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Use of rapid cytochemical staining to characterize fish blood granulocytes in species of special concern and determine potential for function testing

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ABSTRACT

Studies of innate immunity in fish species of special concern are essential for better understanding of their health status during hatchery rearing conditions. The cytochemical and morphological characterizations of blood granulocytes have been used to provide information about phylogenetic differences and determine the potential use of neutrophil function assays. Rapid, simple, cytochemical staining kits used routinely for staining mammalian granulocytes have been used to characterize granulocytes from blood of four fish species: Arctic grayling, cutthroat trout, June sucker, and shovelnose sturgeon. Blood smears were stained with Peroxidase 391 (myeloperoxidase, MPO), alkaline phosphatase (AP), Periodic Acid Schiff (PAS) and Diff-quick stain; examined using bright field and differential interference contrast microscopy. Granulocytes on blood smears were evaluated based on the cell morphology, and presence or absence of the specific chromogen. Presence of lymphocytes, monocytes, platelets/thrombocytes and granulocytes was determined in all fish species. Arctic grayling, June sucker, and cutthroat trout had MPO positive granulocytes, while shovelnose sturgeon heterophils had positive reaction for leukocyte AP, but not MPO. Presence of MPO indicated potential to measure oxidative burst and degranulation of neutrophil primary granules in Arctic grayling, cutthroat trout and June sucker. Absence of MPO in shovelnose sturgeon suggested use of different enzyme marker (AP) in degranulation assay for this species. Standardization of cytochemical techniques allowed for rapid screening of leukocyte types, reducing the number of fish, time and effort to select adequate neutrophil function assays to be used in studies of health status in species of special concern.

1. Introduction

The "species of special concern" category is a legal and societal term that is based on current, or a probability of the near future population decline caused by changes in both biotic and abiotic factors in the environment [1]. The fish species of special concern belong to evolutionary diverse families, but have one common characteristic: all require active restoration programs to rescue or maintain their population in their respective habitats [2]. During the restoration efforts, the fish broodstock is often collected from the wild, artificially spawned stream side or in the hatchery, and offspring reared in a hatchery. Culturing of these species require specialized rearing conditions for each species and can be difficult to replicate at different facilities. Therefore, the broodstock manipulation and offspring rearing procedures in the facilities can be stressful to fish and cause physiological changes resulting in decreased survival of the fish in hatcheries and in the wild [3].

Fish leukocytes have been classified using criteria that primarily apply to mammalian counterparts [4] and several studies suggested that many fish leukocytes show morphological resemblance and functional similarities with mammalian cells [5–7]. Quantification of blood leukocytes is an indispensable clinical tool during health assessments of mammalian species, and leukograms have been used in clinical evaluation of stress and diseases of fish [8]. The diversity of fishes is reflected in variable leukocyte morphology [9], indicating a need for additional characterization to better classify cell types prior to clinical evaluations and determination of potential for function testing. While major aquaculture, laboratory and pet fish species leukocytes have been successfully characterized, there is limited information regarding leukocytes in majority of other fishes [10]. Furthermore, fish species of special concern usually have additional legal restrictions and limited access to

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reared or wild populations, further reducing opportunity to collect information about their leukocyte characteristics.

Neutrophils are an important component of host defense against many bacterial, viral and fungal infections. Evaluation of neutrophil function is valuable for assessment of the health status of individuals and animal populations. Neutrophil populations have been purified to study cell function and to investigate the pathogenesis of many human and some animal diseases [11]. Most studies document that fish neutrophils have very similar histochemical staining properties to mammalian neutrophils, and can be distinguished by the presence of myeloperoxidase (MPO) in their cytoplasmic granules, but some fish species can have heterophilic granulocytes instead [4,12,13]. Neutrophils are found in fish kidney, spleen and blood, and are commonly increased in inflammatory lesions [14,15]. There is evidence for phagocytic, chemotactic, and bactericidal functions of fish neutrophils, including intense respiratory burst and neutrophil extracellular trap release [12,16–18]. Neutrophil function parameters can be used for fish health status assessment [19].

Different granule types within the mammalian neutrophils contain a variety of antimicrobial substances that are released into phagosomes or to the cell exterior during degranulation [20]. Measuring exocytosis of myeloperoxidase from primary neutrophil granules *in vitro* is a direct, rapid and quantitative method to assess the degranulation process in neutrophils. The assay based on MPO – H_2O_2 oxidation of 3,3′, 5,5′ – tetramethylbenzidine (TMB) is considered the least toxic of the peroxidase sensitive substrates, and was successfully used in fish [17,21,22]. The absence of myeloperoxidase in bird and some fish species heterophils suggests use of alternative enzymatic assays to measure degranulation [23–25]. Leukocyte alkaline phosphatase (AP) is present in both tertiary granules of mammalian neutrophils, as well as in some fish species neutrophils and heterophils [4,11]. The use of *p*-Nitrophenyl Phosphate as substrate for leukocyte AP has been described for mammals [26], and has been adapted in this study for use in sturegon heterophil degranulation assay.

Changes in the environment can affect numerous fish species within an ecosystem but often only a few species appear to suffer long term consequences while other closely related and ecologically similar species do not [27]. For this study, four species of special concern that are part of the regional or national conservation programs in the U.S. were selected: Arctic grayling (*Thymallus arcticus*), cutthroat trout (*Oncorhynchus clarki lewisi*), June sucker (*Chasmistes liorus*), and shovelnose sturgeon (*Scaphirhynchus platorynchus*). The shovelnose sturgeon were used as surrogates for the endangered pallid sturgeon (*Scaphirhynchus albus*).

The information about effects of captivity and spawning stress on innate immunity in species of special concern is limited to species also connected with commercial aquaculture (salmon, trout, bass), and basic characterization of granulocytes in the above selected species has not been performed. The limited population and high value of individual specimens poses a limitation to the sample size to be used in optimization and determination of the appropriate function assays in evaluation of the innate immune status of the fish. Cytochemical staining of granulocytes was performed with commercially available leukocyte staining kits to determine presence or absence of enzymes characteristic for granulocytes in other vertebrate animals. The objective of this study was to use rapid cytochemical staining kits for human leukocytes to characterize neutrophilic granulocytes in diverse fish species of special concern and determine if cytochemical characteristics can serve as an indication of functional capabilities of neutrophils, assist in selection of the assays, and reduce number of fish used to determine appropriate function assays.

2. Materials and methods

2.1. Fish

Leukocytes from four fish species of special concern (Arctic grayling, cutthroat trout, June sucker, and shovelnose sturgeon) were evaluated. The fish were maintained at the Bozeman Fish Technology Center (BFTC) research facility, U.S. Fish and Wildlife Service (USFWS), Bozeman, Montana, USA. Fish were held in 300–1000 L tank recirculation systems supplied with conditioned and filtered spring water at adequate temperature for the species and fed daily with pelleted diet prepared in BFTC fish nutrition department, according to hatchery standards. Fish were cared for in accordance with approved USFWS/BFTC animal care guidelines.

2.2. Blood smear preparation and cytochemical evaluation

Three fish from each species were caught in a net and immediately killed by immersion in buffered 1 g L^{-1} tricaine methylsulphonate (pH 7.4; MS 222, Finquel, Argent Chemical Laboratories) solution. Blood was collected in heparinized microhematocrit tubes from severed caudal peduncle, and six blood smears per fish were prepared. Blood smears were stained with Peroxidase 391 (myeloperoxidase, MPO), leukocyte alkaline phosphatase (AP), Periodic Acid Schiff (PAS), and Diff quick (all from Sigma–Aldrich, Saint Louis, Missouri, USA) according to manufacturer's instructions, with minor modifications [13]. Granulocytes were examined using bright field and differential interference contrast microscopy (magnification of 400× and 1000×). Cell morphology and presence or absence of the specific chromogen were evaluated using bright field and differential interference contrast (DIC) microscopy (Zeiss Axioscope with Axiocam digital camera, magnification 1000×). Digital images of leukocytes were processed and figures prepared using imaging software (Adobe Photoshop CS4, Adobe 2007; FreeHand 10.0, Adobe 2004).

2.3. Kidney leukocyte separation

Fish from each species were caught in a net and immediately killed by immersion in MS 222 as described above. Anterior kidney tissue from 16 (8 fish per day, two separate days) individual fish of each species was aseptically collected and granulocyte enriched fraction was separated using a previously described technique [13,17]. Briefly, anterior kidneys were dissected and collected in Hank's balanced salt solution without Ca, Mg and phenol red ($HBSS^{CMF}$, Mediatech – CellGro, AK, USA), homogenized in a 15 mL tissue grinder (Wheaton, USA) and pelleted for 15 min at 250 *g*. The cell pellet was resuspended in $HBSS^{CMF}$ and gently placed over separation medium with a specific gravity of 1.078 g mL^{-1} (Lymphocyte separation medium 1078, Mediatech – CellGro, AK, USA). Gradients were centrifuged for 30 min at 400 *g*, the cells at the interface were removed, washed, resuspended in $HBSS^{CMF}$ and total leukocyte counts and viability (trypan blue dye exclusion) were determined using a Neubauer-ruled hemocytometer [13,17]. Cell suspensions were adjusted to a standard concentration of 2×10^7 cells mL^{-1} and the granulocyte to non-granulocyte proportion was determined by differential leukocyte counts (minimum of 200 counted cells per slide) on Hemacolor (Harleco, EM Science, NJ, USA) and Myeloperoxidase (391A staining kit, Sigma) stained cytospin preparations of cell isolates. Independent counts were performed by two trained researchers, and average of two counts was reported. If independent counts differed for more than 10%, a third count of the leukocytes on the slide in question was performed using double-headed scope. Identification and morphological characterization of leukocytes were done using suggested criteria [4,5,7].

2.4. Reagents

For neutrophil function assays working solutions of 3,3′, 5,5′ − tetramethylbenzidine hydrochloride (TMB, Sigma; 2.5 mM in water), hydrogen peroxide (H_2O_2, 5 mM in water), and Cytochrome C from horse heart (Cyt C, Sigma, 490 μg mL^{-1}) were prepared immediately before use. Sulfuric acid (H_2SO_4, Fisher; 2 M) was used as a myelperoxidase reaction stop solution. The detergent cetyltrimethylammonium bromide (CTAB, Sigma; 0.02% in water) was used as a lysing agent for determining total myeloperoxidase and leukocyte alkaline phosphatase content of neutrophils or heterophils. Stock solutions of Cytochalasin B (cyto B, Sigma; 1 mg mL^{-1}), phorbol-myristate-acetate (PMA, Sigma; 1 mg mL^{-1}) and calcium ionophore A23187 (CaI, Sigma; 1 mg mL^{-1}) were prepared in dimethyl sulfoxide (DMSO, Sigma), and stored at −80 °C. Reagents for determination of leukocyte alkaline phosphatase activity were prepared as per manufacturer's instructions (QuantiChrom™ Alkaline Phosphatase Assay Kit, BioAssay Systems, Hayward, CA, USA). Aliquots of the reagents were diluted in HBSS for each assay. Preliminary titrations were used to determine optimal reagent concentrations. The final concentrations of stimulants used in the assays after addition of HBSS and resuspended neutrophils were: cyto B 2.5 μg mL^{-1}, CaI 5 μg mL^{-1}, and PMA 1 μg mL^{-1}.

2.5. Oxidative burst and degranulation of fish granulocytes

Neutrophil oxidative burst was assayed using modifications of a previously described method [28,29]. Detection of extracellular superoxide was based on reduction of cytochrome C (cyt C, Sigma), with the use of PMA as a stimulant in microtiter plates. All samples were tested in duplicate wells. Briefly, 50 μL of HBSS and PMA were added to each test well, followed by 150 μL of Cyt C. Non-stimulated test wells received 100 μL of HBSS and 150 μL of Cyt C. Background wells received all reagents, but no cells. All test wells received 50 μL of cell suspension containing 2×10^7 cells mL^{-1}. After addition of cells, plates were immediately placed in a microtiter plate spectrophotometer (ELx808IU Absorbance Microplate Reader with KC3 data analysis software; BioTek, Vinooski, Vermont, USA) at room temperature, optical density (OD) in each well was determined every minute for 15 min using two wave lengths (OD = V1 − V2; V1 = 550 and V2 = 650 nM), and background values were subtracted from the plate. Total superoxide release was measured as nmol O_2^- produced per 10^6 neutrophils using the following correction formula:

$$O_2^- = [(OD_{av}) \times 100/\%N] \times 15.87$$

O_2^- = nmol of superoxide produced per 10^6 neutrophils; OD_{av} = average measured optical density in two sample wells; %N = percent of neutrophils in cell suspension; 15.87 = correction factor for transformation of OD value to nmol of O_2^- [30].

Release of myeloperoxidase from neutrophils in response to CaI with cyto B was determined using 96-well flat bottom microtiter plates (Fisher, USA). Test wells received 75 μL of cyto B and 50 μL of CaI. Control (background) wells received 125 μL of HBSS. Total MPO content wells received 125 μL of CTAB solution. Total MPO and background values for each trial were determined concurrently with neutrophils exposed to stimuli. All samples were tested in duplicate wells with 25 μL of cell suspension containing 2×10^7 cells mL^{-1} added to each well and incubated at 20 °C for 20 min. After incubation, 50 μL of TMB was added, followed immediately with 50 μL of H_2O_2. The color change reaction was allowed to proceed for 2 min, and 50 μL of 2 M sulfuric acid was added to stop the reaction. Test plates were centrifuged at 600 *g* for 15 min, 200 μL of supernatant from each well was transferred to another plate and optical density (OD) in each well was determined at 405 nM using a microtiter plate spectrophotometer.

The QuantiChrom™ Alkaline Phosphatase Assay Kit was used to quantify release of leukocyte alkaline phosphatase from shovelnose sturgeon heterophils in response to CaI using 96-well flat bottom microtiter plates. Test and control wells were set up as described above for MPO release assay, with modification of cyto B being replaced with HBSS. Plate incubation was in the dark at 20 °C for 20 min, the plate was placed on ice for 2 min to stop the reaction, centrifuged at 4 °C, 600 g, 5 min and 50 μl of supernatant was transferred to a new 96-well plate. A working solution of 200 μl assay buffer, 5 μl Mg Acetate, and 2 μl of pNPP (*p*-Nitrophenyl Phosphate) liquid substrate were added to each sample and control wells, and water and tartrazine were added to 2 blank wells each as assay standards. Plate was immediately read on microplate reader at 405 nm. Percent of release for MPO and leukocyte AP was calculated as described before [17].

$$\%\ \text{release} = [(OD_{\text{stimulated}} - OD_{\text{background}})/(OD_{\text{lysed}} - OD_{\text{background}})] \times 100$$

Stimulated: CaI with cyto B (or CaI alone in AP assay); Background: HBSS; Lysed: CTAB

2.6. Statistical analysis

Data are presented as means ± standard error of the mean (SEM) unless otherwise indicated. The differences in oxidative burst and degranulation of fish neutrophils prior to data transformation were examined by Student's *t*-test (GraphPad Prism 3.00, 1999). $P < 0.05$ was considered significant.

3. Results

Morphological and cytochemical characterization of blood leukocytes in Arctic grayling, cutthroat trout, June sucker and shovelnose sturgeon was performed using rapid staining kits for leukocyte differentiation (Diff quick), detecting the presence of granular enzymes (myeloperoxidase, MPO; leukocyte alkaline phosphatase, AP) and glycogen component of granules. The microscopic analysis of blood smears revealed presence of several leukocyte cell types (lymphocytes, monocytes, thrombocytes, and granulocytes) in all examined fish species (Table 1). Granulocytes in Arctic grayling, cutthroat trout and June sucker have characteristic neutrophil morphology of multi-lobed (Arctic grayling and cutthorat trout) or kidney shaped (June sucker) nucleus, with a moderate nuclear to cytoplasmic ratio and granular appearance of the cytoplasm (Fig. 1A–C). Two major granulocyte types (eosinophils and heterophils) were observed in shovelnose sturgeon blood. Eosinophils have characteristic morphology with kidney shape nucleus, moderate nuclear to cytoplasmic ratio and abundant eosinophilic cytoplasmic

Table 1
Overview of different leukocytes found in blood of four fish species. All species had thrombocytes, monocytes, and lymphocytes present in the blood. Myeloperoxidase (MPO) positive granulocytes were considered neutrophils, and MPO negative granulocytes were considered heterophils. Eosinophils were detected by Diff quick in shovelnose sturgeon, and no basophils were detected in any of the fish.

Blood cell type	Arctic grayling	Cutthroat trout	June sucker	Shovelnose sturgeon
Neutrophils	+	+	+	−/Heterophil
Eosinophils	−	−	−	+
Basophils	−	−	−	−
Thrombocytes	+	+	+	+
Monocytes	+	+	+	+
Lymphocytes	+	+	+	+

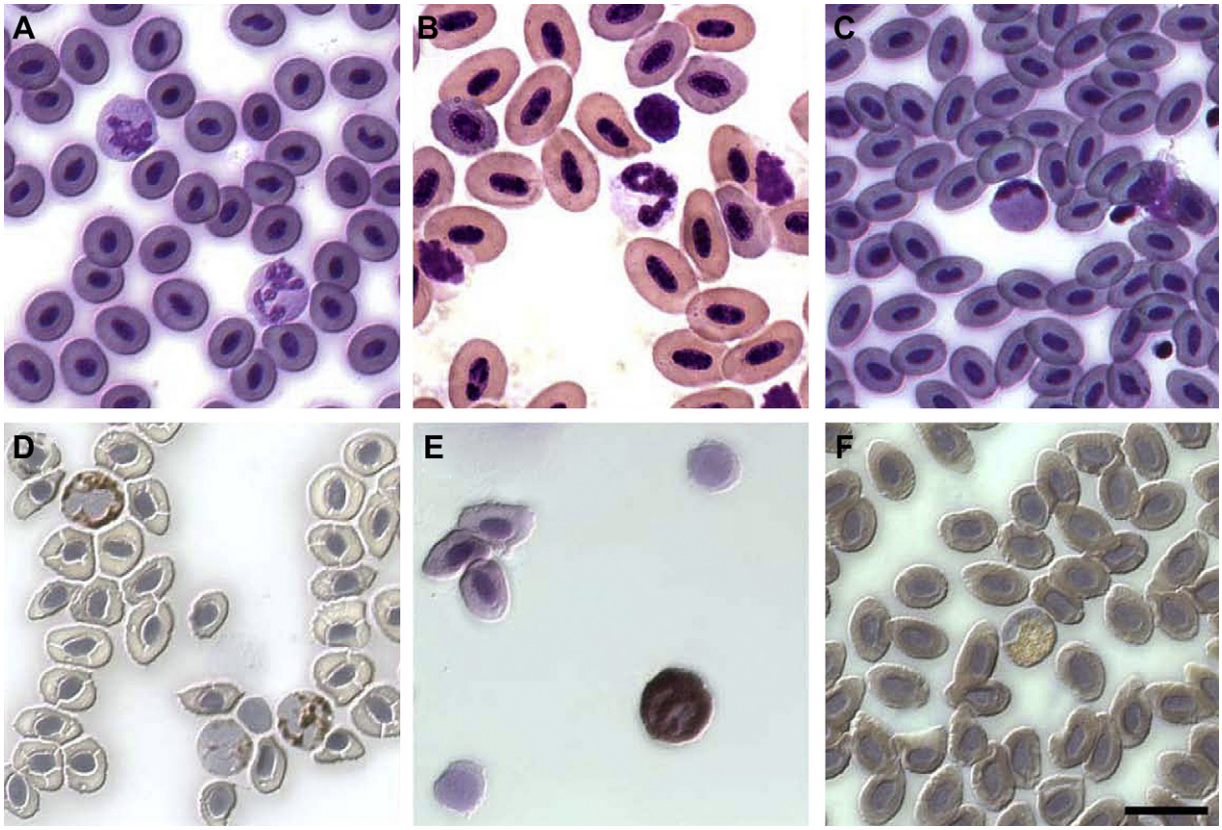

Fig. 1. Characteristic morphology of blood neutrophils. Cells with multi-lobed or indented nucleus with moderate nuclear to cytoplasmic ratio were determined by Diff-quick staining in Arctic grayling (A), cutthroat trout (B), and June sucker (C). Presence of myeloperoxidase in neutrophils from Arctic grayling (D), cutthroat trout (E), and June sucker (F) was determined using Peroxidase 391 staining kit. Bar = 10 μM.

granules. Heterophils have characteristic multi-lobed nucleus, moderate nuclear to cytoplasmic ratio and granular appearance of the cytoplasm (Fig. 2A).

Neutrophil granules in Arctic grayling, cutthroat trout and June sucker stained dark red to golden brown with the peroxidase No 391 staining kit, thereby confirming the presence of myeloperoxidase (MPO) in the cytoplasmic granules of those species (Fig. 1D–F). All observed granulocytes demonstrated strong positive staining with MPO in clear contrast with weak positive and diffuse reaction that was occasionally observed in the cytoplasm of some monocytes, erythrocytes and thrombocytes (not shown). Eosinophilic granulocytes, and multi-lobed, neutral stained granulocytes were detected in shovelnose sturgeon (Fig. 2A). The absence of eosinophil peroxidase and MPO in sturgeon blood and head kidney granulocytes was indicative of heterophils, rather then neutrophils (Table 1, Fig. 2B). The shovelnose sturgeon heterophil granules stained dark blue with the No 85L-2 AP kit, indicating a positive staining reaction and presence of leukocyte alkaline phosphatase in all observed granulocytes, but not other leukocytes (Fig. 2C).

The majority of cells obtained from discontinuous gradient separation of the kidney cell suspension from all species were granulocytes (neutrophils, eosinophils, or heterophils). The final cell isolation after gradient purification had cell viability of >95%, and granulocyte to non-granulocyte proportions of 78.3 ± 7.4%, 82.2 ± 9.1%, 67 ± 10.4%, and 73 ± 8.4% (mean ± SEM; Arctic grayling, cutthroat trout, June sucker, and shovelnose sturgeon, respectively; as determined by microscopic examination of Diff quick and MPO staining of the cytospin preparations). The majority of remaining cells was lymphocytes and thrombocytes, and less than 5% of isolated cells were monocytes, erythrocytes, with occasional kidney epithelial cell. The mean purity of granulocyte isolates (>70%), viability of over 95%, and cellular integrity by microscopic examination support the minimal deleterious effects of gradient cell separation on the granulocytes. In addition, the cytochemical staining reactions were similar for blood and granulocytes isolated from tissues, indicating no change in granule or cytoplasmic contents and enzyme activity.

Presence of MPO in neutrophil granules of Arctic grayling, cutthroat trout, and June sucker indicated functional capabilities of their neutrophils for oxidative burst and degranulation. Significant increase in production of superoxide anion (O_2^-) in PMA stimulated compared to non-stimulated cells was detected in each species except shovelnose sturgeon (Fig. 3A). Absence of detectable peroxidases in shovelnose sturgeon heterophils and eosinophils suggested reduced capabilities for oxidative burst and indicated the need for a different enzymatic marker to be used in the degranulation assay (Fig. 3A–B). The use of leukocyte alkaline phosphatase as a marker in the degranulation assay resulted in the detection of 55% degranulation after 30 min stimulation with CaI (Fig. 3B).

4. Discussion

Microscopic analysis of blood smears from fish species of special concern indicated presence of lymphocytes, granulocytes (neutrophils, eosinophils and heterophils), monocytes, and thrombocytes in studied fish species of special concern (Table 1). Different leukocyte types have been described in fishes, major categories being lymphocytes, granulocytes, monocytes, and thrombocytes [4]. Morphological characterization of leukocyte types has been performed in fish species at different taxonomic levels, revealing significant diversity in

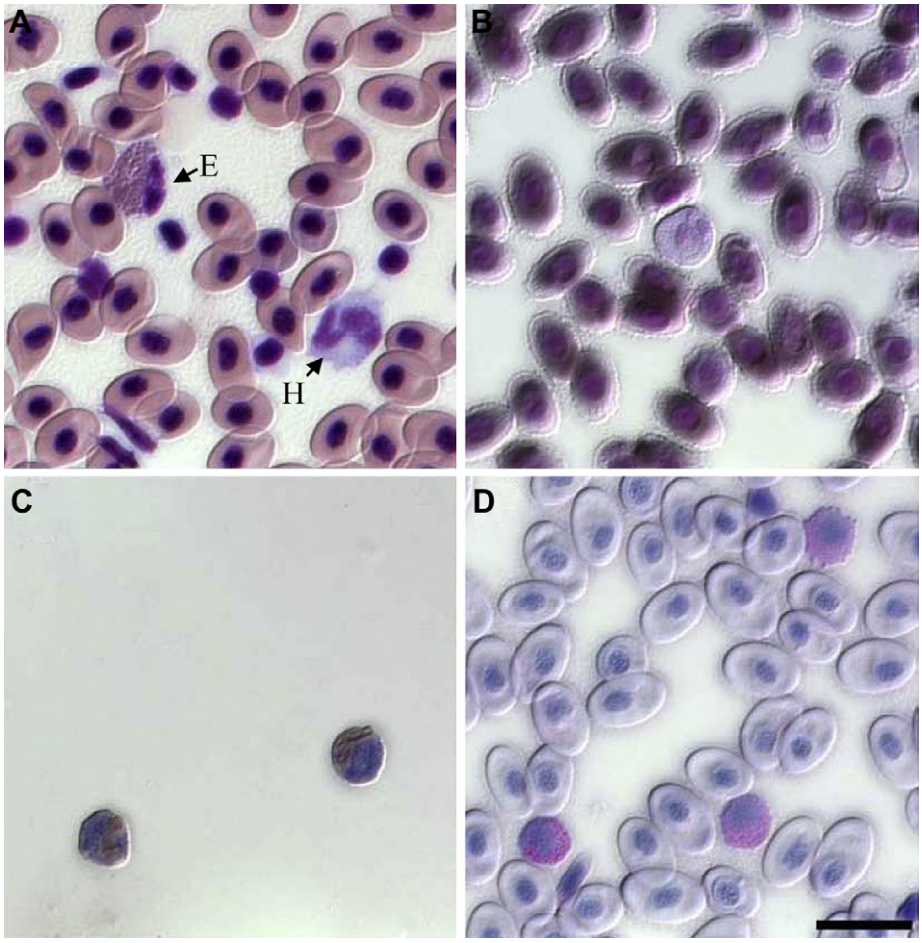

Fig. 2. Characteristic morphology and cytochemical staining of the shovelnose sturgeon blood granulocytes (H = heterophil; E = eosinophil). Presence of eosinophils and heterophils was detected in blood using Diff-quick stain (A). Absence of MPO (B) and presence of AP (C) in granulocytes indicated the potential for use of AP in the degranulation assay. Presence of abundant granules in sturgeon leukocytes was detected by PAS staining (D). Bar = 10 μM.

appearance of cells that belong to each of the leukocyte types [6,10]. The finding of listed leukocyte types in Arctic grayling and cutthroat trout (salmonidae), June sucker (catostomidae), and shovelnose sturgeon (acipenseridae) is in accordance with the literature and established leukocyte characterization criteria [10,15].

The positive myeloperoxidase (MPO) staining of cytoplasmic granules and morphology in Arctic grayling, cutthroat trout and June sucker granulocytes distiguished them as neutrophils (Fig. 1). The lack of peroxidases and presence of characteristic morphology in Diff-quick stained blood smears in shovelnose sturgeon

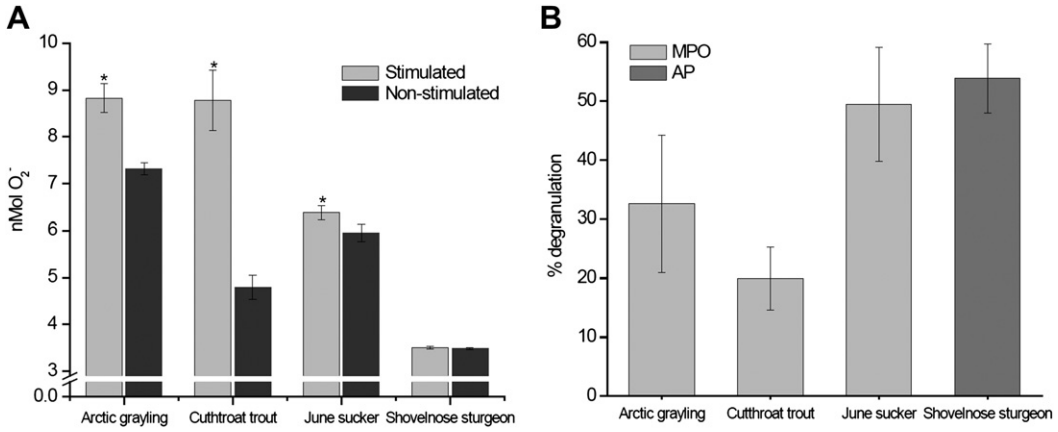

Fig. 3. Oxidative burst (A) and degranulation (B) in granulocytes from four fish species of special concern. A: The oxidative burst was significantly increased in PMA stimulated neutrophils of Arctic grayling, cutthroat trout and June sucker, while shovlenose sturgeon heterophils and eosinophils did not respond to PMA stimulation with increased production of superoxide anion. B: Neutrophil degranulation was observed in Arctic grayling, cuthtroat trout, and June sucker using myeloperoxidase (MPO) and in and heterophil/eosinophil shovlenose sturgeon using alkaline phosphatase (AP). Total of 16 samples (8 samples per day) per fish species were used in granulocyte function assays on two separate days (* denotes significant difference at $P < 0.05$, $n = 16$).

granulocytes indicated presence of eosinophils and heterophils in this species (Fig. 2). Cytochemical characterization of enzymatic content, polysaccharide (glycogen) and lipid components of granules to distinguish granulocyte sub-types has been used extensively in human and veterinary clinical medicine. It has been generally accepted that the presence of peroxidases in granulocytes is a defining characteristic of neutrophils (myeloperoxidase) and eosinophils (eosinophil peroxidase) in mammals, alongside neutrophilic granular staining with Giemsa or Diff-quick blood stains [11]. The absence of peroxidases in avian granulocytes, and heterophilic granular staining observed when conventional blood stains are used, identifies them as heterophils [31].

Multiple granulocyte types have been reported in many fish species, but the diversity of morphological characteristics has often prevented differentiation of granulocytic and monocytic lineages, and terminology (neutrophils vs. heterophils) was not always consistent [32]. In fish granulocytes, the perioxidase enzymatic activity was often co-localized with other less specific markers of neutrophil granules such as lipid content or glycogen (Sudan Black B/SBB/and Periodic acid Schiff/PAS/staining, respectively) [4–7,13]. The presence of MPO gene transcript, protein product, or cytochemical staining in granulocytes was reported in fish species from different families, indicating that this enzyme can be used as distinguishing characteristic of the myelopoietic lineage in teleost fishes [33,34]. Classification of granulocytes as neutrophils in Arctic grayling, cutthorat trout and June sucker is supported by the presence of the myeloperoxidase enzyme and is in accordance with hematology of other teleost species [8,23]. Eosinophilic granulocytes have been reported before in the blood of salmonids, including rainbow trout, and have been associated with histology of parasitic infections in catostomids [35]. In this study, no eosinophils were found in blood smears of Arctic grayling, cutthroat trout, and June sucker. The fish used in this study did not display signs of parasitic infection on necropsy, therefore small number of samples and low numbers of blood eosinophils and basophils could have prevented detecting those granulocyte types in the blood smears of studied species.

Two types of granulocytes (eosinophils and heterophils) were detected in shovelnose sturgeon blood (Fig. 2). The positive PAS staining for glycogen content of granules (Fig. 2D) was in accordance with several studies performed on acipenserid family [35–38]. The lack of myeloperoxidase positive staining in multilobuled shovelnose sturgeon granulocytes prompted their classification as heterophils rather than neutrophils [39]. Investigations of cytochemical characteristics of sturgeons have been focused on lipid or glycogen granular contents, and rarely included enzyme-specific staining [37]. This study is the first to investigate myeloperoxidase and alkaline phosphatase content in granulocytes of the sturgeons from genus *Scaphirhynchus* using cytochemical characterization of leukocytes.

Granulocytes of Arctic grayling, cutthroat trout and June sucker demonstrated the ability to perform respiratory burst and produce superoxide anions (O_2^-) when stimulated with a protein kinase C activator phorbol-myristate-acetate (Fig. 3A). Shovelnose sturgeon granulocytes did not respond to PMA stimulation and no difference in superoxide release was observed between unstimulated and stimulated sturgeon kidney granulocytes (Fig. 3A). One defining functional difference between neutrophils and heterophils is the ability of neutrophils to produce oxidative burst and utilize myeloperoxidase to form halide–oxygen complexes such as hypochlorite (OCl^-) in order to reduce potential damage caused by free oxygen radicals [11,31]. The presence of MPO in Arctic grayling, cutthroat trout and June sucker neutrophils indicated their ability to produce respiratory burst, which was confirmed by significant differences in superoxide production between PMA stimulated and non-stimulated neutrophils detected by cytochrome C reduction assay specific for detection of superoxide [28]. This is in accordance with earlier mammalian and fish studies that defined ability of fish phagocytes to perform oxidative burst [40]. Lack of MPO or eosinophil peroxidase in shovelnose sturgeon granulocytes was predictive of their limited ability to produce superoxide upon PMA stimulation. The avian heterophils lack the active MPO–halide enzymatic system and their respiratory burst responses are minimal compared to mammalian neutrophils [25]. The observed absence of granulocyte peroxidases and minimal respiratory burst that was unresponsive to PMA stimulation suggest functional similarity of sturgeon granulocytes to avian heterophils rather than mammalian or teleost fish neutrophils.

Granulocytes in all species in this study were capable of granule exocytosis upon stimulation (Fig. 3B). Arctic grayling, cutthorat trout and June sucker degranulation was measured using myeloperoxidase exocytosis, while alkaline phosphatase was used to measure degranulation in shovelnose sturgeon. Both MPO and AP have been described in mammalian and fish granulocytes [4,20]. In mammals, AP has commonly been associated with tertiary, while MPO was most abundant in primary granules. The dynamics of exocytosis in mammals usually begins with tertiary granule release, followed by primary granule exocytosis [20]. However, there is limited information if similar granular differentiation and enzymatic distribution is closely followed in fish granulocytes. Electronic micrographs reveal that in some fish species, different size granules are present in neutrophils, allowing for at least potential segregation between MPO and AP at a granular level [14,41]. Neutrophil degranulation in fish has been described before, supporting results of this study [17,22].

The rapid cytochemical staining kits developed for use with human leukocytes have been utilized in veterinary medicine, including fish leukocytes with minor modifications [6,7,13]. This clinical technique does not require extensive equipment and skills, indicating potential use in field or hatchery conditions where rapid assessments of health status of fish populations may be required. The cytochemical characterization of leukocytes appears helpful in prediction of granulocyte function characteristics [20,25]. Unfortunately, sampling of head kidney tissue to be used for collection of granulocyte population implies sacrifice of the studied individual, and it has been reported that significant optimization of the function assays is required for obtaining repeatable results [17]. The lethal sampling of species of special concern is the least acceptable method of health evaluation, but due to limited information about non-lethal health parameters such as blood analysis or immune status, it is often the only approach available. Therefore, use of non-lethal techniques to obtain information that will reduce need for lethal sampling of special concern species was considered. The cytochemical characterization of leukocytes requires only minimal sample volume and non-lethal sampling techniques can be employed in obtaining the blood sample. As demonstrated in this study, the cytochemical characteristics of granulocytes can guide the choice of immune function assays and reduce the need for lethal sampling during initial investigations and method optimization. Furthermore, the information obtained from blood leukocyte analysis can be indicative of fish health status, including acute or chronic stress effects [42,43].

The granulocytes of Arctic grayling, cutthroat trout, June sucker and shovelnose sturgeon were described and characterized in this study. Morphologically, lymphocytes, granulocytes, monocytes and thrombocytes were detected in all studied species. Cytochemical and functional characterization of granulocytes revealed differences between neutrophils (grayling, trout and sucker) and heterophils and eosinophils (sturgeon). It was determined that human leukocyte staining kits can be used in fish species of special concern to rapidly characterize blood leukocyte types, and minimize lethal sampling in future studies of clinical hematology and innate immunity.

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