures (Table 1) was performed using univariate analysis of variance (ANOVA) with Tukey post hoc comparisons. Measures that were found to be significant were then incorporated into discriminate analysis models to determine which could be used to fulfill model assumptions and which were most useful for placing individual into the diet group from which they came. Where necessary, measures were removed based on canonical discriminant functions with stepwise removal of individual measure going from the lowest value upward (step-wise analysis). Total correlations were used to evaluate the potential relatedness of individual measures with values ≥ 0.6 or ≤ -0.6 further assessed using regression analysis. Analysis of variance and regression results were rejected if assumptions of normality or constant variance were not met or if values for the Durbin-Watson D Statistic were outside the range of 1.5–2.5. Analysis of variance and discriminate analysis were performed using Systat 11 (SYSTAT Software Inc., Richmond, CA). Regression analysis was performed

Fig. 1. Typical images of a single smallmouth bass anterior kidney leukocyte from mitogenesis assay with bright field (Ch01), dark field – side scatter (Ch06), AF 647 labeled 5-ethynyl-2′-deoxyuridine associated fluorescence (Ch11) and composite imaging using data from Ch01, Ch06, and Ch11. Data captured using a Flow Sight (Amnis/Luminex) imaging flow cytometer and analyzed using Ideas version 6.2 (Amnis/Luminex). Image masking employed in Ch01, Ch06, and Ch11 using 89% adaptive erode. Artificial coloring used to aid in visualization.

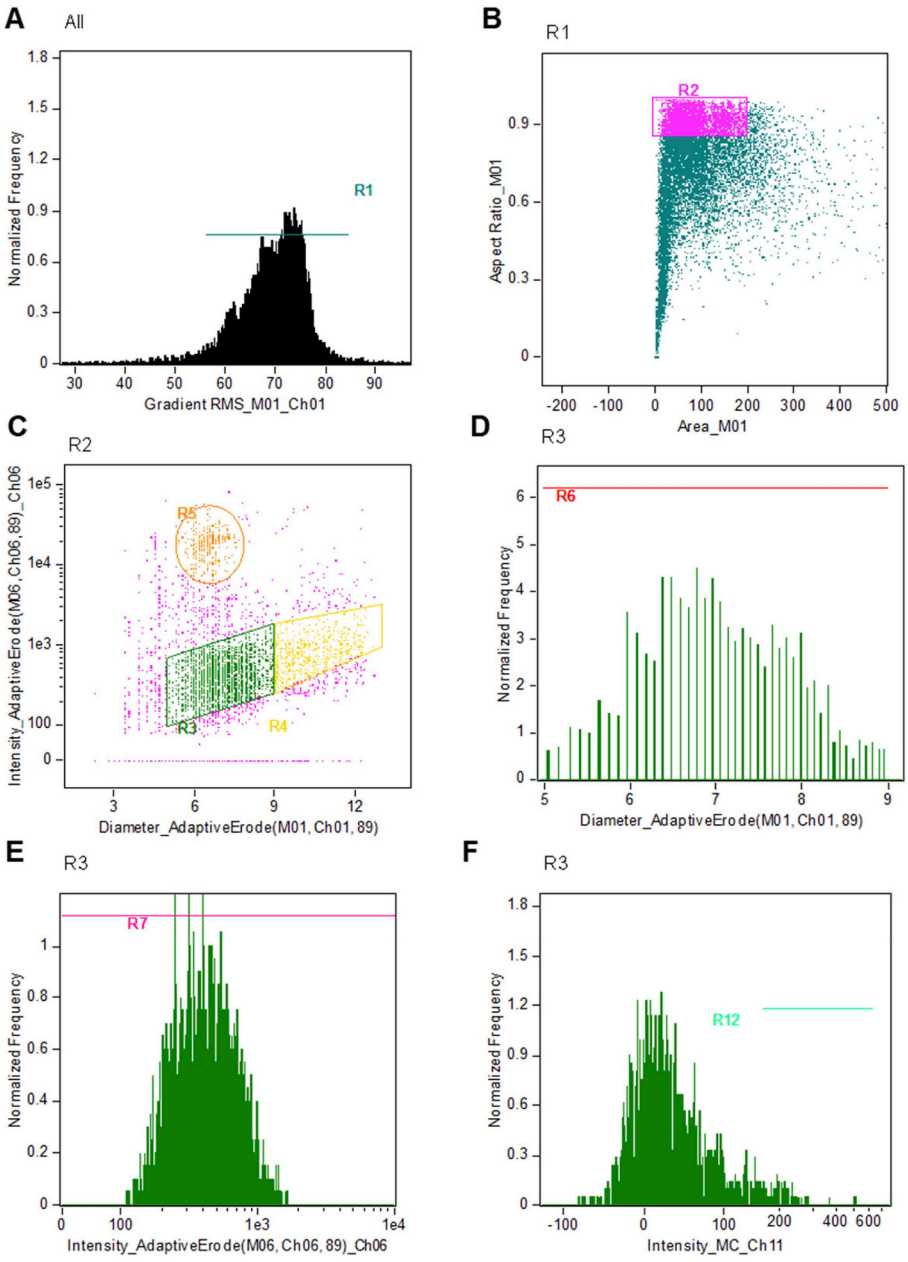

Fig. 2. Data analysis work flow for anterior kidney leukocytes of smallmouth bass assessed for mitogen response using imaging flow cytometry data sets: 1) Cells in focus identified – Region 1 (Ch01, A); 2) Individual, round cells identified from R1 – Region 2 (Ch01, B); Region 2 cell gated based on diameter and side scatter – Regions 3, 4, 5 (C); 3) Median diameter determined for Region 3 cells (Ch01, D); 4) Median side scatter determined for Region 3 cells (Ch06, E); 5) Replication positive cells (AF 647 labeled 5-ethynyl-2′-deoxyuridine (EdU) incorporation) identified in Region 12 (Ch11); 6) Median diameter, side scatter and replication positive cells identified for Regions 4 and 5 as in C, D, and E; 7) Median diameter and side scatter determined for replication positive cells from Regions 3, 4 and 5 as in C and D. Data analysis performed using Ideas version 6.2 (Amnis/Luminex). Values determined for diameter, and side scatter based on image masking (adaptive erode; 89%). Minimum threshold for EdU positive cells based on histogram distribution (F) and visualization of fluorescence in individual cell images (Fig. 1). Channel assignments: Ch01 – bright field; Ch06 – dark field, side scatter; Ch11 – far red fluorescence (642 nm excitation laser).

Table 1
Immunoassay measures modeled for potential diet effects using univariate analysis of variance to identify potentially relevant measures and discriminate analysis to determine the most relevant measures for distinguishing individuals within each diet group. Measures obtained from age-0 smallmouth bass fed an artificial pelleted diet or live fathead minnows.

Assay	Subgroups[a]	Immunoassay Measures
Bactericidal Activity	None	corrected bactericidal activity
Mitogenesis	CON A, PHA-P and LPS treatments R3 and R4 total cells and proliferation positive cells only	median cell diameter, median side scatter, numeric percent of total population, stimulation index
	CON A, PHA-P and LPS treatments R5 all cells and proliferation positive cells only	numeric percent of total population
	Negative Control R3 and R4 total cells and proliferation positive cells only	median cell diameter, median side scatter, numeric percent of total population
	Negative Control R5 all cells and proliferation positive cells only	numeric percent of total population

[a] CON A – Concanavalin A; LPS – Lipopolysaccharide; PHA-P – Phytohemagglutinin-P; R3 – Region 3; R4 – Region 4; R5 – Region 5.

Table 2
Biometric Characteristics of age-0 smallmouth bass fed pelleted or live feed.

Characteristic	Pellet-fed[a]	Live-fed
Weight (gm)	32.8 ± 9.2	31.1 ± 6.4
Length (mm)	146.4 ± 11.9	144.0 ± 9.7
Condition Factor	1.02 ± 0.01	1.03 ± 0.08

[a] Data presented as mean ± standard deviation.

in Sigma Plot 10.0 (SYSTAT Software Inc., Richmond, CA). Test results were considered significant when $p \leq 0.05$. Final statistical analysis was performed using only data from fish from which all relevant measures were obtained.

3. Results and discussion

3.1. Fish and histopathology

During the six-month feeding trial, there was high mortality in the pellet-fed group, particularly during the first three months. Histologic examination of these fish revealed no evidence of infectious disease or parasitic infections. These fish tended to be smaller than other members of the pellet-fed group and the presumptive cause of the morbidity and mortality was failure to feed. Of the initial 100 fish in each group, 64 pellet-fed and 5 live-fed died. A total of 36 pellet-fed and 60 live-fed were sampled. While initial mortality was high in the pellet-fed group, survivors grew well and there was no significant difference between the groups in weight, length or condition factor at the time of sampling (Table 2). As assessed by microscopic pathology, no infectious disease was noted and the major differences between the groups were in spleen and liver tissue (no anterior kidney tissue was available for histopathology). More macrophage aggregates (MA) and accumulation of ceroid/lipofuscin (indicated by presence of yellowish-brown in MA) were noted in splenic tissue of the pellet-fed bass when compared to live-fed as observed in the qualitative assessment (visual inspection) of the tissues (Fig. 3 A and B). Macrophage aggregates are focal accumulations of pigment-containing macrophages in the spleen, anterior kidney and sometimes liver of fishes. An increase in number and/or size has been associated with age, contaminant exposure and other environmental stressors [23,24]. Diet composition has also been shown to impact the occurrence of MA. For example, gilthead seabream (*Sparus aurata*) juveniles exhibited an increase in splenic MA when fed a diet deficient in highly unsaturated fatty acids [25]. Lipofuscin and/or ceroid within MA are derived from peroxidation of cells and organelles. Their presence in MA and other tissues has been shown to increase following exposure to chemicals causing oxidative damage [26,27] as well as due to dietary deficiencies of antioxidants such as vitamin E [28].

Liver tissue of the live-fed bass appeared normal and were comprised of hepatocytes that were highly vacuolated with few to no MA (Fig. 3C). Fish hepatocytes store energy as glycogen and/or lipid, hence highly vacuolated hepatocytes do not necessarily suggest a disease state [29]. The live-fed fish did have to capture their food albeit in a limited space. Conversely, livers from the pellet-fed bass exhibited a range of microscopic changes. Hepatic tissue did not have the normal tubular (cord-like) structure and a few MA were observed (Fig. 3 D). Although livers from the live-fed were highly vacuolated, the cells were of uniform size and nuclei appeared normal (Fig. 3 E). Conversely hepatocytes from pellet-fed bass varied in size and many nuclei appeared shrunken and abnormal (Fig. 3 F).

3.2. Leukocyte yields, viability and use

Leukocytes were isolated from a total of 40 live-fed and 36 pellet-fed fish. Post-isolation cell counts (10^6) for the live- and pellet-fed fish (mean ± S.D.) were 4.05 ± 2.39 and 3.99 ± 2.96 respectively. Post-isolation leukocyte percent viability for the live- and pellet-fed fish (mean ± S.D.) were 96.8 ± 2.3 and 96.5 ± 7.9 respectively. Leukocyte counts ($p = 0.918$) and viability ($p = 0.808$) did not significantly differ based on diet. Insufficient leukocytes were isolated from 13 live-fed and 13 pellet-fed fish to meet all immunoassay requirements, so these fish were excluded from some of the analyses and sample sizes in the final data sets reflect this. The cell isolation methods described here yielded adequate cell counts for the down-scaled assay methods used in most cases; however, this was not the case for all samples, suggesting that a 30 g fish may be the lower size limit for these methods.

3.3. Immune function assessments

3.3.1. Bactericidal activity

Corrected bactericidal activity was significantly higher ($p = 0.009$) in pellet-fed smallmouth bass relative to their live-fed counterparts (Fig. 4). Discriminant analysis correctly assigned individuals into their known diet treatment group in 78% of cases. Assignment of fish in the pellet-fed group was slightly better than the live-fed group and jackknifed values were identical to the primary classification matrix (Table 3).

3.3.2. Mitogenesis

Diet significantly altered the mitogen response of the smallmouth bass. Effects were primarily associated with LPS stimulation, but some effects were also seen with CON A and PHA-P. When total mitogenesis was evaluated, a significant difference was observed in the LPS response with leukocytes from live-fed fish exhibiting significantly more proliferation ($p = 0.043$) than their pellet-fed counterparts (Table 4). A similar relationship was observed with CON A, but the results were not significantly different ($p = 0.065$). Responses to PHA-P stimulation did not significantly differ between diets ($p = 0.170$). The percent replicating cells in negative controls was significantly elevated ($p = 0.003$) in the leukocytes from pellet-fed fish relative to those from the live-fed treatment.

When diet effects were evaluated by individual region, CON A and LPS treatment produced significantly elevated stimulation index values in the R3 and R4 leukocytes of live-fed fish (Fig. 5 A; CON A R3 $p = 0.015$; CON A R4 $p = 0.005$; LPS R3 $p = 0.046$; LPS R4 $p = 0.007$). The negative control (unstimulated) R3 and R4 leukocytes from the pellet-fed fish exhibited significantly more replication than observed in the corresponding live-fed fish (Negative Control R3 $p = 0.027$; R4 $p < 0.001$). The amount of cell proliferation in the R4 leukocytes of live-fed fish was also elevated in the PHA-P treated cells but this difference was not enough to produce a significant difference in stimulation index values ($p = 0.074$). The percent of apoptotic/necrotic-like leukocytes (R5) in the total (replicating and non-replicating) negative control leukocyte population was significantly elevated in the pellet-fed fish (Fig. 5 B; $p = 0.007$). In contrast, the percent population of replicating apoptotic/necrotic-like cells following LPS treatment was significantly elevated in the live-fed fish ($p = 0.015$). Median cell diameters observed in replicating cells from pellet-fed fish were significantly greater in R3 cells following CON A treatment ($p = 0.009$) and in R4 cells treated with LPS ($p = 0.008$) relative to similarly, treated leukocytes from live-fed fish (Fig. 5C). Median side scatter in the replicating R4 leukocytes from live-fed fish was significantly elevated in the PHA-P treated group ($p = 0.021$) relative to the pellet-fed fish but no other significant differences in side scatter were observed (Fig. 5 D). Discriminant analysis correctly placed individuals into their known diet groups in 100% of cases (jackknifed total value of 83% correct; Table 3; Fig. 5). Assignment to the correct diet was better in the live-fed fish (86%) compared to pellet-fed (78%) as indicated by the jackknifed results. Canonical discriminant functions (Fig. 5) indicated that most relevant factors for separating the fish on the two diets were

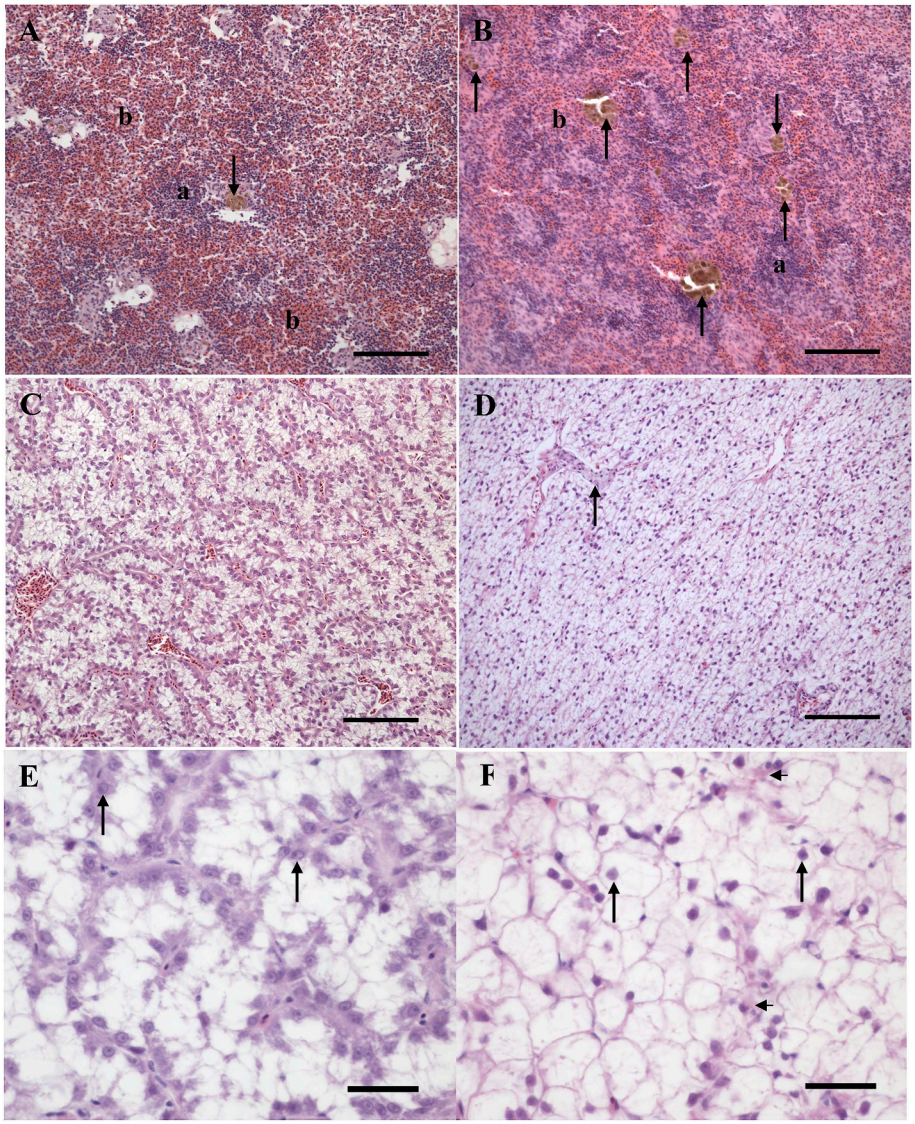

Fig. 3. Microscopic pathology of spleen and liver tissue from artificial pellet-fed and live-fed smallmouth bass. A. Spleen tissue of a live-fed smallmouth bass with normal white (a) and red (b) pulp and few macrophage aggregates (arrow). Scale bar equals 100 μm. B. Spleen tissue of a pellet-fed smallmouth bass with moderate number of macrophage aggregates (arrows). Scale bar equals 100 μm. C. Liver tissue of a live-fed smallmouth bass illustrating the normal tubular structure of hepatocytes which are highly vacuolated. Scale bar equals 100 μm. D. Liver tissue from a pellet-fed smallmouth bass. Normal hepatic structure is not evident, hepatocytes are highly vacuolated and a few macrophage aggregates (arrow) are present. Scale bar equals 100 μm. E. Higher magnification of liver tissue from live-fed smallmouth bass. Hepatocytes are highly vacuolated but of uniform size and with normal nuclei (arrows). Scale bar equals 30 μm. F. Higher magnification of liver from a pellet-fed smallmouth bass. Hepatocytes vary in size and nuclei (arrows) appear small and shrunken. Some cells (arrowheads) contain ceroid/lipofuscin in the cytoplasm. Scale Bar equals 30 μm. Hematoxylin and eosin stain.

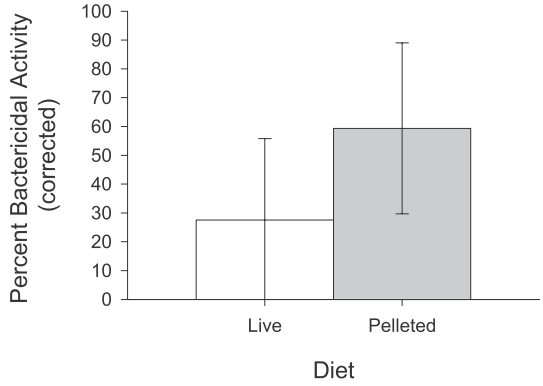

Fig. 4. Effects of live (n = 13) and artificial pelleted (n = 14) diets on corrected bactericidal activity in age-0 smallmouth bass highly-adherent anterior leukocytes. Values expressed as mean ± standard deviation. Measures significantly different at $p \leq 0.05$ as determined by univariate analysis of variance with Tukey post-hoc tests. Discriminate analysis final model contained percent bactericidal activity as single predictor. Model Multivariate Test Statistics: Wilks' lambda, Pillai Trace and Hotelling-Lawley trace: F statistic = 8.116, df = 1, 25, p = 0.0087.

Table 3
Diet-dependent classification matrices for age-0 smallmouth bass determined using standard discriminate analysis based on measures identified as significant relative to diet effects using classic discriminate analysis. Listed parenthetically after each value is the corresponding jackknifed value. Data from fish used in the evaluation of bactericidal activity evaluated separately from that generated in the mitogenesis assay (separate sets of fish).

Assay	Classification Matrix			
Bactericidal Activity[a]	Diet	Live	Pelleted	% Correct
	Live	10 (10)	3 (3)	77 (77)
	Pelleted	3 (3)	11 (11)	79 (79)
	Total	13 (13)	14 (14)	78 (78)
Mitogenesis[b]	Diet	Live	Pelleted	% Correct
	Live	14 (12)	0 (2)	100 (86)
	Pelleted	0 (2)	9 (7)	100 (78)
	Total	14 (14)	9 (9)	100 (83)

[a] Multivariate statistics as per Fig. 3.
[b] Multivariate statistics as per Fig. 4.

LPS-associated stimulation indices in R3 and R4 as well as the percent of apoptotic/necrotic-like cells in the total leukocyte populations. When treatment data were pooled, a negative association was observed between replicating leukocytes in negative control R4 and stimulation indices from CON A R4, LPS R3 and LPS R4 (Fig. 6 A). Results from live-

Table 4
Region independent (total) mitogenesis and negative control percent replication positive results for combined values for regions 3, 4, and 5 obtained from anterior kidney leukocytes of age-0 smallmouth bass fed an artificial pelleted diet or live fathead minnows. Asterisk indicates a statistically significant difference as determined using analysis of variance with Tukey pot-hoc comparison.

Treatment[a]	Pellet-fed[b]	Live-fed
CON A	0.43 ± 0.21	0.72 ± 0.51
PHA -P	0.76 ± 0.36	1.41 ± 1.42
LPS	0.43 ± 0.17	2.04 ± 2.22*
Negative Control	11.34 ± 4.08	6.95 ± 2.70*

[a] Abbreiviations as per Table 2.
[b] Data presented as mean ± Standard deviation

and pellet-fed fish exhibited little overlap because pellet-fed fish exhibited higher replication in negative control/unstimulated R4 leukocytes. A similar relationship was also observed with CON A R3 but the value for the Durbin-Watson D Statistic (1.2454) failed to fall within the acceptable range. Using this same pooled data set, positive associations were observed between the percent of apoptotic/necrotic-like cells (R5) in the negative control total cell population and the percent of replicating cells in negative control R3 and R4 (Fig. 6 B), but the same was not observed for any of the treated groups. The relationships exhibited in the pooled data strongly suggest that the observed differences in mitogen-associated stimulation indices were the result of unstimulated/background cell replication and that a larger percentage of replicating leukocytes from pellet-fed fish were transitioning into an apoptotic/necrotic-like life phase. The latter should partially offset the impact of high background cell proliferation on stimulation index because the calculations were based on direct comparisons to specific cell regions and higher relative numbers of apoptotic/necrotic-like cells reduce the number of replicating negative control cells in R3 and/or R4. Any impact this transition may have had was not enough to prevent the detection of significant differences in mitogen stimulated proliferation. Similarly, the identification of unstimulated cell replication as a discriminatory factor was not impacted by the transition to apoptotic/necrotic like cells.

Detailed leukocyte typing, and cell-cycle analysis was beyond the scope of this study because validated specific reagents and methods for these processes are lacking for smallmouth bass. Imaging flow cytometry is used here in the place of other detection methods (i.e. microplate readers) for the detection of leukocyte replication. The added benefit of using imaging flow cytometry is the additional information gained on individual cell diameter and side scatter. These data allow for analysis based on regions and thus create the potential for additional resolution in the mitogenesis measures. Leukocytes were considered as a single group of cells with subsets that responded differently to specific mitogens and divisions made based on cell diameter and side scatter only (R3, R4, and R5). Treatment of leukocytes as a generalized population limits analysis specificity; however, even in the absence of specific subtype and cell cycle data, some basic interpretation is possible based on long established cell-cycle norms and the commonalities known to occur in teleost leukocyte ontogeny across multiple taxa [30–32].

The relatively higher CON A response in live-fed fish should produce more small daughter cells. An overall median cell diameter decrease in R3 was observed which would be consistent with this hypothesis. However, the LPS mitogenic response in the live-fed fish was significantly higher but no difference in R3 was observed like that seen with CON A. The median diameter of replicating R4 cells from pellet-fed fish was significantly higher than that observed in the live-fed fish which is opposite of what might be anticipated. This, however, assumes homogeneity in responses to different mitogens and that diet-related impacts are limited to the cell-cycle process. The elevated R4 side scatter in the PHA-P stimulated leukocytes from the live-fed group is also difficult to interpret. The elevation could be the result of the greater cell complexity resulting from mitosis in association with the

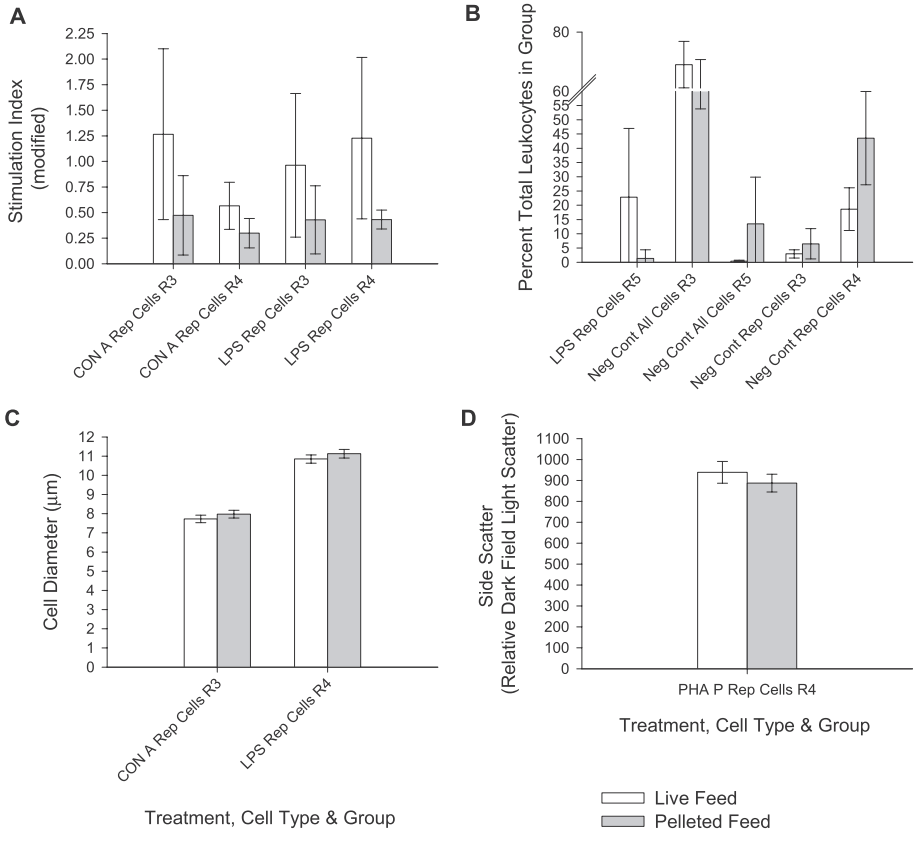

Fig. 5. Effects of live (n = 14) and artificial pelleted (n = 9) diets on stimulation indices (A), relative numbers of replicating cells (B), median cell diameter (C) and median side scatter (D) in two groups of anterior-kidney leukocyte from age-0 smallmouth bass. Leukocytes treated with concanavalin A (CON A), phytohemagglutinin-P (PHA-P) and lipopolysaccharide (LPS) and responses compared to those of untreated negative-control leukocytes (Neg Cont). Data presented, and relevant measures identified as per Fig. 3. Leukocytes regions defined as per Fig. 1. R3 – region 3; R4 – region 4; R5 – region 5; All Cell – All cells within identified region; Rep Cells – Cells that have either completed or in the process of replication as indicated by incorporation of 5-ethynyl-2′-deoxyuridine into their DNA. Model Test Statistics: Wilks' lambda, Pillai Trace and Hotelling-Lawley trace: F statistic = 6.475, df = 12, 10, p = 0.003. Canonical discriminate function (standardized by within variances): Stimulation Index; CON A replicating cells R3 = −0.791, CON A replicating cells R4 = 0.202, LPS replicating cells R3 1.611, LPS replicating cells R4 = −1.279; Percent Replicating Leukocytes; LPS replicating cells R5 = −0.504, Negative control all cells R3 = 0.929, Negative control all cells R5 = 1.60, Negative control replicating cells R3 = −0.544, Negative control replicating cells R4 = 0.853; Cell Diameter; CON A replicating cells R3 = 0.540, LPS replicating cells R4 = 0.852; PHA-P replicating cells R4 = −0.246.

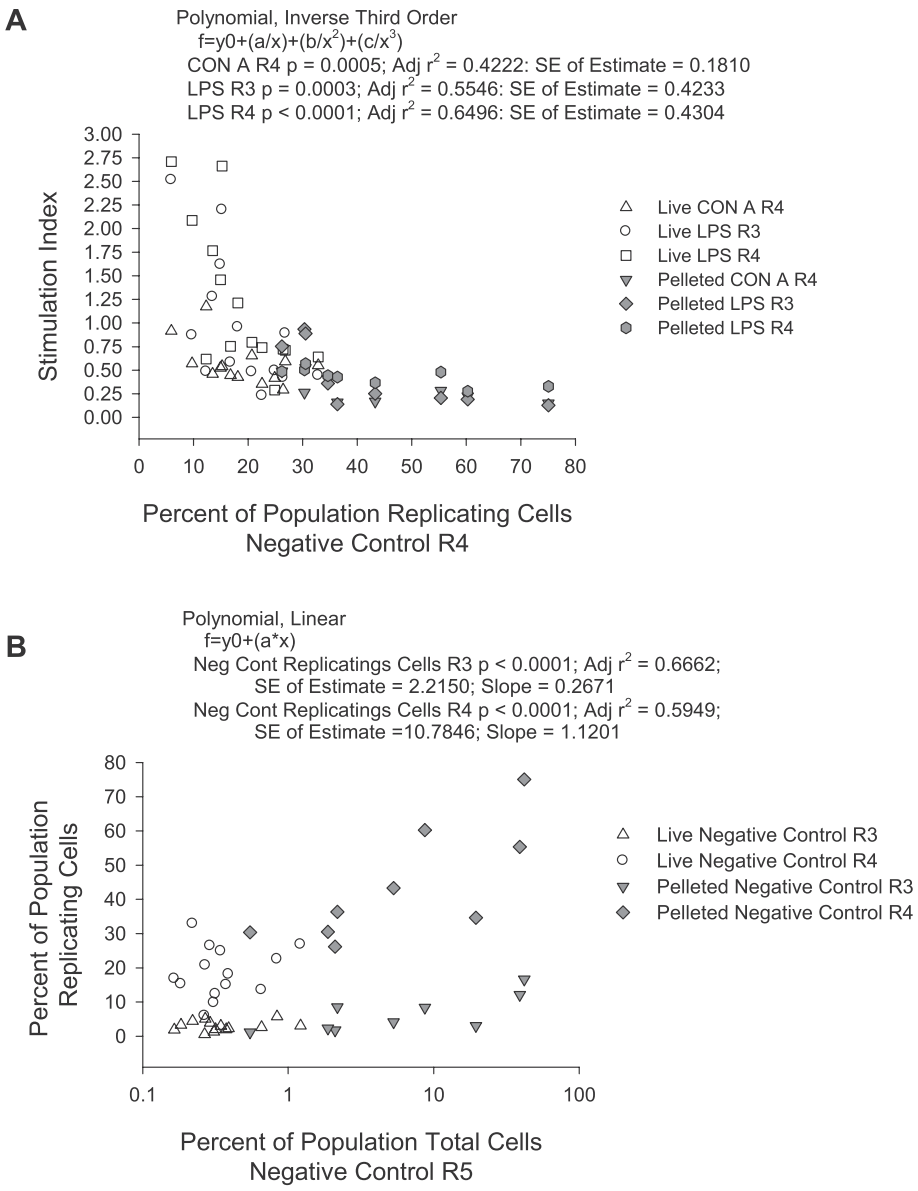

Fig. 6. Pooled data sets examining the relationships between total negative-control replicating leukocytes in R4 relative to SI values associated with concanavalin A (CON A) and lipopolysaccharide (LPS) as well as the association of the relative number of negative control apoptotic/necrotic cells (R5) to negative control cell proliferation (B) in anterior kidney leukocytes from age-0 smallmouth bass fed either a live or an artificial-pelleted diet. Regression model and results presented above each graph. Leukocyte regions defined as per Fig. 1.

elevated stimulation values observed, but this is difficult to substantiate. Differences in salmonid mitogen response kinetics have been previously reported [33]. Thus, there is experimental evidence that teleost leukocyte subpopulations respond to different mitogens at different rates.

Assuming such variation in mitogen response kinetics are occurring in the smallmouth bass leukocyte populations, these differences would make the detection of changes based on cell diameter or side scatter across mitogen treatment more difficult. Cells that are early in the mitogenesis process will have incorporated EdU, but their diameter and side-scatter properties may be only slightly different from none-replicating cells. Such cells are counted for stimulation index calculation but offer little information for more detailed analysis.

Cell replication was measured for all mitogen treatments at the same time point. This time point was sufficient to detect diet-related size changes in response to CON A as well as detecting differences in CON A and LPS stimulation index values but may not have been optimal for detecting all potential differences across mitogen treatments. The resolution provided in the mitogen assessment can be improved, but it was adequate for the characterization of diet-related effects on this aspect of leukocyte function.

Given that LPS-associated stimulation indices were significantly elevated in the live-fed fish as were the percent of apoptotic/necrotic-like replicating leukocytes following LPS treatment, these results suggest that a greater number of LPS-sensitive replicating leukocytes transitioned to apoptotic/necrotic-like cells in the live-fed fish. Lipopolysaccharide is a well-established inducer of inflammatory responses and has been used for this purpose in multiple studies involving teleosts. The induction of apoptosis using LPS has been reported in some teleost leukocyte populations [34,35] but not in others [36]. The diet related differences in LPS response do suggest that leukocytes from fish on the two diets respond differently to pro-inflammatory stimuli. The significant elevation of apoptotic/necrotic-like untreated leukocytes and the relative number of replicating cells observed in this region from the pellet-fed fish also suggests that cells from the fish on the two diets were responding differently in their *in vitro* environment. This

observation may also help to explain why LPS and potentially CON A responses differed based on diet.

4. Conclusions

Compositional analysis of the diet was not conducted in this study. However, the purpose of this study was to evaluate practical diets that could be used to maintain smallmouth bass to best approximate wild fish responses in a laboratory setting. Based on the ability to accurately assign individual fish into their known diet group using the immune function endpoint (Table 3) and the observed diet-related histologic differences (Fig. 3), a diet effect clearly occurred. Additionally, early survival was also influenced by diet.

The use of an artificial diet is attractive because it eliminates the need for maintaining additional fish to serve as a food source saving money, space and staff time. In addition, the use of an artificial diet provides a more consistent and defined nutritional source which can aid in minimizing the number of potentially confounding variables in an experimental design. The results presented here suggest the possibility that the use of the commercial artificial diet led to generalized oxidative stress by an unknown mechanism. When the fundamental culture conditions of the fish induce effects that could also potentially be produced by an experimental treatment, the task of characterizing treatment effects can become problematic. Currently, the use of the live feed for the laboratory maintenance of smallmouth appears to be the best alternative.

Author contributions

Conceptualization, C.O., V.B.; Methodology, C.O., C.S., V.B.; Investigation, C.O., C.S., V.B.; Writing Original Draft, C.O.; Writing and Reviewing & Editing, C.O., C.S., V.B.; Funding Acquisition, V.B., C.O.

Declaration of competing interest

None.

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