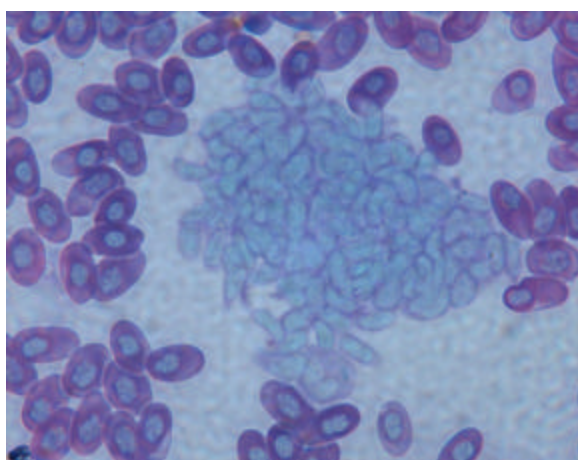




National Wetlands Research Center in Cooperation with U.S. Fish and Wildlife Service, Southeast Region, Natchitoches National Fish Hatchery, Natchitoches, Louisiana

Pallid Sturgeon in the Lower Mississippi Region: Hematology and Genome Information

by Jill A. Jenkins



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OFR 03-406

**U.S. Department of the Interior
U.S. Geological Survey**

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Outside front cover photographs:

Pallid sturgeon number 0121617596 was sampled in November 2001 at the Old River Control Structure Complex in Concordia Parish, Louisiana. Morphological measurements including lengths of rostrum, barbels, head, and tail fork were recorded. Photograph by Jan Dean, USFWS.

Pallid sturgeon whole blood was smeared on a microscope slide and stained with Wright Giemsa. Red blood cells are nucleated and surround a gathering of white blood cells. Microscopic image is 1000X magnified.

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Preface

This project (Project 1448-43270-2M-002) has been coordinated through the Natchitoches National Fish Hatchery (NNFH) and the U.S. Geological Survey's National Wetlands Research Center (NWRC). From November 2001 to April 2002, over 280 sturgeon of the genus *Scaphirhynchus* (including pallid sturgeon, shovelnose, and their hybrids) were sampled from the outflow channel of the Old River Control Structure Complex (ORCC) in Concordia Parish, La. In the overall project, several datasets were collected (see Appendix), including species identification by using microsatellites and morphometric characters, food habits, physical anomalies, information on blood cells, and pathologic evidence of iridovirus – the first indication in the lower Mississippi population of pallid sturgeon. In this study, data on blood cells were obtained from the sturgeon collected monthly from approximately 20 different animals at each sampling time.

This report presents preliminary information on differential blood cell identifications in sturgeon, data on comparative genomic DNA content and DNA degradation, and summaries and interpretations of data collected in light of available scientific literature addressing blood parameters of fish and sturgeon, in particular. Results obtained from collection and examination of blood and body fluids are often essential in establishing the health of fish (Blaxhall, 1972; Fange, 1992). Blood cells and sperm cells can be obtained nondestructively from fishes, even from small specimens that weigh less than 100 g (Stoskopf, 1992a). For flow cytometry assays, whereby cells are analyzed individually in a fluid stream, less than 1 : L of blood is needed. Examinations of blood by microscopy and flow cytometry were performed at NWRC in assisting in the efforts directed at recovery of the pallid sturgeon population in the Lower Mississippi River Basin.

Introduction

Hematological factors, particularly the cellular parameters, of sturgeon are not well studied. Serum glucose, lactate, and osmolality (Kieffer and others, 2001) as well as various other hematological features have been used to evaluate the physiological responses of fishes (Clark and others, 1979; Leonard and McCormick, 1999; Martinez-Alvarez and others, 2002). Species-specific normal ranges of such parameters can be established as useful guidelines for interpreting stress-induced physiological changes (Clark and others, 1979; Roche and Boge, 1996) with the recognition that variances may be due to genetic makeup, early life history, nutritional status, and the fish's environment.

In the study of fish blood, there are inconsistent rules of nomenclature and procedures as compared to mammalian hematology (Stoskopf, 1992b). Generally, the nomenclature in fish hematology has followed the nomenclature used in classifying mammalian cells. A comprehensive review on fish leukocytes (white blood cells or WBC) (Ellis, 1977) presents the major types as lymphocytes, thrombocytes, granulocytes (or neutrophils or heterophils), and monocytes.

The English scientific literature is not abundant with regard to pallid sturgeon, and incomplete information is available with regard to sturgeon hematological parameters (Alyakrinskyay and Dolgova, 1984). What can be gleaned are the facts that immature sturgeon have high amounts of hemoglobin, and that by sexual maturity, the total blood volume has lessened to approximately 3% of their body weight (Alyakrinskyay and Dolgova, 1984). The hemoglobin proteins are identical during the sea and river periods of life, unlike salmonids (Luk'yanenko and others, 2002b), yet the albumins do express heterogeneity (Luk'yanenko and others, 2002a). Investigations into serum electrolyte concentrations showed that sodium concentration is independent of the aquatic environment, but potassium levels change with season, and that the overall serum ionic composition of the body fluids remains stable in both salt water and freshwater environments (Natochin and others, 1975; Hunn and Christenson, 1977). Notable differences in osmolality between sturgeon species and within animal compartments (serum versus milt) have been seen with lake, pallid, and shovelnose sturgeon (Wayman, 2003).

Blood cells are the mediators of defense mechanisms in animals, and WBCs are key components of innate immune defense (Jenkins and Ourth, 1993) where defense responses are measurable and influenced by stressors (Adams, 2002). In response to stressors in the aquatic

environment, an overall drop in WBCs could indicate immunosuppression. An overall increase could mean infection or response to stressors mediated by cortisol hormone response. In all teleosts, cortisol is the major corticosteroid produced under stress, and it has been indicated as the major factor that mediates the suppressive effects of stress on reproduction (Consten and others, 2002). Circulating levels of cortisol are often used as an indicator of the degree of stress experienced by fish (Adams, 2002). Numbers of the neutrophil cell type, in response to increased circulating cortisol, is often indicative of stressful conditions or infectious disease (Ellsaesser and others, 1985; Ellsaesser and Clem, 1986). Hybrid Russian sturgeon (*Acipenser gueldenstaedtii* x *Huso huso*) fingerlings fed increased dietary protein and lipid showed a distinct decrease in neutrophil numbers and an increase in lymphocytes (Gershanovich and Kiselev, 1993). Significant differences were found in the differential leukocyte counts between stellate sturgeon *Acipenser stellatus* and beluga *Huso huso* sturgeons at age ~200 days, where 68.0-73.5% were lymphocytes, 21.8-25.1% were neutrophils, and 3.0-4.6 were eosinophils (Palikova and others, 1999). In 6-year-old Persian sturgeon *Acipenser persicus*, the leukocyte count was 10.3% with 20% neutrophils, and in the 6-year-old beluga the leukocyte count was 7.9% with 33.9% neutrophils (Bahmani and others, 2001).

The *Scaphirhynchus* sturgeon stock at the ORCC is comprised of pallid sturgeon (an endangered species), shovelnose sturgeon *S. platyrhynchus*, and their hybrids. Spawning, propagation, and recovery of pallid sturgeon are complicated by morphological similarity between pallid sturgeon and their hybrids with sympatric shovelnose sturgeon. Differentiation of species by morphometric and meristic characters alone is problematic (Wills and others, 2002). Because fish red blood cells (RBC) are nucleated, DNA is readily available for measuring genome content with flow cytometric methods (Shapiro, 1993). Sturgeon blood analyzed in concert with a standard of known genome size (Tiersch and Chandler, 1989) can be used in an attempt to distinguish notable genomic size differences between animals, thereby assisting with species determination efforts for pallid sturgeon, shovelnose sturgeon, and their hybrids. This approach has been attempted with pallid and shovelnose sturgeon, where no significant differences were noted between the genome sizes ($P = 0.9333$) (Wayman, 2003). For Eurasian sturgeon species, DNA content differences were detected by flow cytometry (Birstein and others, 1993).

Genomic DNA alterations or fragmentations are widely used in physiological, genetic, and toxicological studies. Under normal circumstances, all nonreplicating normal diploid cells are in

the G₀ and G₁ phases of the cell cycle, and these nuclei should have the same DNA content. In addition to increased DNA coefficient of variation (CV), deviations from normal diploid histograms can be noted in cells from animals exposed to contaminants (Lamb and others, 1991; Lengenfelder and others, 1997). These are very sensitive and reliable endpoints that detect deviations in ploidy from the normal 2N. Because the acquisition of data on potential alterations in CV or aneuploidy detection does not call for additional sample manipulation beyond DNA content acquisition, samples were analyzed in this study for possible indications of loss of DNA integrity.

Alterations observed in blood cell characteristics (number, shape, components, etc.) are diagnostic indicators of environmental stress on fish (Llorente and others, 2002). Overall, the goal of this project was to obtain baseline data on differential blood cell counts, on comparative DNA content, and on DNA integrity. By sampling the sturgeon nondestructively, the additional data was provided on the sturgeon collected in a pallid sturgeon recovery effort in the Lower Mississippi River region in 2001 and 2002.

Materials and Methods

Fish were bled without anticoagulant, blood smears were fixed, and differential counts were performed later. The remaining blood was added to acid citrate dextrose (ACD) anticoagulant (~9 parts blood to 1 part ACD) for analysis of genome size and DNA integrity within 24 hours.

Blood Collection

In order to study the differential cell count and the genome size, the following NWRC standard operating procedure was employed for the monthly blood collections at Natchitoches National Fish Hatchery (NNFH):

BLOOD COLLECTION OFFSITE

Each month, blood was collected from 20 different sturgeon. The whole blood was analyzed for genome size and possible aneuploidy by flow cytometry, and the smears were used for differential cell counts.

Supplies Needed

100 microscope slides	25 microcentrifuge tubes w/ 1 drop ACD
microcentrifuge tube box	25 3-ml syringes w/ 25G5/8 needle
methanol	formaldehyde
staining jar	transfer pipettes
permanent markers & pencil	distilled water in squirt bottle
latex gloves	microscope slide box
cooler and ice pack	notebook
paper towels	

METHODS

USFWS personnel drew blood from behind the anal fin during physical inspections of the fish. Four microscope slides were prepared for each fish by labeling twice with a marker with the fish number (sequential in the notebook with corresponding recorded pit tag number) and “M” or “F”, so that there were 2 methanol-fixed and 2 formaldehyde vapor-fixed slides per fish. Blood smears were made using a pinpoint of blood. Blood was gently smeared with the edge of another slide, without smashing cells (rest the slide on your finger and drag it across the smear slide). Smears were air-dried.

In the notebook, all possible information was recorded, remembering the fish ID number along with the corresponding pit tag number so the data could be cross-referenced.

For methanol-fixed smears, 2 slides were fixed for 30 seconds with methanol using a transfer pipette. Rinse with squirt bottle of water, then excess water was shaken off. For formaldehyde vapor-fixed smears, 2 slides were fixed over formaldehyde vapors for 10 minutes in staining jar. (The bottom of jar had a small amount of formaldehyde in it, and the blood smears did not come into contact with liquid.) Slides were stored in a labeled microscope slide box.

The rest of the blood was placed in a microcentrifuge tube containing a drop of ACD. The tube was labeled twice (on the cap and side) with the fish number. Tubes were inverted once. The blood was stored in a cooler with an ice pack, and cells were not allowed to freeze.

Differential blood cell counts. Two of the methanol-fixed blood smears were stained by Wright Giemsa (WG), and two of the formaldehyde vapor-fixed blood smears were stained by Sudan Black B (SB) (Ellsaesser and others, 1984; Jenkins and Ourth, 1993), and counterstained with WG or methyl green. Sudan Black B stains the lipids in neutrophils. Images of cells are presented (figs. 1-5). The WG staining allows differentiation between WBC and RBC (figs. 1-2, 4). The SB-stained slides were used to differentiate neutrophils from other WBC and RBC (figs. 3 and 5). No attempts were made at further differentiation of WBC into categories such as eosinophils, thrombocytes, monocytes, or lymphocytes. In order to collect data for cell counts, at least 300 total cells (RBC + WBC) per slide were counted in duplicate. For SB slides, neutrophils per total WBC (at least 100 WBC) were also scored in duplicate.

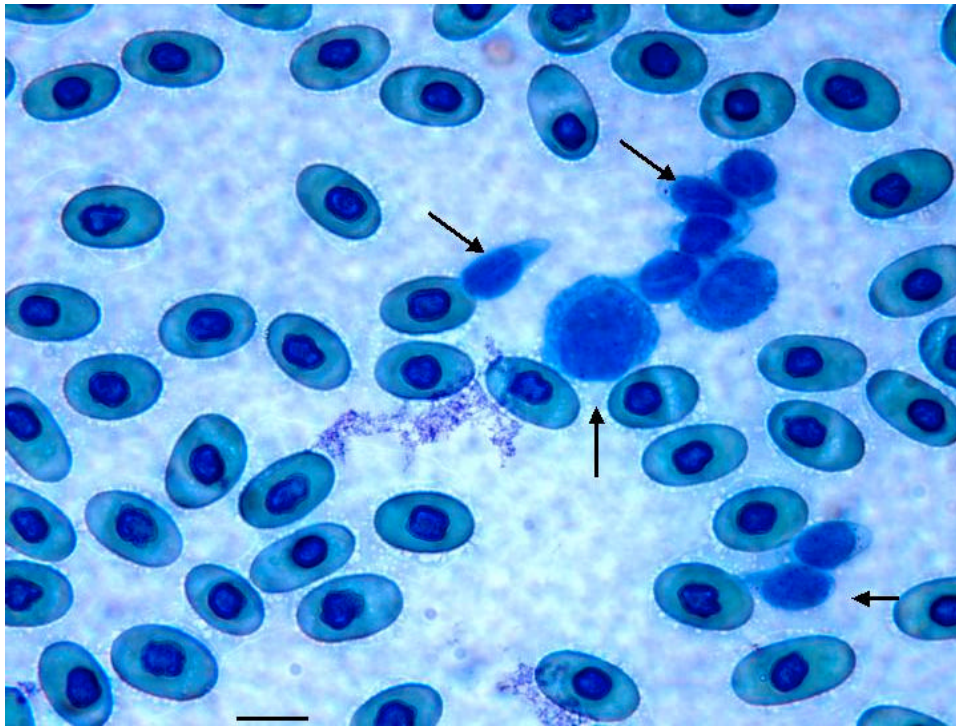


Fig. 1. Pallid sturgeon whole blood stained with Wright Giemsa. Red blood cells are nucleated and arrows point to multiple types of white blood cells. Bar is equal to 10 : m.

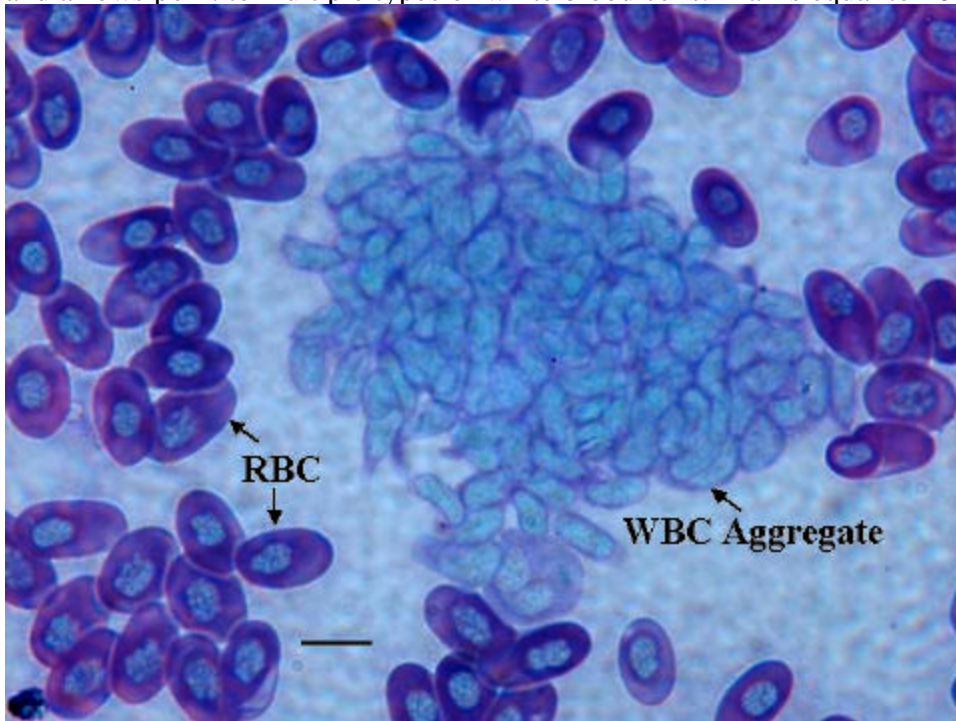


Fig. 2. Pallid sturgeon (fish 37) whole blood stained with Sudan Black B and counterstained with Wright Giemsa. Leukocytes, including thrombocytes, are congregated in the center of the field of view, indicating an ongoing peripheral blood response. RBC is red blood cells; WBC is white blood cells. Bar is equal to 10 : m.

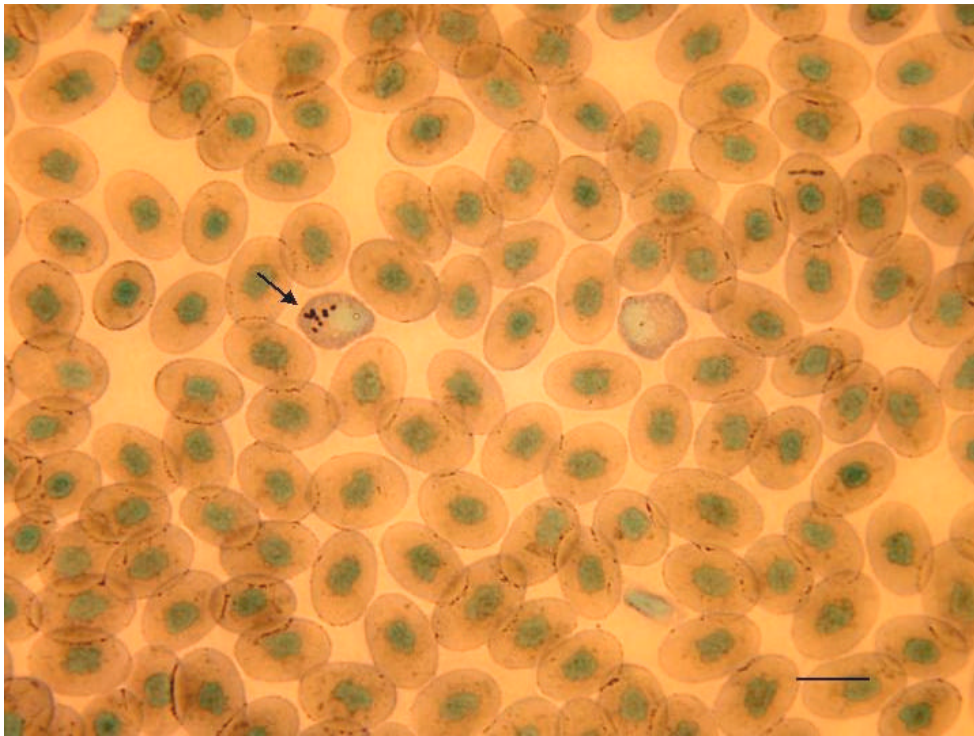


Fig. 3. Pallid sturgeon (fish 72) whole blood stained with Sudan Black B and counterstained with methyl green. Neutrophils (arrow) can be identified from other WBC by darkly staining cytoplasmic granules. WBC is white blood cells. Bar is equal to 10 F m.

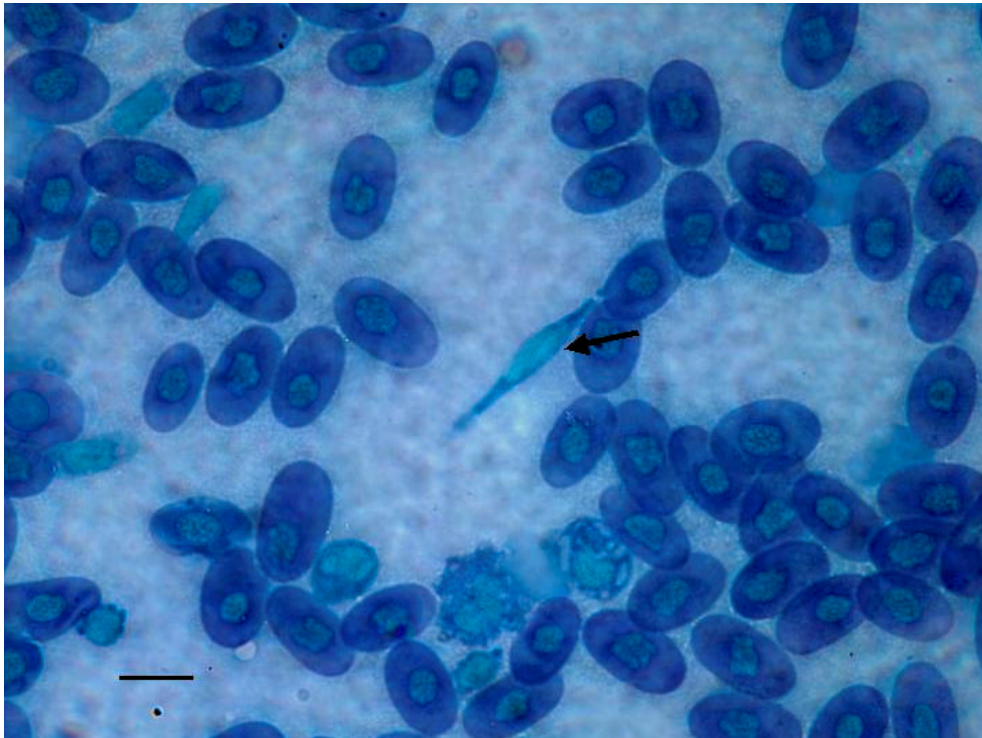


Fig. 4. Pallid sturgeon whole blood stained with Wright Giemsa. Multiple cell types are noted, and the arrow points to a fusiform shaped thrombocyte. Bar is equal to 10 : m.

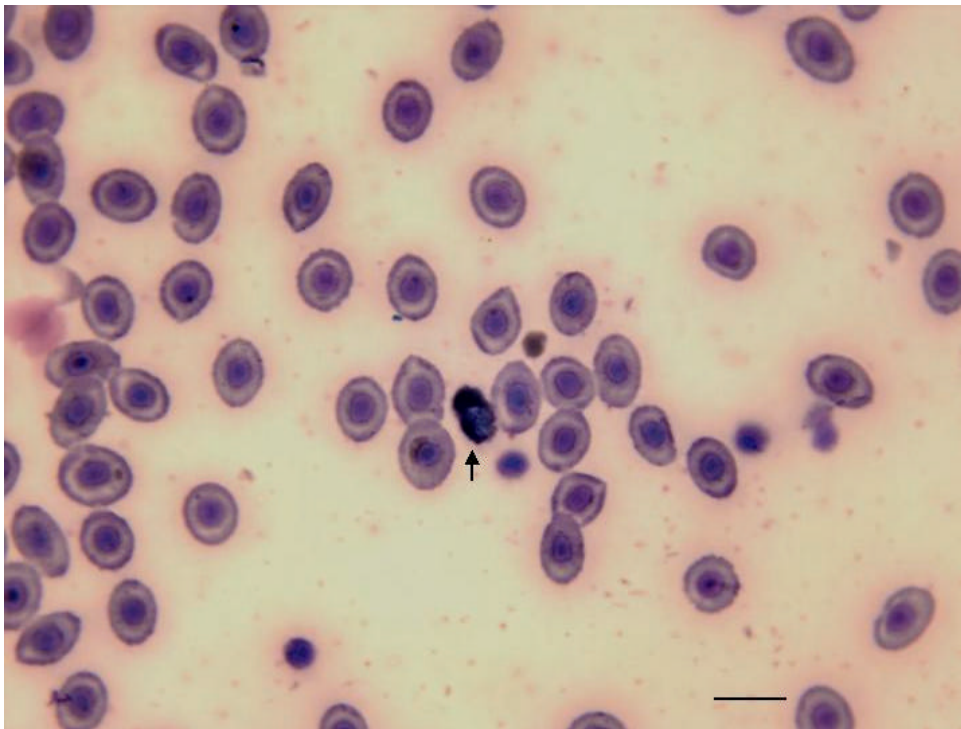
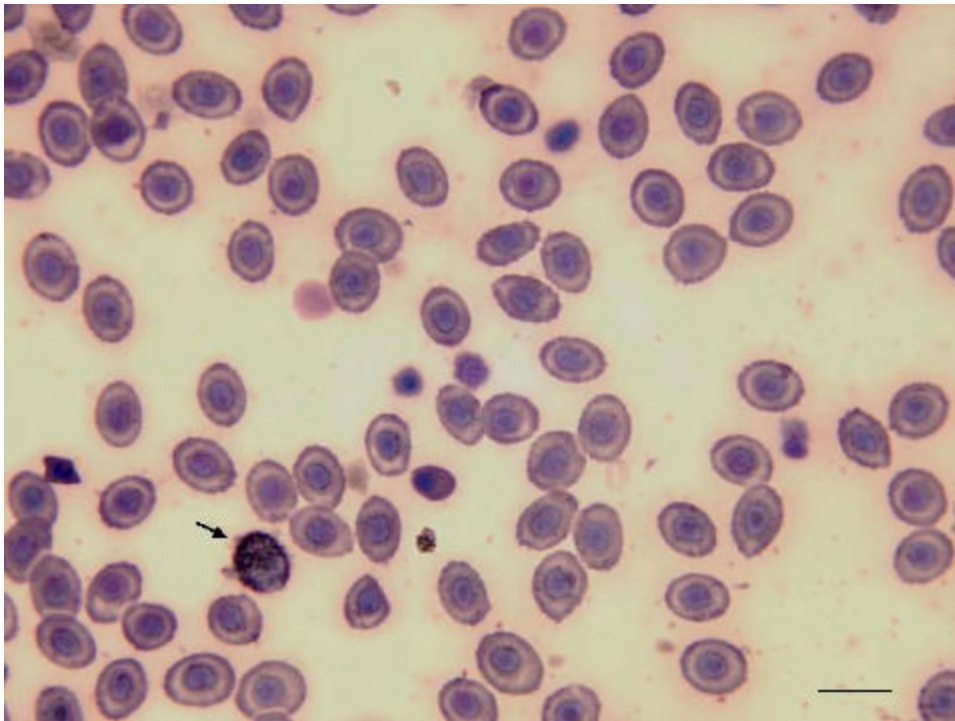


Fig. 5. Whole blood of pallid sturgeon stained with Sudan Black B and counterstained with Wright Giemsa (top and bottom micrographs). Neutrophil (arrows) can be identified from other cells by darkly staining cytoplasm. Bar is equal to 10 Fm.

Statistics. Since the fish collected every month were not the same, differences between the two WBC staining methods over the 6 months were analyzed by a two-way ANOVA for staining

methods and month effects including their interaction, using the General Linear Means procedure (PROC GLM) (SAS Institute Inc., 1990). Ratios were scored as proportions and were arcsine (sqrt) transformed for analysis (Zar, 1984). For the significant main month effect, the Duncan's Multiple Range Test was applied at the level of significance $\alpha = 0.05$.

Flow Cytometry of Blood

Cells were stained with equal volumes of 0.112% sodium citrate containing 50 $\mu\text{g/mL}$ propidium iodide (PI), RNase A at 1 $\mu\text{g/mL}$, and 0.1% (v/v) Triton X-100 for 30 min at 24° C. Stained cells were filtered through a 30 μm nylon mesh (Small Parts, Miami Lakes, Fla, USA) and the distribution of nuclei in the G_0/G_1 phase of the cell cycle was analyzed with a flow cytometer (FACScan; Becton Dickinson Immunocytometry Systems, San Jose, Ca [BDIS]). Nuclei were analyzed at 1×10^6 per mL at a rate of 300 per second, and 5-10 K events were collected by using a 1024-channel FL2 parameter at 340 linear, with linear size and scatter parameters, and doublet discrimination mode. Samples were run in triplicate. Histograms, dot plots, and density plots were generated by using CellQuest software (BDIS), with each sample analyzed in four ways: FSC versus SSC, FL2A histogram, FL2A versus FSC, and FL2W versus FL2A. These analyses allowed degraded samples to be distinguished from intact samples (Alanen and others, 1989; Zbieranowski and others, 1993). Nuclei occurring in the G_0/G_1 peak were analyzed for distance from chicken nuclei internal standard, and the CV was calculated at the full width of the peak at half the maximum height (Shapiro, 1993). ModFit LT or Cell Quest software (BDIS) were employed for analyses of flow cytometry data.

Results

In the preliminary analyses, fork length and fish weight were found to not influence the cell counts, in that they were not significant as covariates in the model. For WBC per total cells, no significant differences were noted in staining methods for detecting WBC and RBC, as well as in the interaction between month and method ($p \geq 0.05$). Significant differences were noted between the months ($p < 0.0001$), where Duncan's test grouped March, April, and November as similar and significantly higher than February and December, and January being significantly lower than all (figs. 6 and 7). Neutrophils were plotted in relation to RBC (fig. 8).

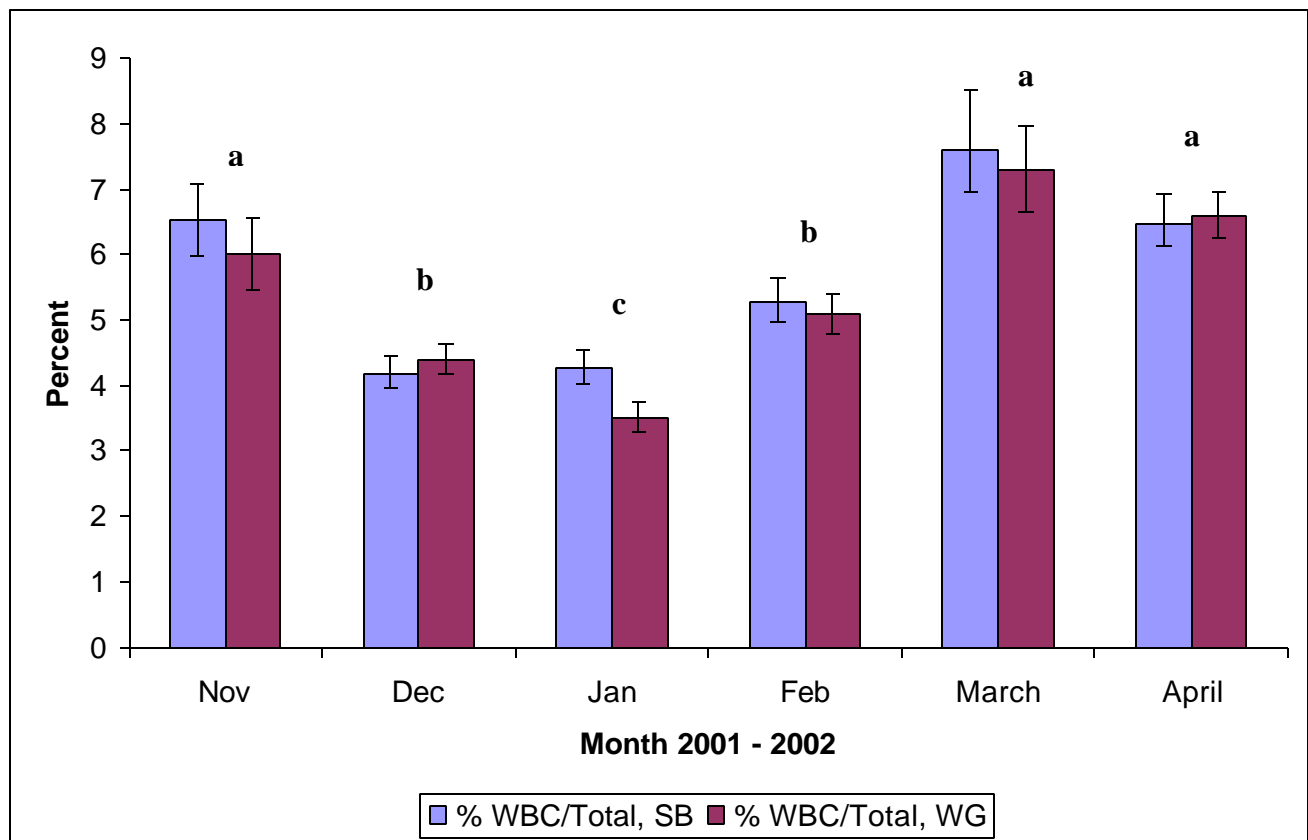


Fig. 6. White blood cells (WBC) as percent total cells in differential blood cell counts (+SE). Blood was collected from ~20 different sturgeon per month in 2001-2002. WBC are white blood cells, total count is red blood cells plus WBC, SB is Sudan Black B staining method, and WG is Wright Giemsa staining method. Different letters designate significant differences.

For neutrophils per WBC counts, significant differences were noted between months, with April significantly highest (62.2%), followed by February (48.3%) and March (46.3%), and then the group November (35.3%), December (30.0%), and January (28.7%) (fig. 7).

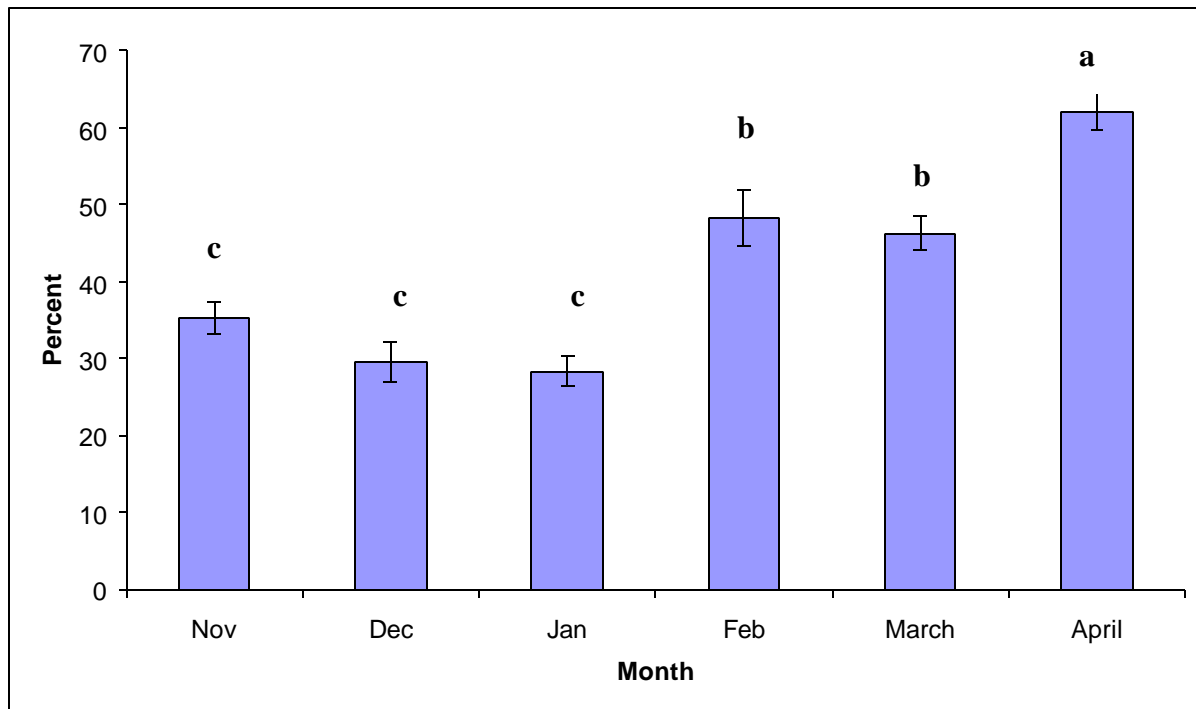


Fig. 7. Neutrophils as percent white blood cells in differential blood cell counts (+SE). Blood was collected from ~20 different sturgeon per month in 2001-2002. Cells were stained with Sudan Black B and counterstained with methyl green or Wright Geimsa. Different letters designate significant differences.

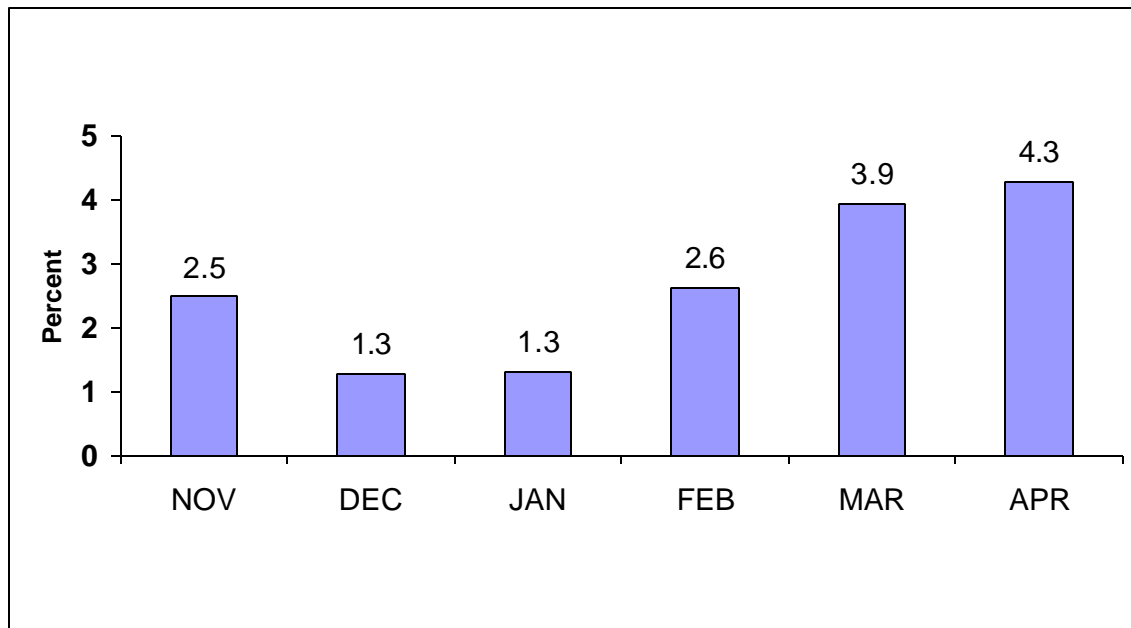


Fig. 8. Average of neutrophils as percent red blood cells in differential blood cell counts. Blood was collected from ~20 different sturgeon per month in 2001-2002. Cells were stained with Sudan Black B and counterstained with methyl green or Wright Geimsa.

The graphs that follow represent data per fish by month (figs 9 – 14).

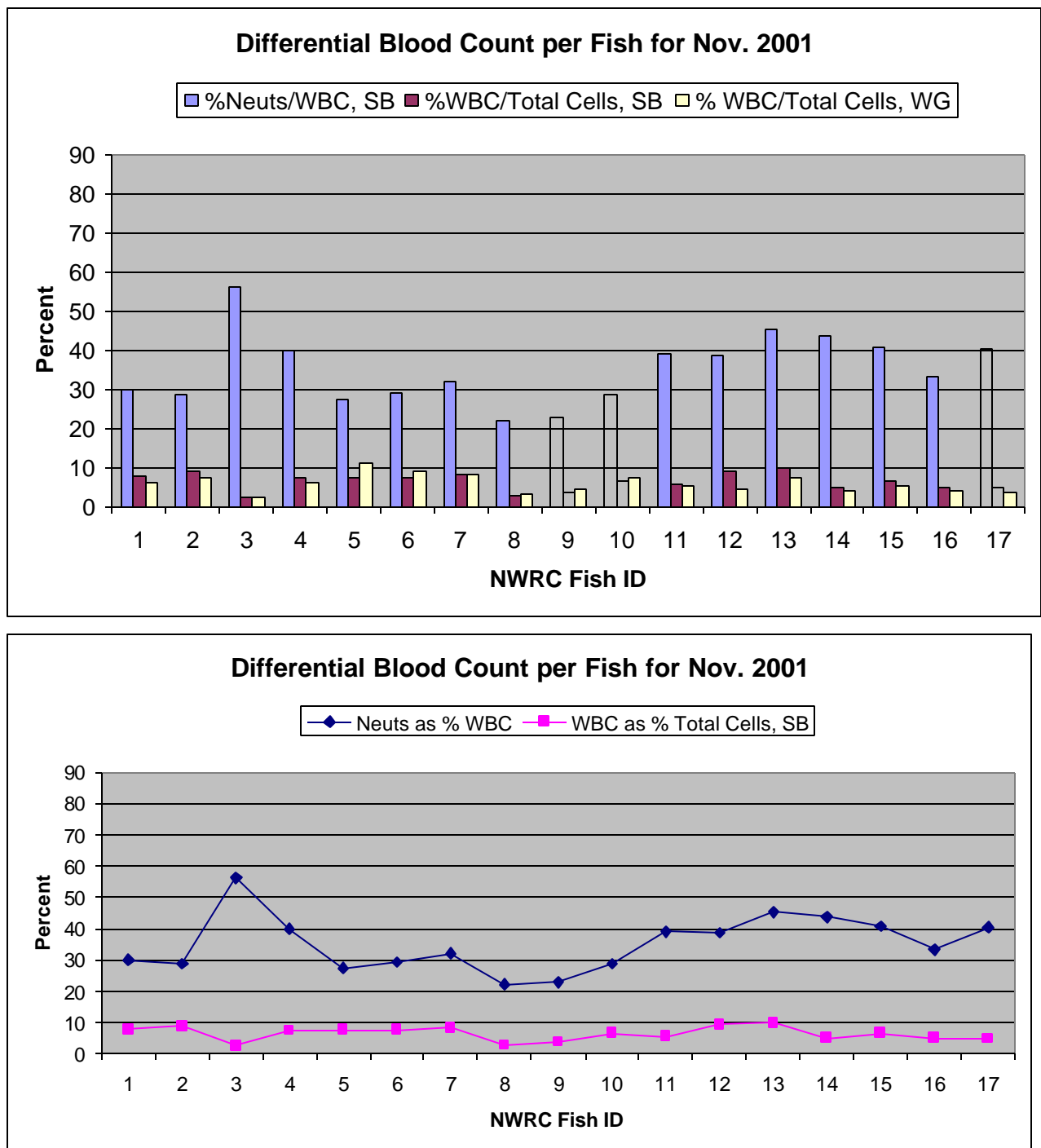


Fig. 9. Differential blood cell counts using Sudan Black B (SB) and Wright Giemsa (WG) staining with sturgeon blood. Striped bars indicate fish had a morphological abnormality (top). Note fish 3 had elevated neutrophils (Neuts) and a decreased total white blood cell (WBC) count.

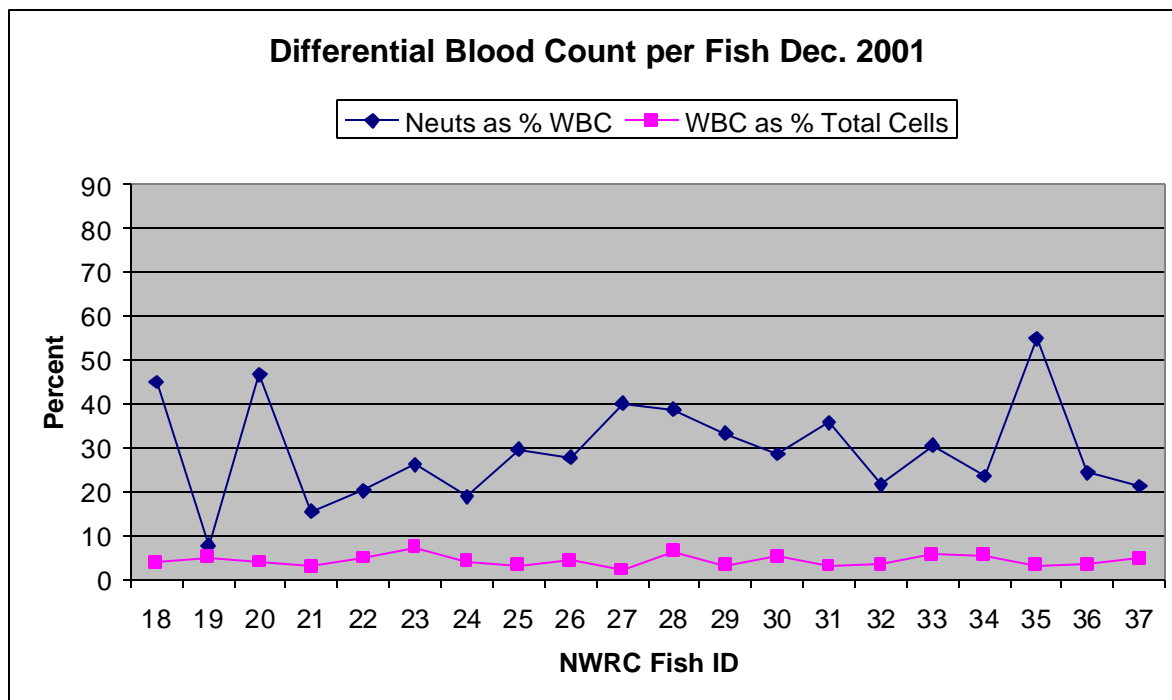
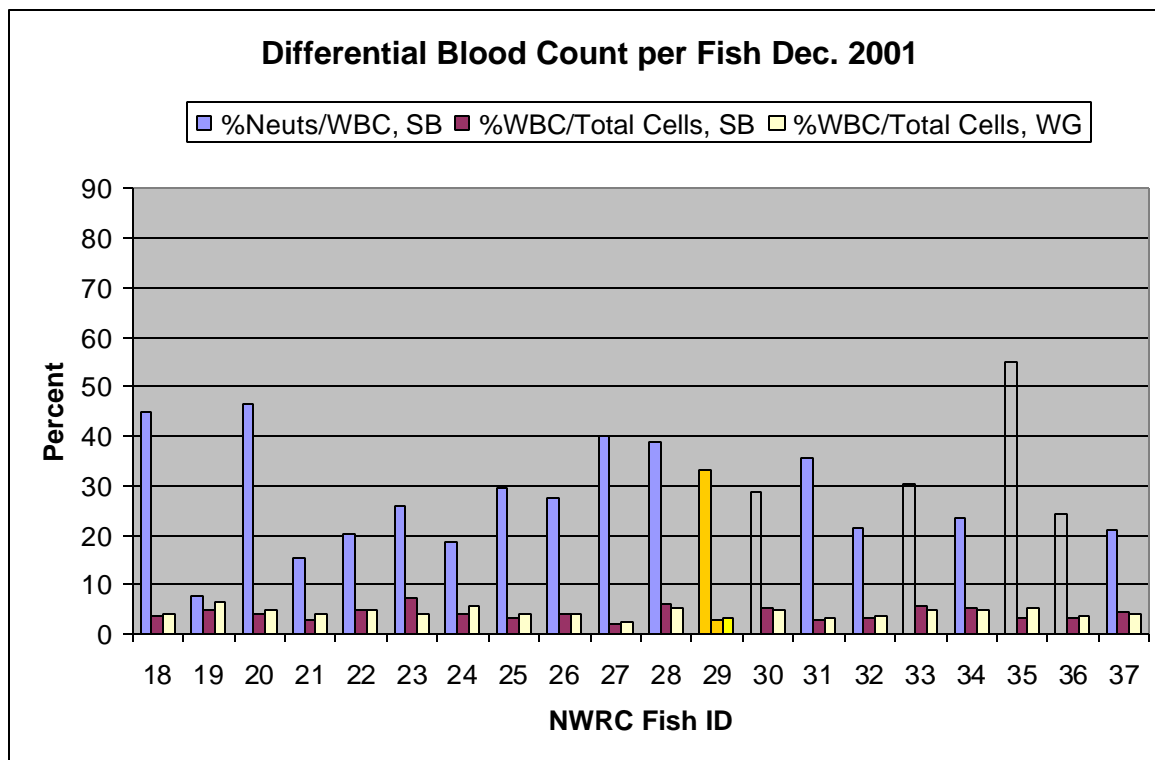


Fig. 10. Differential blood cell counts using Sudan Black B (SB) and Wright Giemsa (WG) staining with sturgeon blood. Bars in yellow indicate iridovirus suspected in the fish, and striped bars indicate fish with a morphological abnormality (top). Note low neutrophils (Neuts) for fish 19, and the elevated neutrophil count for fish 35. WBC is white blood cells.

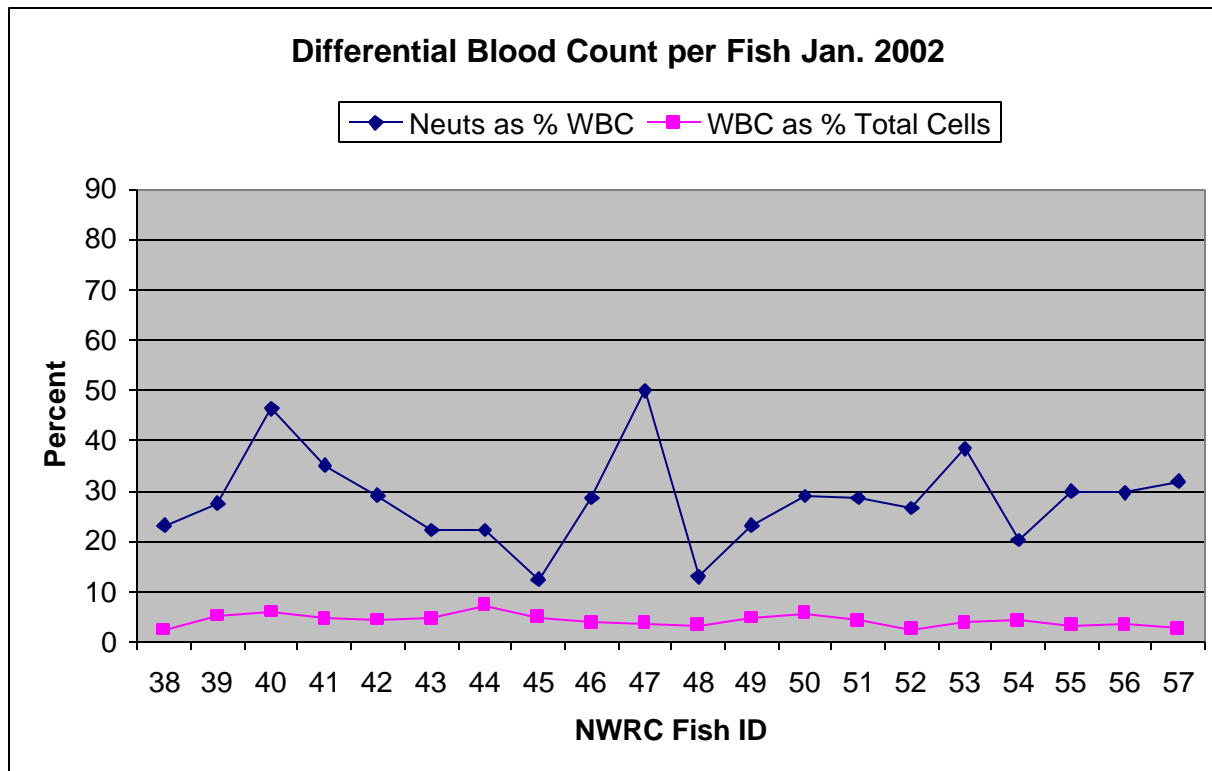
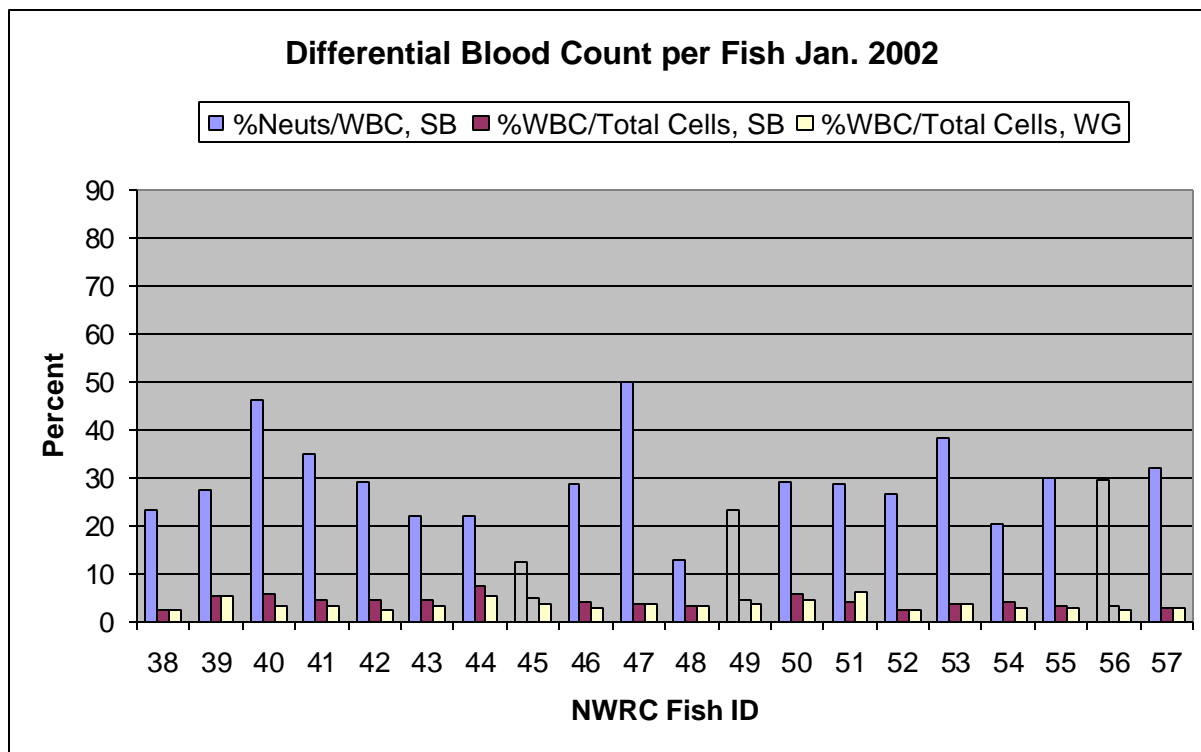


Fig. 11. Differential blood cell counts using Sudan Black B (SB) and Wright Giemsa (WG) staining with sturgeon blood. Striped bars indicate fish had a morphological abnormality (top). Note low percent neutrophils (Neuts) for fishes 45 and 48, and elevated neutrophils for 40 and 47. WBC is white blood cells.

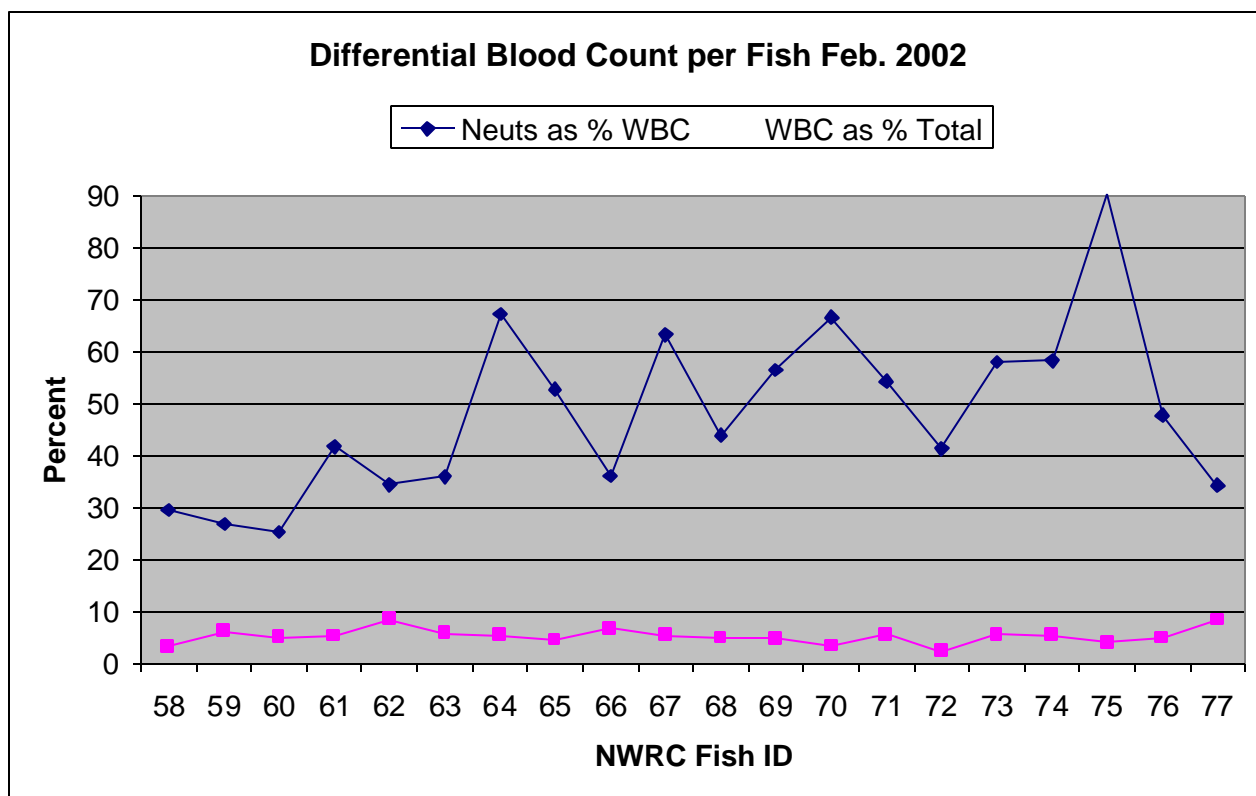
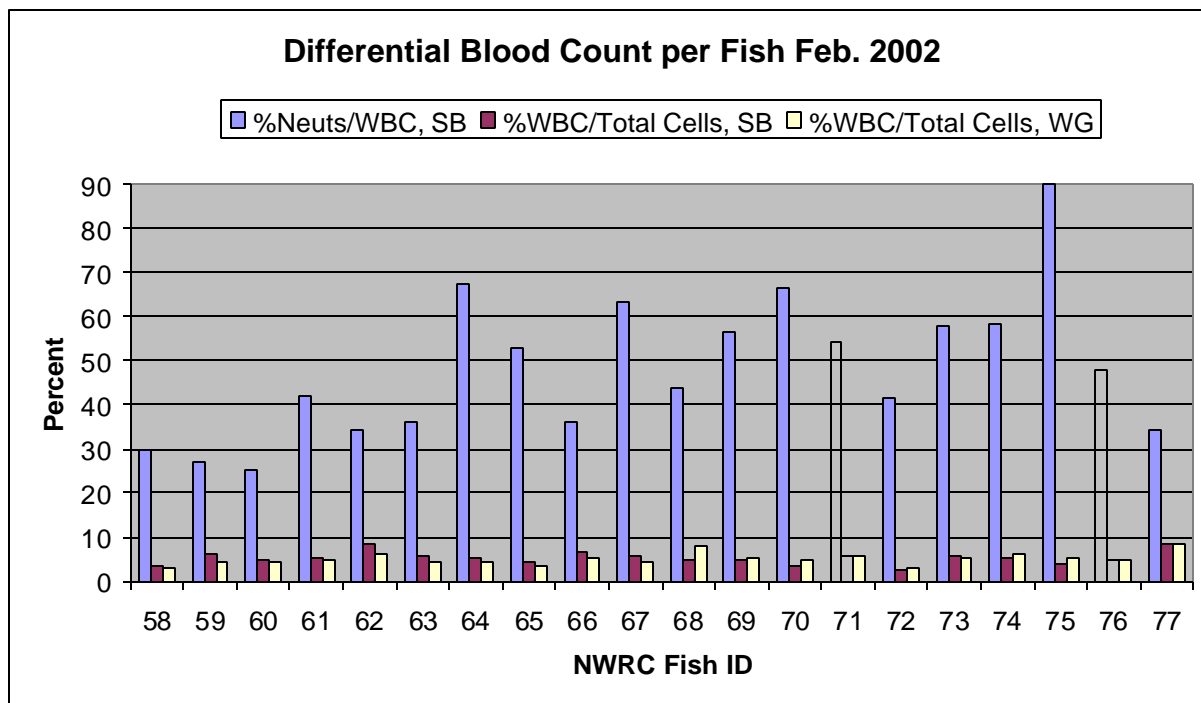


Fig. 12. Differential blood cell counts using Sudan Black B (SB) and Wright Giemsa (WG) staining with sturgeon blood. Striped bars indicate fish had a morphological abnormality (top). Note the elevated percent neutrophils (Neuts) for fish 75. WBC is white blood cells.

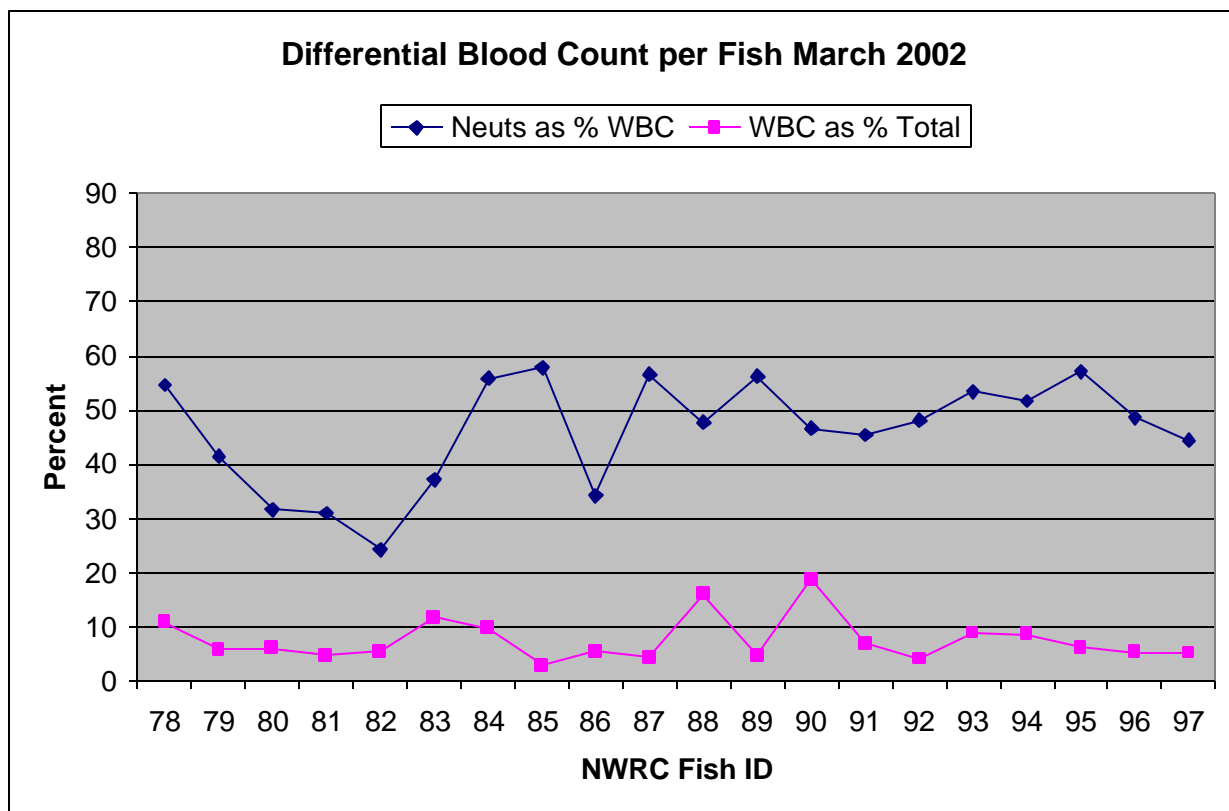
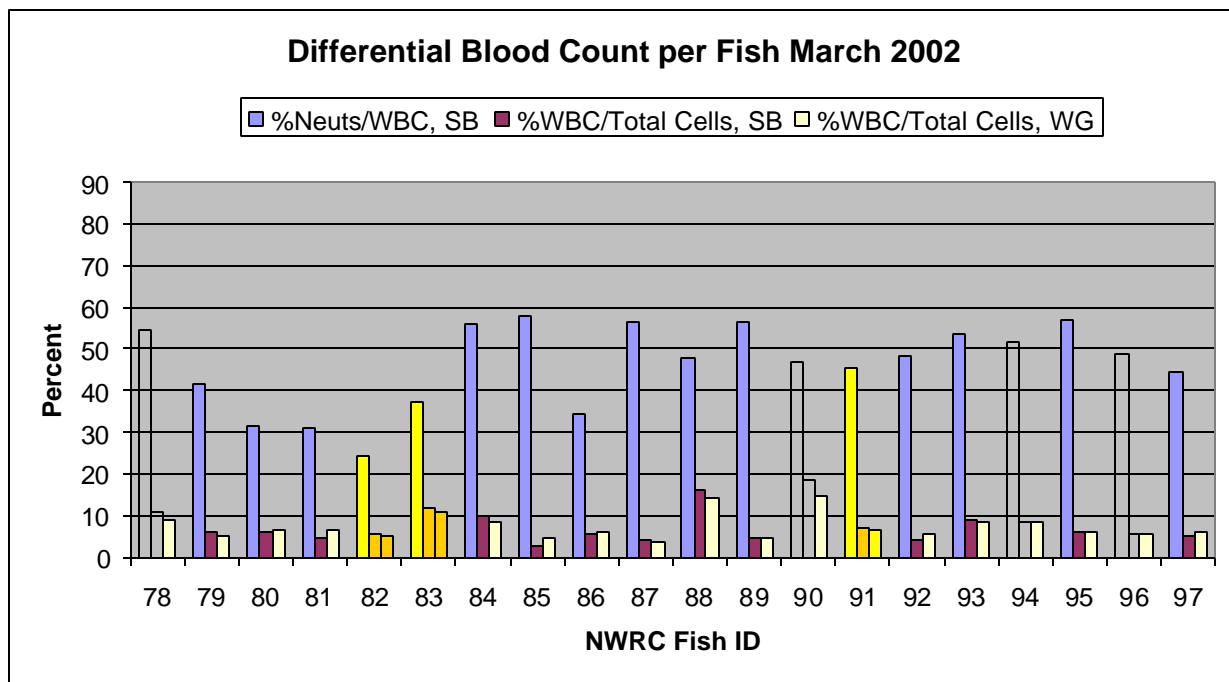


Fig. 13. Differential blood cell counts using Sudan Black B (SB) and Wright Giemsa (WG) staining with sturgeon blood. Bars in yellow indicate iridovirus detected, and striped bars indicate fish had a morphological abnormality (top). Note low number of percent neutrophils (Neuts) in fish 82. WBC is white blood cells.

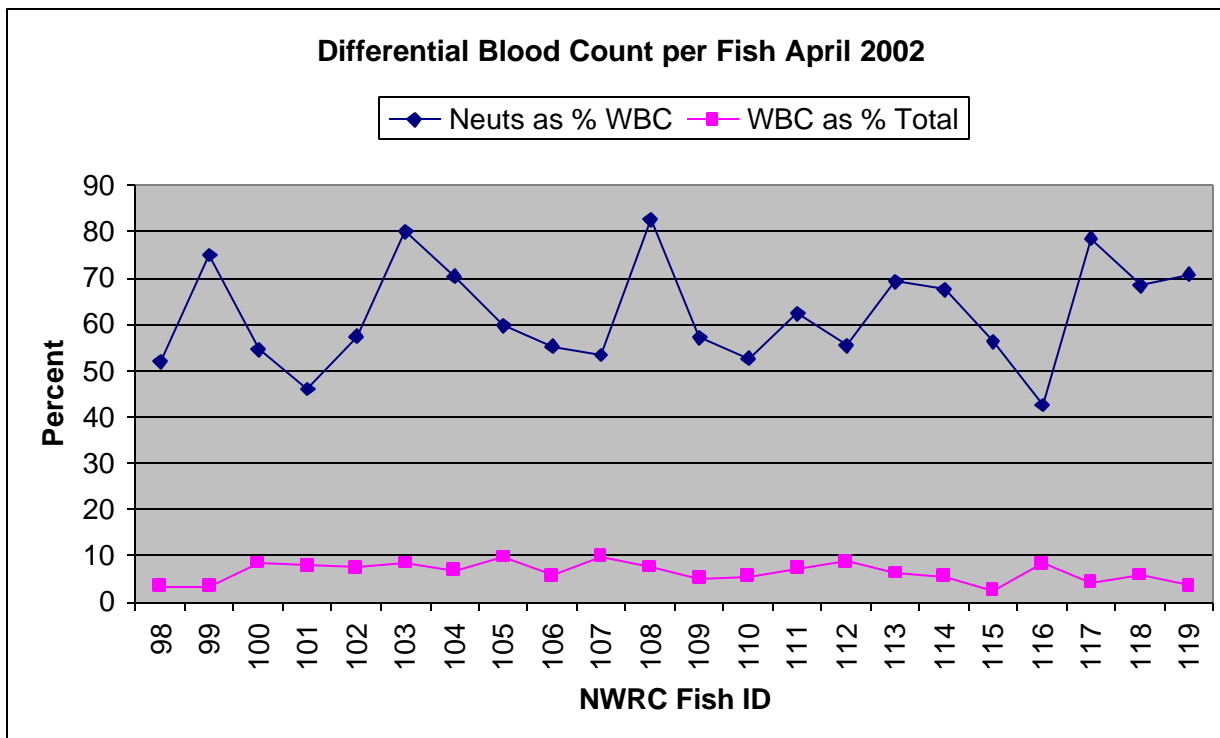
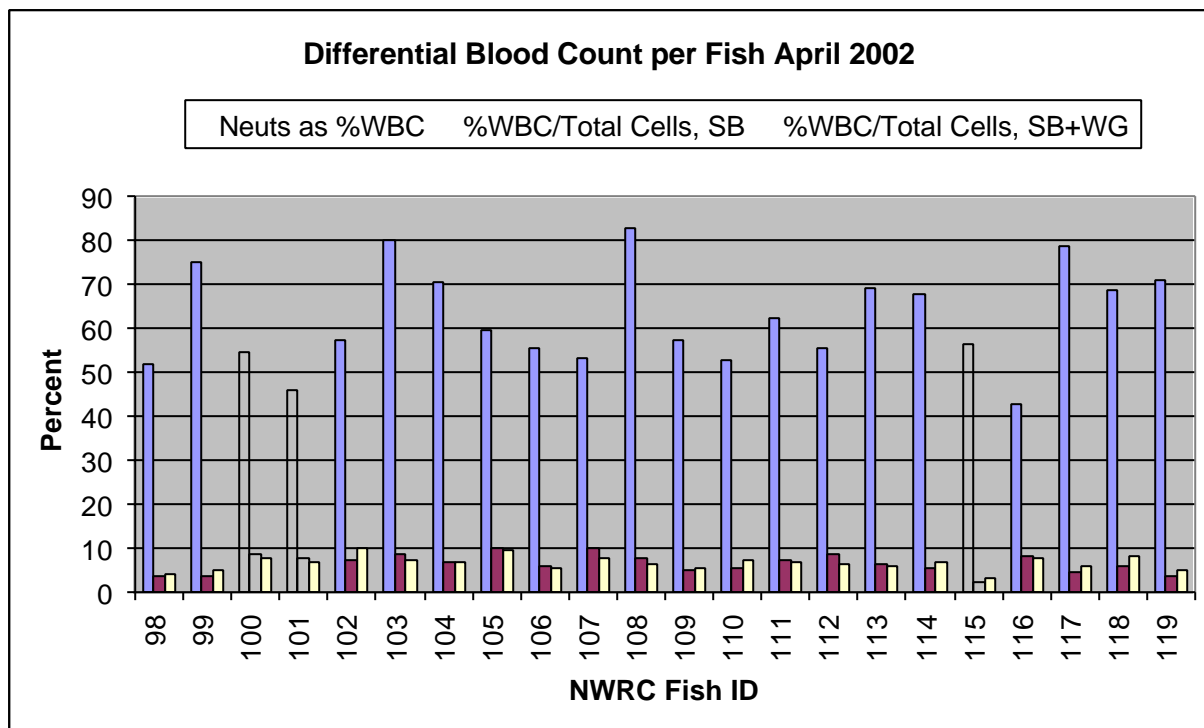


Fig. 14. Differential blood cell counts using Sudan Black B (SB) and Wright Giemsa (WG) staining with sturgeon blood. Striped bars indicate fish had a morphological abnormality (top). Neuts is neutrophils; WBC is white blood cells.

A summary of the pit tag and NWRC fish identification numbers, along with morphological anomalies and iridovirus evidence, are provided in table 1.

Table 1. Pit tag numbers and corresponding NWRC I.D. numbers*.

Fish ID #	NWRC#	Fish ID #	NWRC#	Fish ID #	NWRC#
Nov-01		Jan-02		Mar-02	
131969654A	1	115224797A	38	131674651A	78
131817271A	2	115325597A	39	131866730A	79
131734772A	3	132618723A	40	131752577A	80
131827211A	4	114953621A	41	131679652A	81
131752511A	5	132623093A	42	131659350A	82
131866280A	6	133116374A	43	131959356A	83
131715473A	7	132937351A	44	131762337A	84
131911640A	8	115225144A	45	131811445A	85
131833172A	9	132579385A	46	131946460A	86
012617596b	10	132939735A	47	131727293A	87
132271352A	11	132937135A	48	131736514A	88
131859460A	12	114955317A	49	131914283A	89
131872521A	13	133114772A	50	132232192A	90
131666630A	14	115225461A	51	131931454A	91
131751561A	15	115266243A	52	131874240A	92
131672570A	16	132622264A	53	131934170A	93
131679567A	17	132968535A	54	131659165A	94
Dec-01		132936457A	55	131966597A	95
		132939522A	56	131736643A	96
		115233754A	57	131877246A	97
		Feb-02		Apr-02	
132246640A	18	133112621A	58	131872595A	98
131669367A	19	132625123A	59	131725116A	99
131939285A	20	132632254A	60	131736095A	100
131661364A	21	133109513A	61	131728690A	101
131767627A	22	132945647A	62	131733112A	102
131736763A	23	132962160A	63	131847356A	103
131758560A	24	132953096A	64	131871656A	104
131728246A	25	132616172A	65	131916532A	105
131663525A	26	132973235A	66	131677091A	106
132269194A	27	132616151A	67	131677545A	107
131713140A	28	132619753A	68	132613227A	108
132268445A*	29*	133118654A	69	131869212A	109
012611022b	30	132622346A	70	131768653A	110
131751652A	31	133119277A	71	131865137A	111
131865516A	32	132968145A	72	131746133A	112
131674596A	33	132944766A	73	131662656A	113
131936091A	34	132939226A	74	131869692A	114
131914717A	35	132962694A	75	131722447A	115
132272560A	36	133117761A	76	131674497A	116
131925643A	37	132932547A	77	131917221A	117
				131715522A	118
				131659517A	119

*Yellow highlights indicate iridovirus detected or suspected in the samples by histopathology (see Appendix). Pink highlights indicate fish had a morphological anomaly such as developmental abnormality (see fig. 18) from predator/prey interaction, or lesion, etc. Most abnormalities were accompanied by redness of tissue, indicating an inflammation reaction.

A nonlinear analysis performed on the cumulative frequency distribution (Frank Manheim, personal communication) resulted in the following graphic (fig. 15):

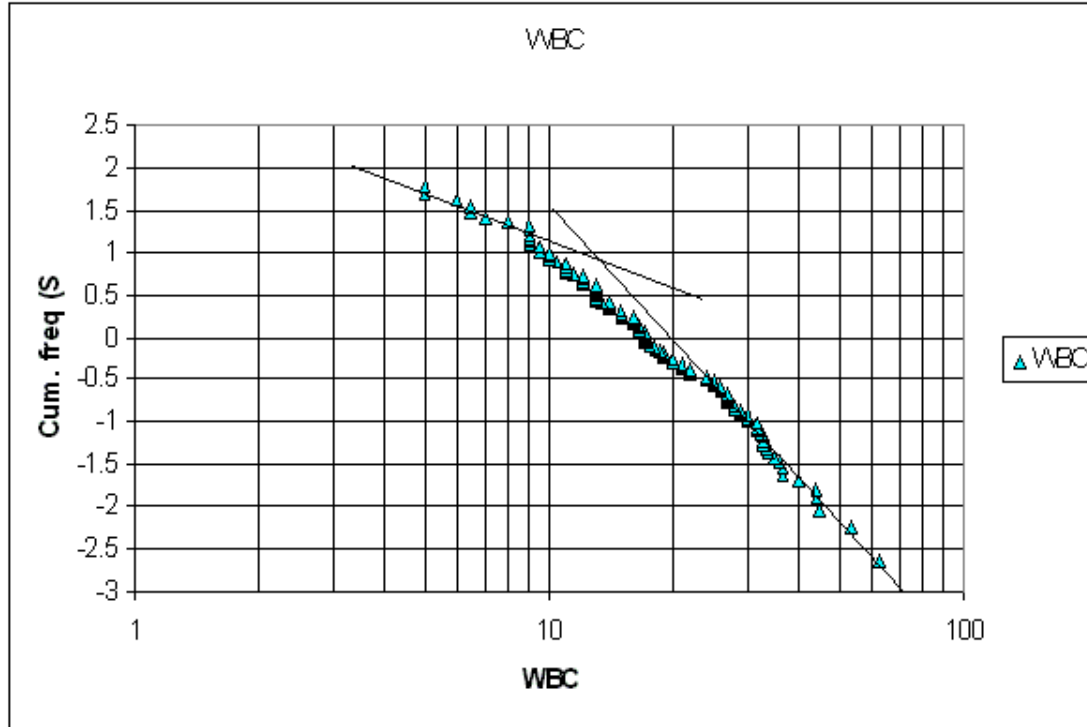


Fig. 15. This graphic depicting a nonlinear data analysis on the cumulative frequency distribution (y axis) of white blood cells (WBC) suggests that animals are not showing equivalent numbers of WBC. Most samples had an average value, while a few animals displayed many or few WBC.

A trend in increased numbers of WBC was noted with increased temperature (figs. 16 and 17).

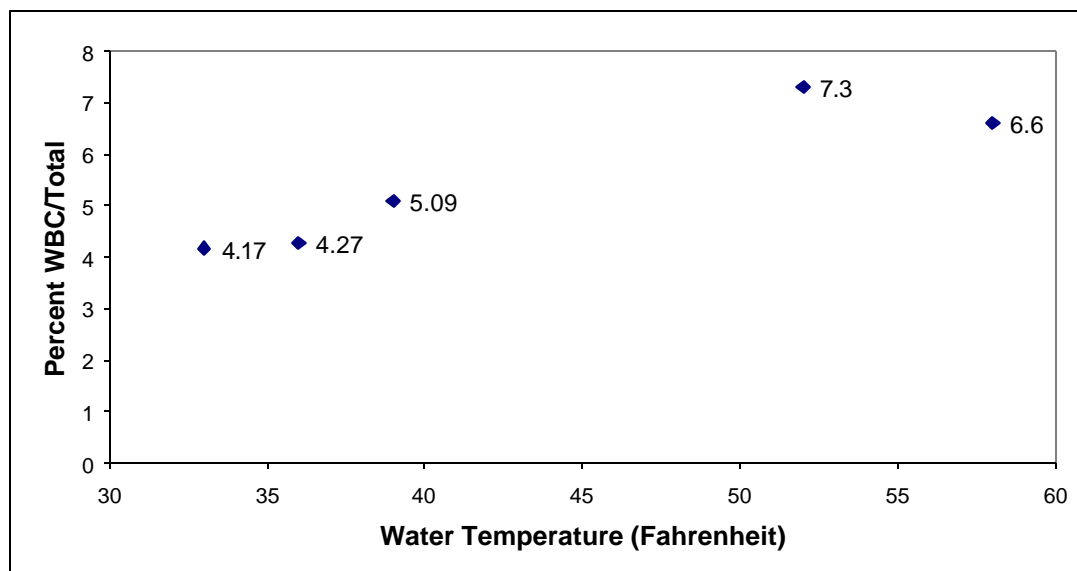


Fig. 16. Water temperature at the sturgeon sampling site, the Old River Control Structure Complex on the Mississippi River, versus the percent white blood cells (WBC) in the total cell population.

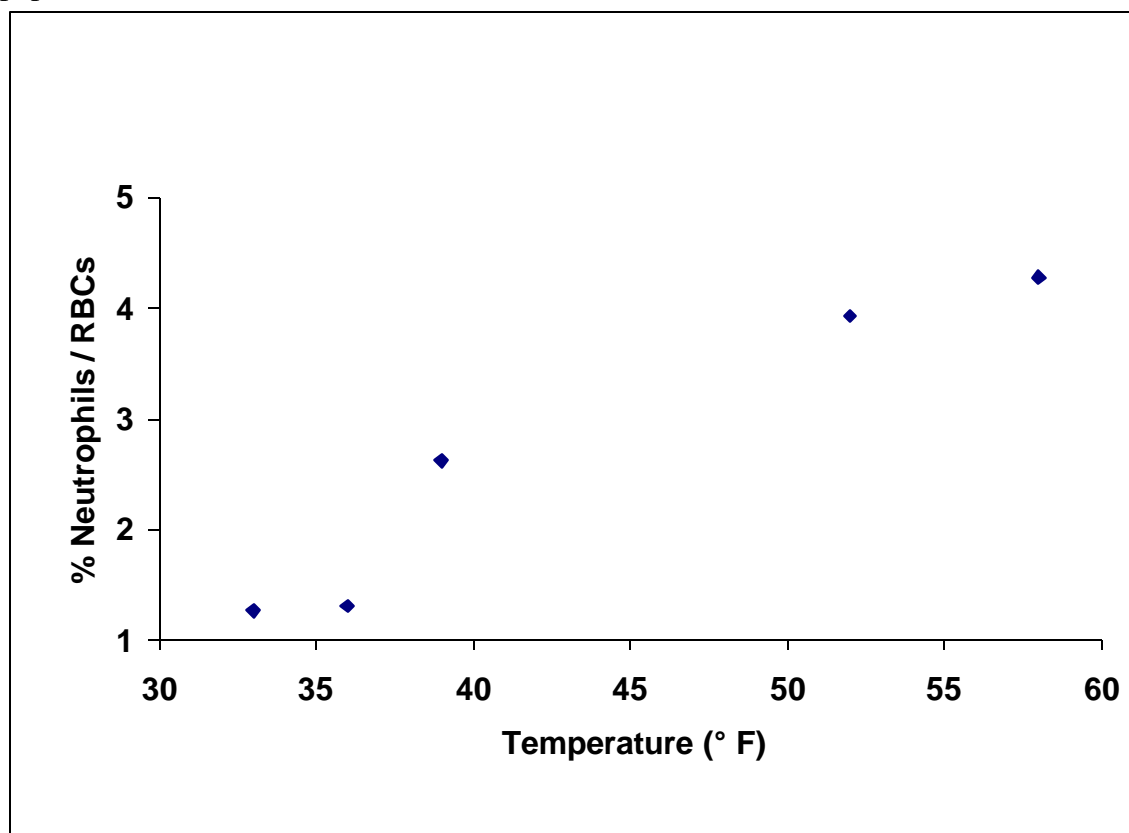


Fig. 17. Water temperature at the sturgeon sampling site, the Old River Control Structure Complex on the Mississippi River, versus the percent neutrophils relative to the RBC counts. RBC is red blood cells.

Results of flow cytometry:

Blood was analyzed for relative genome size and DNA integrity. The results are summarized per month.

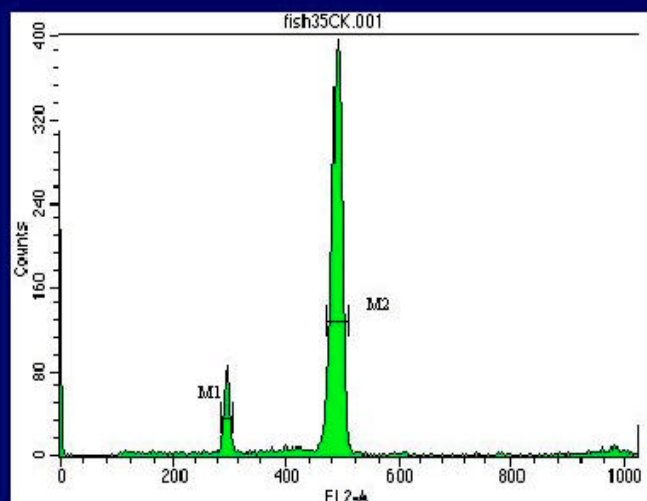
November: Normal profile for DNA content and DNA integrity.

December: Normal profile for DNA integrity.

NWRC Fish 35 (pit tag 131914717A), having physical anomalies (fig. 18) and having been identified as a shovelnose sturgeon, had greater DNA content than the other fish (fig. 19).



Fig. 18. Digital photograph of fish 131914717A pit tag, or NWRC 35. Note the malformed pectoral fin.

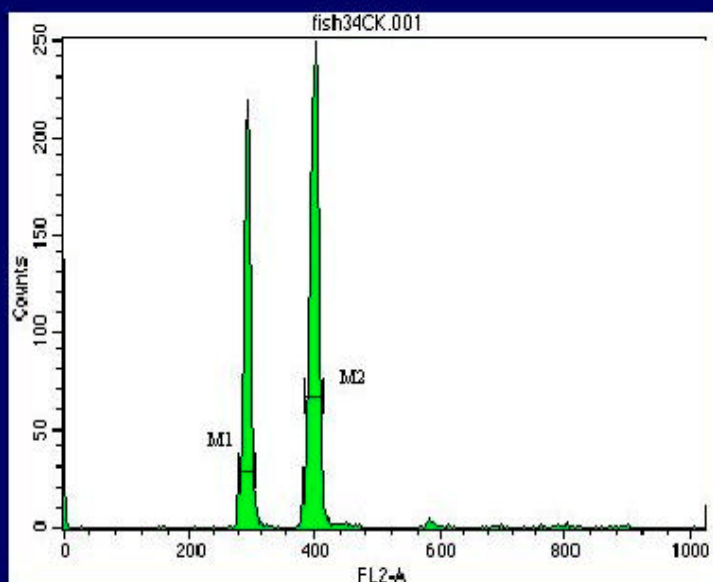


Histogram Statistics

File: fish35CK.001
 Log Data Units: Linear Values
 Acquisition Date: 17-Dec-01
 Gate: No Gate
 Total Events: 10000
 X Parameter: FL2-A (Linear)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	10000	100.00	100.00	444.48	323.56	35.50	486.00	491
M1	285, 307	846	8.46	8.46	295.32	295.29	1.47	295.00	296
M2	473, 512	7244	72.44	72.44	489.16	489.10	1.50	489.00	491

Fig. 19. Flow cytometry frequency histogram indicating DNA content of blood from sturgeon NWRC ID 35 (peak at 491) and internal chicken blood standard (peak at 296). Peaks are designated with markers (M).



File: fish34CK.001									
Log Data Units: Linear Values									
Acquisition Date: 17-Dec-01									
Gate: No Gate									
Total Events: 7395									
X Parameter: FL2-A (Linear)									
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	0, 1023	7395	100.00	100.00	321.52	180.29	43.20	390.00	0
M1	279, 307	2419	32.71	32.71	292.98	292.95	1.62	293.00	293
M2	384, 415	3826	51.74	51.74	398.65	398.61	1.50	399.00	400

Fig. 20. Flow cytometry frequency histogram indicating DNA content of blood from sturgeon NWRC ID 34 (peak at 400) and internal chicken blood standard (peak at 293). This histogram, indicating the distance between the peaks, was the most common among all the samples. Peaks are designated with markers (M)

The nuclear DNA content of chicken erythrocytes is about 2.5 pg/cell (Tiersch and Chandler, 1989), and clearly less than the DNA content of sturgeon 34 and 35 (and all other samples) (figs. 19 and 20). Although detailed analyses of geometric means were not performed for each sample, the distance between peaks was measured linearly per sample. Sturgeon 35 was clearly different from the other samples, where the genome content for sturgeon 35 is estimated at 4.2 pg (fig. 19) and the other samples were estimated at 3.4 pg DNA.

January: Fish 51 (pit tag 115225461A) appeared to have a hyperdiploid peak (fig. 21 top). Upon closer inspection by the following flow dot plot (fig. 21 bottom), the peak was not due to increased

DNA amount, but to degraded DNA. This is indicated by the pattern of increased stain binding to smaller nuclei (Alanen and others, 1989; Zbieranowski and others, 1993).

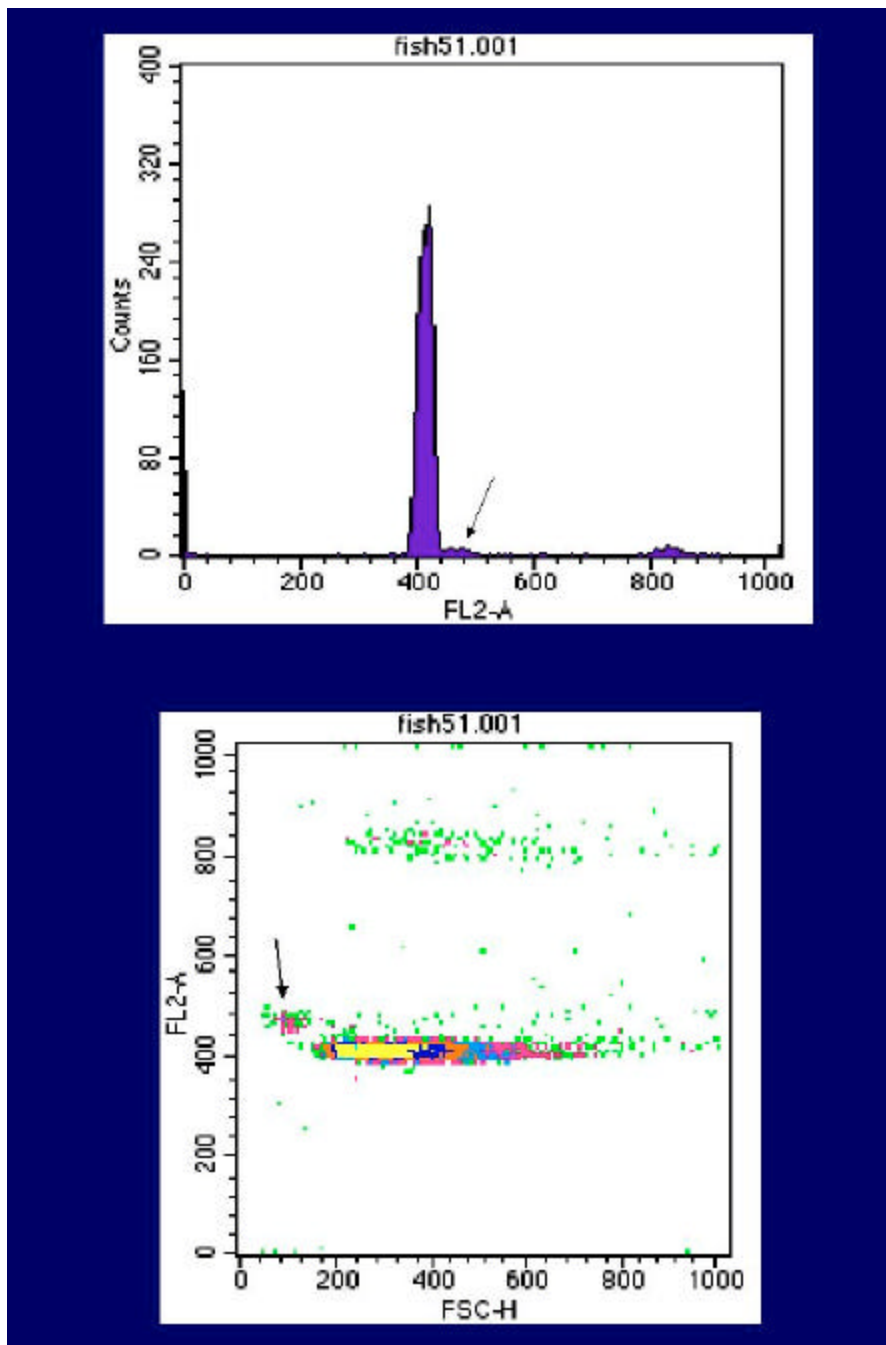


Fig. 21. Flow cytometry histogram and density plot of DNA content of nuclei from blood from sturgeon NWRC ID 51. A hyperdiploid peak is noted (arrow) (top). When size (FSC-H) is compared with DNA staining (FL2A), the position of the subpopulation of nuclei (arrow) indicates a degraded sample, not being an indication of DNA fragmentation (bottom).

February: Fish 63 (pit tag 1133109513A), having two different species designations in FWS spreadsheet), had two diploid peaks close to each other (fig. 22 top). Fish 68 (pit tag 132619753A) had a similar profile as fish 63, except the peaks were more clearly separated (fig. 22 bottom). Fish 68 also was noted as having two species designations in the FWS spreadsheet (Jan Dean, personal communication; see Appendix).

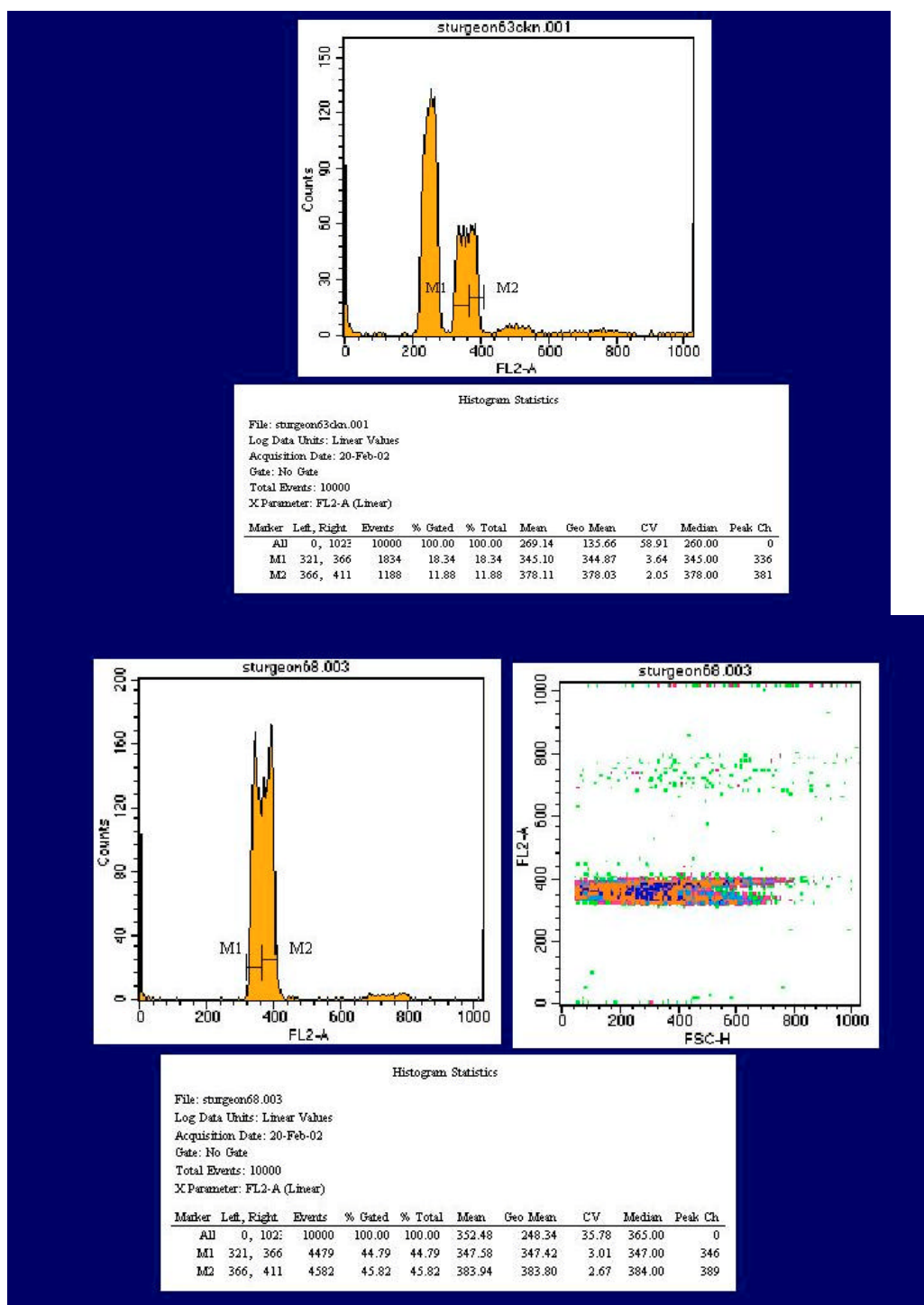


Fig. 22. Flow cytometry histograms of DNA content of sturgeon (right peak, Marker 1 and Marker 2) with internal chicken standard (left peak) (top). Bottom histogram without internal chicken control shows two peaks (Marker 1 and Marker 2) clearly, and the size (FSC-H) versus DNA content (FL2A) does not indicate the sample was degraded and shows two fluorescent subpopulations of different sizes. There was a 45 channel difference in DNA amount between diploid peaks for Fish 63, and a 43 channel difference in DNA amount between diploid peaks for Fish 68. The doublet peaks may indicate a hybrid sturgeon.

March: Normal profiles for DNA content and DNA integrity.

April: Normal profiles for DNA content and DNA integrity.

Discussion

In light of efforts to restore depleted populations of sturgeon, these data add to the physiological information needed for better understanding pallid, shovelnose sturgeon, and their hybrids. There are multiple reasons for studying blood of endangered and threatened species. Firstly, the use blood provides a nondestructive sample, thereby not decreasing animal numbers. Moreover, the data are obtained from the focus species, and not a surrogate. Secondly, species determinations can be assisted by the study of stained blood cell DNA content. Thirdly, loss of DNA integrity could point to possible exposure to anthropogenic compounds. Fourthly, baseline normal values for differential blood cell counts can establish ranges of blood cells numbers that reflect average values for the species. In our study, sampling blood over the course of six months and obtaining resultant differential cell counts have begun to provide a basis for delineating the normal ranges of blood cell values for the pallid sturgeon. Because the percentages of neutrophils for the final three sampling months appeared high (fig. 7), data will be compared to both a second year of sampling (planned 2003 – 2004 study) and a recount on the slides from this 2001-2002 sampling season. Finally, additional valuable information can be obtained from blood, such as a measure of stress by serum cortisol concentrations, or measures of sex steroids or ions that can be used to assist with gauging breeding readiness.

Overall, fish did not show extremely high or low values of WBC counts (fig. 6), and no fish showed DNA integrity loss (fig. 21). In a previous study of baseline contaminant levels in shovelnose sturgeon from the same study area, whole body levels of organochlorines and toxaphene indicated exposure to environmental contamination (Conzelmann and others, 1997). The results of this study found no evidence of genotoxicity.

Under normal circumstances, all nonreplicating normal diploid cells are in the G_0 and G_1 phases of the cell cycle, and these nuclei should have the same DNA content. By the inclusion of a sample with a known DNA content with the unknown sample, one can measure the DNA content of the unknown relative to the known (Tiersch and Chandler, 1989). One fish had a notably higher DNA content than the others (figs. 19 and 20), and two fish presented two diploid peaks each (fig. 22).

The range of the heterophil or neutrophil numbers in fish blood is quite wide, but this cell type is usually not the predominant leukocyte in peripheral circulation (Stoskopf, 1992b). The increase in this cell type is a relatively rapid response in fish – within 24 hours – and has appeared to be independent of temperature (Stoskopf, 1992b). Stress was likely induced due to gillnet capture. An increase in WBC and neutrophil counts appeared directly or indirectly related to temperature (figs. 16 and 17). Fathead minnows, *Pimephales promelas*, were shown to have increased numbers of leukocytes during their spawning season during a time of increased temperatures, where the count tripled (Thomas and others, 1999). In stressed channel catfish *Ictalurus punctatus*, neutrophils approximated 30% of the circulating leukocytes, and in non-stressed catfish levels were about 4% (Ellsaesser and Clem, 1986). In fish, an increase of neutrophils due to stress responses is frequently associated with a decreased overall leukocyte number (Slicher, 1961) and may be seen in instances of chronic stress (Adams, 2002). In this study, some individuals exhibited this pattern (figs. 9 and 10). Again, additional monthly sampling of pallid sturgeon will be illustrative.

Thrombocytes are responsible for clot formation and are considered to be distinct and unrelated to lymphocytes. Thrombocytes were noted to clump (fig. 2). For the purposes of this study, although some were distinct by their elongated, elliptical, or fusiform shape (fig. 4), they could not be reliably separated from the other WBC types. Counting thrombocytes as WBC may have inflated the overall WBC counts.

The use of either WG or SB showed that each is a reliable method for distinguishing WBC from RBC for pallid sturgeon. In the upcoming study for 2003 – 2004, the flow cytometry data from the 2001 – 2002 season will be analyzed for DNA content rather than simply noting relative genetic differences among the population studied. Additionally, another replicate of neutrophil counts will be performed on the 2001 – 2002 microscope slides. Data obtained from the blood of pallid, shovelnose sturgeon, and their hybrids from 12 months over two field seasons of sampling will provide a solid basis for assisting in delineating sturgeon health and assist with distinguishing between the species and their hybrids.

Acknowledgments

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Appendix

Notes derived from minutes of Pallid Sturgeon Lower Basin Work Group as recorded by Karen Kilpatrick, November 2002

Over 280 sturgeons of the genus *Scaphirhynchus*, including pallid and shovelnose sturgeon and their hybrids, have been collected via gill nets from the outflow channel of the Old River Control Structure Complex in Concordia Parish, La., during November 2001 through April 2002. Fish number collected was about 20 fish each month.

At the waterside, data were collected on at least 20 fish. Most were implanted with passive integrated transponder (PIT) tags and released into the same waters near their capture site.

Datasets and Preliminary Conclusions:

1 Mark-recapture population estimate. Using the recaptured fish, a mark-recapture population estimate was made for the study area.

An assumption for the Schnabel mark-recapture population estimate is that the population is closed.

2. Age: on X number of animals that were euthanized or that died.
3. Species identification.
 - a. Visual observations using digital pictures of the ventral side, head area, and lateral view of the heads. Morphometric character indices, too. By visual observations, the populations comprised: 10% pallids, 35% hybrids, and 55% shovelnose. Pallid percentages were 32 and 26% in Nov. and Dec.
 - b. Fin clips are being used for genetic (allozyme) analyses.
4. Iridovirus. Out of 263 samples, four confirmed and one suspected incident were found, but none were in pallids. This is the first documentation of iridovirus in wild sturgeon from the Lower Mississippi population.
5. Physical anomalies or damage. 46 fish positive.
6. Gastric lavage. Food habits.
7. Differential blood cell counts. Hematological characteristics.
8. Genome size.
9. Ploidy. Two fish were classified as aneuploids and one fish had slightly more DNA than the others.

J. Dean presented this Jan. 03 at: the Middle Basin Pallid Sturgeon Recovery Work Group meeting in St. Louis. "For my presentation on the 6-month study, I plan to mention your work and show a couple of slides from your data (relative no. of red and white blood cells)."
10. Vocalizations.
11. Evaluation of MS-222 as an anesthetic for sturgeon.

Habitat diversity and managing to increase population is the best management practice for restoring all the sturgeon species, as decided by the Work Group.

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