Comparative Medicine
Copyright 2017
by the American Association for Laboratory Animal Science

Vol 67, No 3
June 2017
Pages 263-269

Original Research

Stress Leukogram Induced by Acute and Chronic Stress in Zebrafish (*Danio rerio*)

Agata K Grzelak,[1] Daniel J Davis,[1] Susan M Caraker,[2] Marcus J Crim,[2] Jan M Spitsbergen,[3] and Charles E Wiedmeyer[1,*]

The use of zebrafish (*Danio rerio*) as an animal model for experimental studies of stress has increased rapidly over the years. Although many physiologic and behavioral characteristics associated with stress have been defined in zebrafish, the effects of stress on hematologic parameters have not been described. The purpose of our study was to induce a rise in endogenous cortisol through various acute and chronic stressors and compare the effects of these stressors on peripheral WBC populations. Acutely stressed fish underwent dorsal or full-body exposure to air for 3 min, repeated every 30 min over the course of 90 min. Chronically stressed fish underwent exposure to stressors twice daily over a period of 5 d. After the last stressful event, fish were euthanized, and whole blood and plasma were obtained. A drop of whole blood was used to create a blood smear, which was subsequently stained with a modified Wright–Giemsa stain and a 50-WBC differential count determined. Plasma cortisol levels were determined by using a commercially available ELISA. Endogenous cortisol concentrations were significantly higher in both stressed groups as compared with control fish. Acutely stressed fish demonstrated significant lymphopenia, monocytosis, and neutrophilia, compared with unstressed, control fish. Chronic stress induced lymphopenia and monocytosis but no significant changes in relative neutrophil populations in zebrafish. The changes in both stressed groups most likely are due to increases in endogenous cortisol concentrations and represent the first description of a stress leukogram in zebrafish.

Abbreviation: GR, glucocorticoid receptor

A CBC analysis is one of the most common diagnostic tests performed in clinical medicine. This test can detect a variety of ongoing changes throughout the body by measuring multiple components of blood, including the concentrations of RBC and WBC. In this study, we sought to measure the specific changes in peripheral WBC populations caused by acute and chronic stressors. These changes have already been established as a 'stress leukogram' in many mammalian models and a few teleost species.[35] Altered levels of circulating leukocytes reflect a physiologic response to a rise in endogenous glucocorticoid concentrations, such as cortisol, that often occurs after a stressful event. As in other species, studies have shown that zebrafish respond to stressful stimuli with a rise in cortisol.[12,21,23] However, zebrafish studies to date have not investigated the specific effects of cortisol on WBC populations.

In mammals, stress-induced glucocorticoid secretion is regulated by the HPA axis. This axis consists of 3 major endocrine glands that share a complex set of interactions involving positive and negative feedback mechanisms. Studies have shown that fish regulate stress-induced glucocorticoid secretion similarly to mammals but by the hypothalamic–pituitary–internal axis.[1,31] This axis is analogous to the mammalian HPA axis and shares extensive homologies between the different components, including many of the main endocrine glands involved in mammals.[14,27] Although zebrafish do not have a separate adrenal gland, their head kidney tissue is analogous to the adrenal cortex in mammals, and glucocorticoids are synthesized and secreted by the interrenal tissue.[31] One large advantage of using zebrafish in stress research is that the final product of this stress-regulating system is cortisol, which is the primary glucocorticoid in humans.[27] In contrast, murine models synthesize and secrete corticosterone, a weak glucocorticoid in humans that primarily serves as an intermediate in the formation of aldosterone.[27]

Although both the HPA and hypothalamic–pituitary–internal axes play a major role in cortisol secretion during stress, endogenous cortisol concentrations are also mediated by 2 important intracellular corticosteroid receptors that are expressed in both mammalian and teleost tissues, the mineralocorticoid receptor and the glucocorticoid receptor (GR). Most fish contain 2 isoforms of the GR, thought to have arisen from a whole-genome duplication event occurring 350 million years ago.[18] Zebrafish, in contrast, are similar to mammals in that they contain only a single GR isoform.[1] This similarity is important from the standpoint that duplicated genes can acquire new functions,[3] making zebrafish an even more favorable model organism for stress studies.

The purpose of the current study was to examine the defining morphologic characteristics of individual WBC types (lymphocytes, monocytes, neutrophils, eosinophils) and ultimately analyze the effects of acute and chronic stressors on peripheral WBC populations. To illustrate a direct link between rises in endogenous cortisol and changes in peripheral WBC, we

Received: 04 Aug 2016. Revision requested: 23 Sep 2016. Accepted: 21 Nov 2016.
[1]Department of Veterinary Pathobiology, University of Missouri, and [2]IDEXX Bioresearch, Columbia, Missouri, and [3]Department of Microbiology, Oregon State University, Corvallis, Oregon
**Corresponding author. Email: wiedmeyerc@missouri.edu*

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-28

used ELISA to measure plasma cortisol concentrations in both stressed and nonstressed groups of fish. We show that acute and chronic stressors increase plasma cortisol concentrations and subsequently alter WBC populations in circulating blood. These results have important implications, not only in the progression of clinical zebrafish pathology but also in the use of zebrafish as an animal model for human and mammalian stress and hematologic research.

Materials and Methods

Animals and husbandry. Adult wild-type zebrafish were purchased from Aquatica BioTech (Sun City Center, FL). All fish were acclimated to the facility for 1 wk prior to any treatment and were maintained in recirculating 3-L tanks at 28 ± 2 °C. Fish were kept on a 14:10-h light:dark cycle and fed a commercial fish diet (TetraMin Tropical Flakes, Tetra Spectrum Brands, Blacksburg, VA) twice daily. All experimental procedures were approved by the University of Missouri's IACUC and were performed according to the guidelines set forth in the *Guide for the Use and Care of Laboratory Animals.*[15]

Acute stress protocol. After a 1-wk acclimation period, 11 adult fish were randomly selected to undergo a series of 2 alternating 3-min long acute stressors performed every 27 min (total time, 108 min). Stressors comprised full-body exposure to air by suspension in a net (air stress) and swimming at extremely low water levels (low-water stress), such that the dorsum of the fish was exposed to air (Figure 1). Before the application of the stressor, 11 adult fish were sampled and served as controls. At 108 min after the initial start of the protocol (15 min after the last stressor was performed), all fish were euthanized by using previously described techniques.[8] Fish were individually placed in a 50-mL conical tube containing 20 mL 0.1% (100 mg/L) clove oil in sterile water.

Chronic stress protocol. A chronic unpredictable stress paradigm was modified from a previous study.[5] Zebrafish were exposed to stressors twice daily for 5 d. A detailed list of the stressors used is provided in Figure 2. To avoid potential habituation to stressors, the specific stress paradigm and its timing were chosen randomly each day (Figure 2).

Blood collection and hematologic analysis. Immediately after euthanasia of each fish, blood was collected into a heparinized capillary tube by using cardiocentesis. This method was modified from a technique described previously.[26] Blood smears were prepared from whole blood and stained with Wright–Giemsa (Hematek Slide Stainer, Siemens Health Care Diagnostics, Tarrytown, NY). The slides were examined and leukocyte differentials performed under oil immersion at 100× magnification.

Serum collection and cortisol ELISA. Blood was collected by sharp transection of the caudal peduncle a few millimeters cranial to the caudal fin. Fish were then placed in a fenestrated microtube (0.6 mL) nested within a 1.5-mL microfuge tube for centrifugation at 400 × *g* for 5 min at room temperature. The 1.5-mL tubes containing the blood samples were centrifuged at 13,800 × *g*, 4 °C for 15 min. Supernatants were recovered, and the serum was stored at –80 °C until analysis. Serum cortisol concentrations were determined by using a cortisol ELISA kit (Salimetrics, Carlsbad, CA) according to the manufacturer's instructions. The sensitivity of the assay is less than 0.007 µg/dL, and cortisol concentrations were read on a plate reader (SpectraMax M3, Molecular Devices, Sunnyvale, CA).

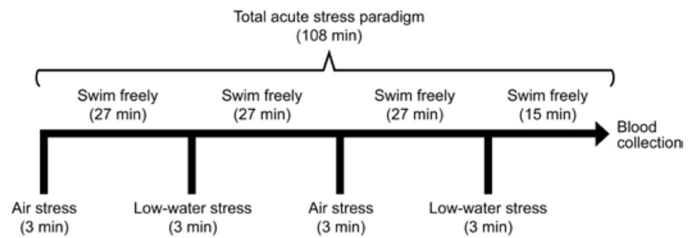

Figure 1. Acute stress paradigm.

	Morning	Evening
Day 1	Chasing (8 min)	Tank changes (x6)
Day 2	Over-crowding (60 min)	Dorsal body exposure (2 min)
Day 3	Social isolation (30 min)	Tank changes (x6)
Day 4	Dorsal body exposure (2 min)	Chasing (8 min)
Day 5	Tank changes (x6)	Dorsal body exposure (2 min)

Figure 2. Chronic stress paradigm.

Statistics. Data were analyzed by using Prism (GraphPad Software, La Jolla, CA). Statistical significance was set at a *P* value of less than 0.05. Leukocyte counts and cortisol data were analyzed by using 2-way ANOVA, with treatment (control compared with stress) and type of stressor (acute compared with chronic) as the 2 factors. A Student–Newman–Keuls posthoc test was performed for pairwise comparisons.

Results

Characterization of zebrafish leukocytes based on morphology. According to morphologic characteristics, 4 WBC types were present in peripheral blood of adult zebrafish (Figure 3). Differential counts indicated that lymphocytes were the predominant leukocyte, followed by lower percentages of monocytes and neutrophils (Table 1). Eosinophils comprised the smallest percentage of circulating leukocytes and were rarely found on peripheral blood smears (Table 1).

Lymphocytes varied in shape and size but mainly had a round appearance, with sporadic pseudopodia lining the cytoplasmic border (Figure 3 A through D, J, and K). Occasionally, lymphocytes morphed around surrounding cells (Figure 3 C). The lymphocyte nucleus generally remained round and occupied more than half of the cytoplasm. The cytoplasm of lymphocytes varied from light blue containing small pink granules (Figure 3 C and D) to deeply basophilic (Figure 3 J and K). In addition, sporadic vacuoles were dispersed throughout the cytoplasm. The morphologic differences in the lymphocytic lineage most likely reflect the different types of lymphocytes present in peripheral blood. The differentiation of plasma cells, T cells, and natural killer cells according to morphologic characteristics alone has not been described for zebrafish. Although thrombocytes might be mistaken for small lymphocytes,[16] examining cellular characteristics by using Wright–Giemsa stain revealed the smaller size and clear cytoplasmic border of thrombocytes, allowing them to be distinguished from small lymphocytes in zebrafish.

Monocytes, at times, had a similar appearance to lymphocytes but were distinguished by their larger size, irregularly ovoid nucleus, smaller nuclear:cytoplasmic ratio, and increased amounts of vacuoles throughout a lighter cytoplasm (Figure 3 E through G).

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-28

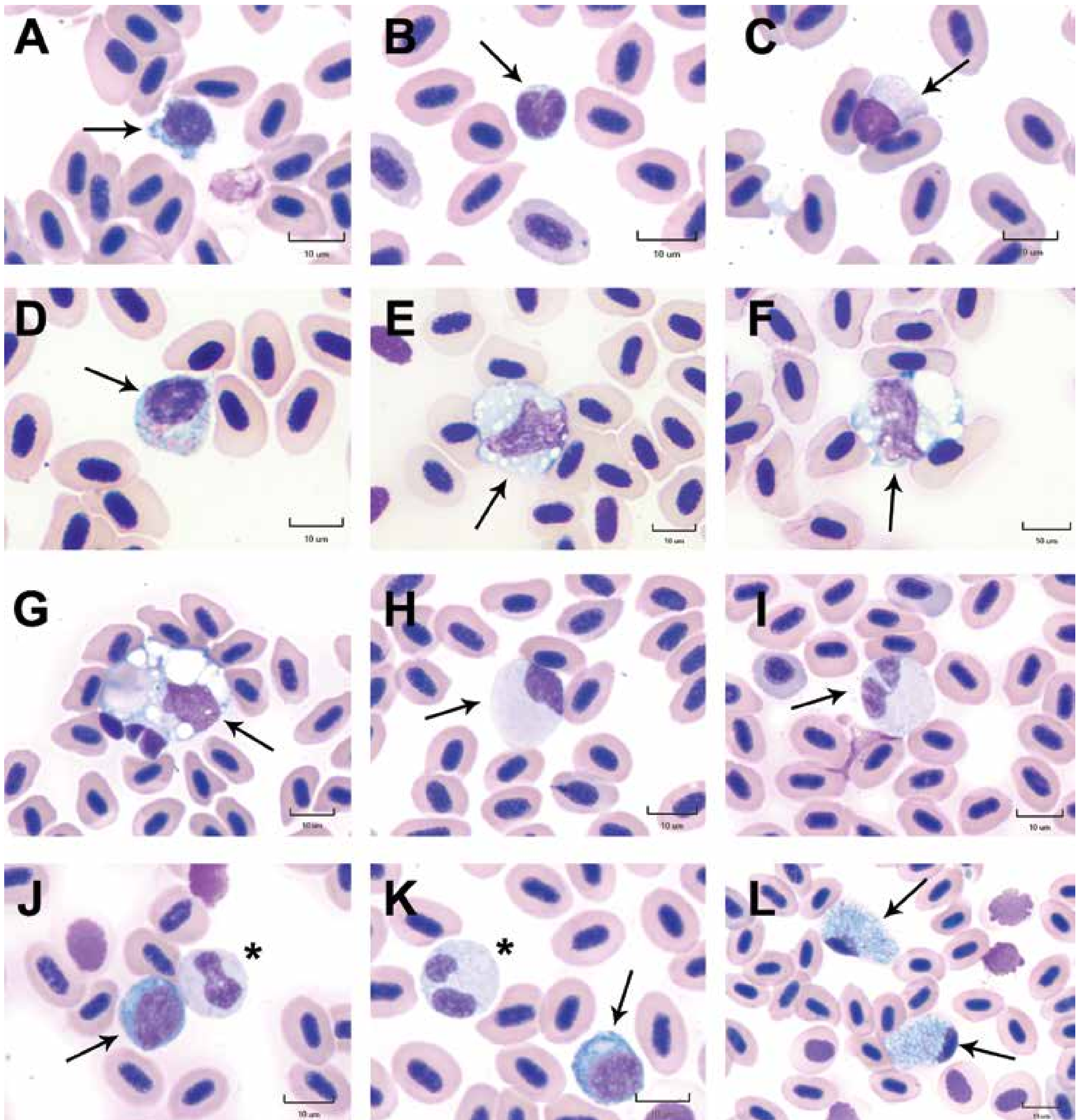

Figure 3. Microstructure of peripheral blood cells in zebrafish. (A–D) Lymphocyte (arrow). (E and F) Monocyte with vacuoles in cytoplasm (arrow). (G) Monocyte engulfing material (arrow). (H) Neutrophil with peripheral nucleus (arrow). (I) Neutrophil with segmented nucleus (arrow). (J and K) Lymphocyte (arrow) and monocyte (asterisk). (L) Eosinophils (arrows). Wright–Giemsa stain; bars, 10 µm.

Occasionally, they appeared exceptionally large and contained engulfed material within phagosomes (Figure 3 G).

Neutrophils, also known as heterophils, were well differentiated from other leukocytes, with a grainy, clear to pale-blue cytoplasm (Figure 3 H through K). The nucleus of neutrophils varied, taking on a segmented, band, or round appearance. Eosinophils, the other granulocytic cell in zebrafish, were larger than neutrophils, and, unlike in mammals, circulating eosinophils contained basophilic cytoplasmic granules on Wright–Giemsa staining (Figure 3 L). Zebrafish eosinophils had a peripheral

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-28

Table 1. WBC differential counts (%) in control and acutely and chronically stressed zebrafish

	Acute				Chronic			
	Control group (n = 11)		Stressed group (n = 11)		Control group (n = 7)		Stressed group (n = 7)	
	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range	Mean ± SEM	Range
Lymphocytes	76.36 ± 2.48	64–92	54.55 ± 3.25	38–74	80.00 ± 2.69	70–90	59.29 ± 4.17	42–74
Monocytes	11.64 ± 2.32	4–32	22.55 ± 2.99	10–42	8.57 ± 1.94	2–16	30.14 ± 3.36	18–46
Neutrophils	10.91 ± 1.93	2–18	23.64 ± 4.87	8–62	10.57 ± 2.89	4–22	9.14 ± 2.86	2–22
Eosinophils	0.91 ± 0.41	0–4	0.18 ± 0.18	0–2	0.86 ± 0.40	0–2	1.43 ± 1.43	0–10

nucleus with a dense chromatin pattern, but nuclear borders were often obscured by the overwhelming vacuolization within the cytoplasm.

Serum cortisol levels. Serum cortisol was measured in control fish and in both groups of stressed fish (Figure 4). Compared with levels in controls, serum cortisol concentrations were significantly higher in acutely and chronically stressed fish ($F_{1,56}$ = 29.58, P = 0.00586; $F_{1,56}$ = 29.58, P = 0.0000268, respectively; Figure 4). A trend toward greater serum cortisol concentrations in the acutely stressed group compared with the chronically stressed group was noted ($F_{1,56}$ = 29.58, P = 0.067; Figure 4).

Leukocyte differentials. The relative percentages of circulating leukocytes in peripheral blood were significantly altered after either acute or chronic stress paradigms (Figure 5). Acutely stressed fish exhibited a relative decrease ($F_{1,32}$ = 43.10, P = 0.0000319) in lymphocytes (Figure 5 A) and increase in both monocytes ($F_{1,32}$ = 32.73, P = 0.00920; Figure 5 B) and neutrophils ($F_{1,32}$ = 4.058, P = 0.0246; Figure 5 C) compared with percentages in control fish. Chronically stressed fish shared a similar relative decrease ($F_{1,32}$ = 43.10, P = 0.00129) in lymphocytes (Figure 5 A) and increase ($F_{1,32}$ = 32.73, P = 0.000122) in monocytes (Figure 5 B) but did not exhibit a change in neutrophils compared with the control group (Figure 5 C). Relative eosinophil counts did not differ in either acutely or chronically stressed fish (Figure 5 D). However, one chronically stressed fish with no apparent disease had a higher proportion of eosinophils, consisting of 10% of the leukocyte population.

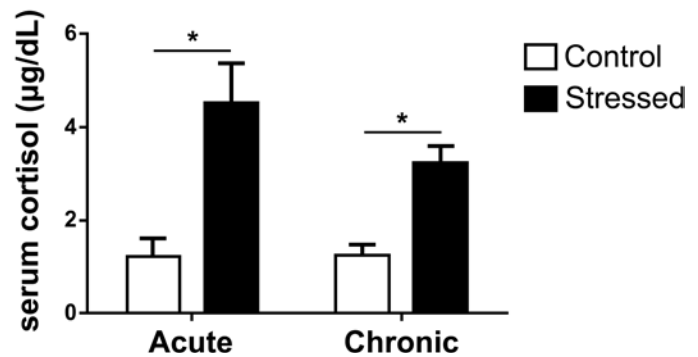

Figure 4. Serum cortisol concentrations (µg/dL) in zebrafish from control and acutely and chronically stressed groups. Serum cortisol levels were increased in stressed zebrafish compared with their control groups. Data are shown as mean ± SEM (n = 7 each in acute control and stressed groups; n = 11 each in chronic control and stressed group). *, P < 0.05 (2-way ANOVA).

Discussion

The use of zebrafish as models for stress research has been increasing rapidly in recent years. Studies have already outlined the mechanisms of cortisol secretion and regulation in zebrafish, mechanisms that share extensive homologies with mammalian models.[12,20,23,27] Like humans, zebrafish respond to stressful stimuli by activating the hypothalamic–pituitary–internal axis and ultimately increasing endogenous cortisol secretion. Our study evaluated this response one step further and illustrated the changes in peripheral leukocyte populations that accompany increased serum cortisol concentrations. These changes can be identified as a stress leukogram in zebrafish.

Stress leukograms vary among species but are typically characterized by lymphopenia and neutrophilia, with occasional monocytosis and eosinopenia. Our findings in both acute and chronic stress groups were consistent with mammalian models, demonstrating a relative decrease in circulating lymphocytes in all stressed subjects. The exact mechanisms of cortisol and its effects on circulating leukocytes have not been well defined. Lymphopenia is the result of an immediate shift of lymphocytes from the circulating blood to other tissues, but the specific location is unknown. Glucocorticoids can induce apoptosis of lymphoid cells in both mammals and fish,[24,30,33] and chronic exposure to glucocorticoids can lead to lymphotoxicity, lymphoid hypoplasia, and decreased lymphopoeisis.[17,28]

In addition, monocytosis was present in both our chronic and acute stress groups. This effect is most likely the result of a shift from a marginalized to a circulating population of monocytes. The direct mechanisms of this shift are still unknown but may be due to changes in the expression of adhesion molecules and chemotactic cytokines to interfere with the trafficking of leukocytes into tissues.[6] Neutrophilia occurred only in our acute stress group, and similar to the situation with monocytes, a relative shift of neutrophils into circulating blood most likely contributed to this change. However, in vitro studies have also shown that cortisol can inhibit neutrophil apoptosis in carp, an effect that is mediated specifically by glucocorticoid receptors.[32] The fact that circulating neutrophils did not increase in our chronically stressed group might be due to the downregulation of different glucocorticoid receptors during chronic exposure to increased cortisol concentrations. Cortisol concentrations are regulated by these receptors, which are located intracellularly in various tissues, including blood leukocytes. One study demonstrated that chronic elevation in plasma cortisol downregulates corticosteroid receptor concentrations in the gills of coho salmon.[25] Another study illustrated the downregulation of mRNA levels of glucocorticoid receptors in the brain of carp as a result of prolonged exposure to stress.[29] It is clear that glucocorticoid receptors and cortisol concentrations have an extensive relationship based on various feedback mechanisms, but the role they have on circulating WBC populations in zebrafish has yet to be explored.

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-28

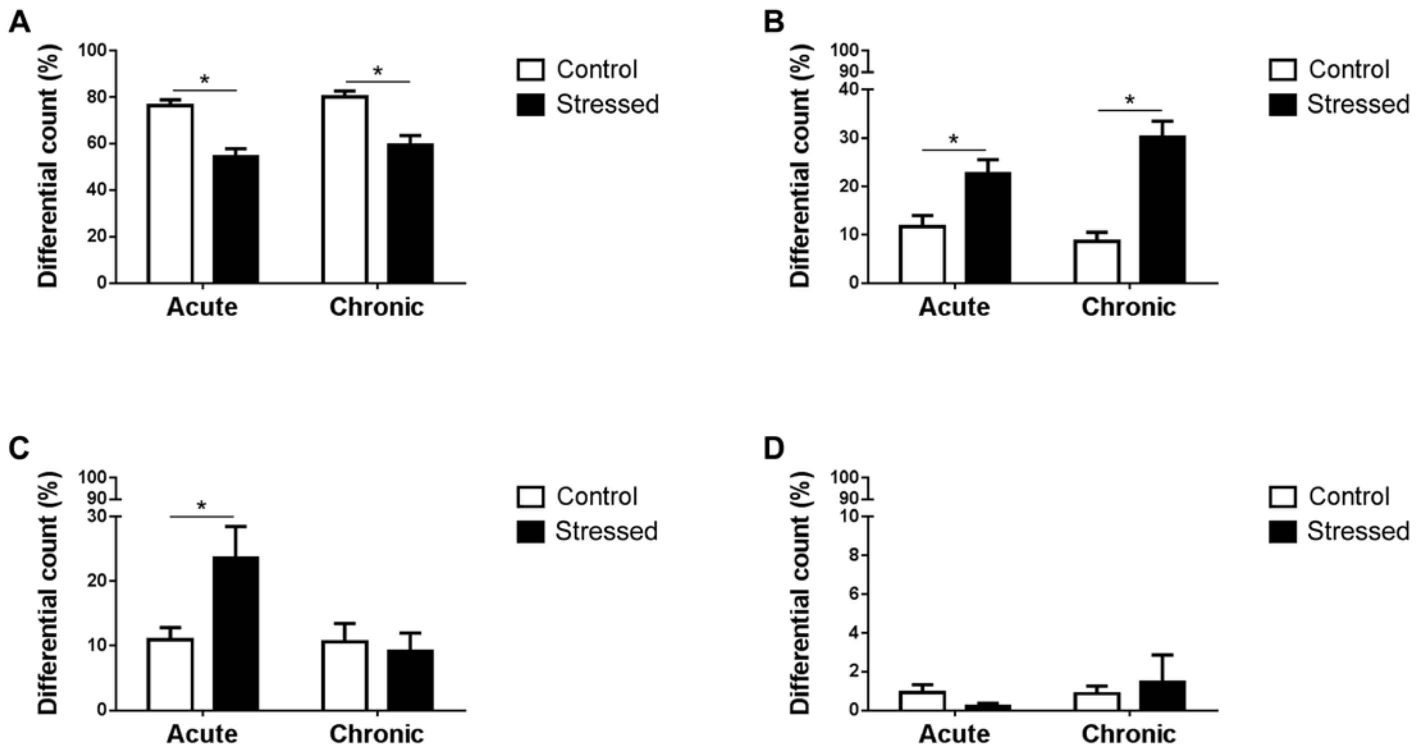

Figure 5. WBC differential counts (%) in acutely and chronically stressed zebrafish and their controls. (A and B) WBC differential counts reveal that acutely and chronically stressed fish have (A) significant reductions in peripheral lymphocytes and (B) significant increases in peripheral monocytes compared with their respective control groups. (C) Peripheral neutrophil numbers are significantly increased in acutely stressed fish but are not altered in chronically stressed fish, compared with their respective control groups. (D) Eosinophil differential counts remained unaltered in both acutely and chronically stressed fish compared with their respective control groups. Data are shown as mean ± SEM ($n = 7$ each in acute control and stressed groups; $n = 11$ each in chronic control and stressed groups). *, $P < 0.05$ (2-way ANOVA).

One of the most interesting aspects of a stress leukogram is the variation between individuals within the same species. Although our data display intragroup variability, ranges from our control groups are consistent with a study that published the first reference intervals for WBC differential counts in zebrafish.[19] These ranges are most likely due to normal variations within the blood leukocyte populations of a single species. Therefore, it is important to consider the differences between individuals when analyzing hematologic parameters.

Zebrafish hematology is still at its infancy, and many hematologic parameters are unknown. Routine procedures in other model systems, such as making blood smears, can be challenging in small teleost models, especially when working with minute quantities of blood that rapidly clot and undergo hemolysis. Studies have been able to work around blood collection altogether by using transgenics to tag individual WBC with different fluorescent markers.[34] Using these techniques has allowed researchers to visualize leukocyte trafficking in vivo in genetically modified transparent zebrafish.[13] Although the information gained from these innovative studies is highly useful in mapping the mechanisms of zebrafish immunity, these methods require specific materials and tools that are not accessible to all laboratories. Therefore, we wanted to establish methods that are easily reproducible and can be done with the most basic approach. Although our study specifically focuses on the relative WBC populations found in peripheral blood, our method allows for the potential of assessing many other hematologic parameters. Changes in these parameters are not limited to the effects of stress. Many diseases and neoplasms can be evaluated through blood analysis. One study has already identified eosinophilia in zebrafish exposed to helminth infections.[2]

One of the largest limitations in the development of zebrafish clinical pathology is that blood collection has ultimately been lethal. Repeated blood collections from individual zebrafish have been attempted only in one study,[36] but the damage induced and the resulting secondary physiologic effects have yet to be determined. It would be advantageous to explore the potential for using larger mutants or relatives, such as the giant danio (*Devario aequipinnatus*), of the common laboratory species of zebrafish, which would provide greater quantities of blood with the possibility of repeated blood collections. In doing so, studies might track hematologic changes over time. Regardless, sampling blood from a few zebrafish by using current methods can still be useful clinically, such as for assessing the health status of a population sharing the same water source. However, further development in finding viable blood collection methods likely would provide even more opportunities in the field of zebrafish clinical pathology.

Similar to the variety of blood collection methods published in zebrafish research, cortisol collection and measurement techniques have differed in zebrafish stress studies. Much of the current literature describes methods for extracting whole-body cortisol and using cortisol-specific radioimmunoassays to measure cortisol concentrations per gram of fish.[22,23] Others have used commercial enzyme immunoassay kits on a variety of samples, including trunk cortisol[20] and gill filaments.[11] One study in particular used RIA to measure cortisol in plasma collected from

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-28

Table 2. Comparison of baseline and peak cortisol concentrations in zebrafish after exposure to stressors according to collection and quantification methods gtw, trunk weight in grams; EIA, enzyme immunoassay, MS222, tricaine methanesulfonate; RIA, radioimmunoassay

Stressor type	Sample	Cortisol quantification method	Cortisol concentration		Reference
			Baseline	Peak	
1) Acute net handling and air exposure 2) Chronic unpredictable stress (5 d)	Plasma	Human salivary cortisol ELISA kit	1) 1.22 ± 0.39 µg/dL 2) 1.25 ± 0.36 µg/dL	1) 4.51 ± 0.86 µg/dL 2) 3.228 ± 0.36 µg/dL	Current
1) Crowding (3 h) 2) Crowding (5 d)	Whole-body cortisol extraction	Cortisol-specific RIA	1) 3.2 ng/g fish	1) 11.7 ng/g fish 2) 14.3 ng/g fish	22
Acute net handling	Whole-body cortisol extraction	Cortisol-specific RIA	4–6 ng/g fish	27–35 ng/g fish	23
Social hierarchy: dominant compared with subordinate	Plasma	Cortisol-specific RIA	Dominant fish: 75.66 ng/mL (7.57 µg/dL)	Subordinate fish: 115.95 ng/mL (11.595 µg/dL)	10
1) Acute net handling and air exposure 2) Crowding 3) Background color	Trunk cortisol extraction	Commercial cortisol EIA kit	1) <2.0 ng/gtw 2) <2.0 ng/gtw 3) 4.8 ± 1.1 ng/gtw	1) 11.87 ± 2.46 ng/gtw 2) 6.6-22.4 ng/gtw 3) 37.8 ± 6.7 ng/gtw	20
Acute stressor	Plasma Gills	Commercial cortisol ELISA kit	Plasma: 17.2 ± 4.2 ng/mL Gills: <0.2 ng/mg protein	Plasma: 108.0 ± 30.7 ng/mL Gills: 0.5–0.8 ng/mg protein	11
Euthanasia in clove oil compared with MS222	Plasma	Human salivary cortisol ELISA kit	Euthanasia in clove oil: <1.0 µg/dL	Euthanasia in MS222: 1.5–3.5 µg/dL	7
Chronic unpredictable stress	Plasma	Human salivary cortisol ELISA kit	<2.0 µg/dL	3.0–4.0 µg/dL	8

zebrafish blood.[10] Similarly, our study evaluated plasma from peripheral blood but instead used a human salivary ELISA kit to measure cortisol concentrations. This particular human salivary ELISA kit has previously been used on zebrafish plasma to quantify changes in cortisol after both acute and chronic stressors.[7,8] Although the overall cortisol values we obtained were lower than those in one previous study,[10] they were consistent with those in others.[7,8] In addition, a human salivary ELISA kit was used to measure whole-body cortisol in a pharmacologic study[9] and was described by other authors as a highly sensitive yet simple and inexpensive method of measurement.[4] Comparing cortisol levels between different measuring techniques would be to establish well-defined baseline cortisol concentrations in zebrafish. In addition, it would be interesting to compare a variety of acute and chronic stressors by using a single collection and measurement technique. Doing so would make it possible to relate types of stressors to peak cortisol concentrations. We have provided a table of comparison between the various stressors, methods, and changes in cortisol concentrations published in the studies we described earlier (Table 2).[7,8,10,11,20,22,23] However, the ability to detect changes in cortisol concentrations is the most important factor and is demonstrated in all of the cited studies. We chose to use a human salivary ELISA kit to measure cortisol concentrations in plasma in view of its simplicity and reliability, as shown in other studies.[7,8]

Hematologic analysis is one of the most common diagnostics used in veterinary and human medicine. The findings provide indications of physiologic and pathologic change and can be used to assess various tissues and organs before any outward manifestation of disease occurs. Establishing various hematologic changes that occur in zebrafish is crucial in assessing their health and also provides the opportunity to expand the use of zebrafish as models for human disease. Eliciting a stress leukogram in zebrafish only further exemplifies their importance as a research model that shares many of the conserved physiologic mechanisms found in higher vertebrate species.

Acknowledgments

We thank Miriam Hankins for assistance with animal husbandry and Elizabeth C Bryda, Aaron C Ericsson, Karen M Clifford, and Marina M Hanson for technical assistance and additional consultations.

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-28

References

1. **Alsop D, Vijayan MM.** 2008. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am J Physiol Regul Integr Comp Physiol **294:**R711–R719.
2. **Balla KM, Lugo-Villarino G, Spitsbergen JM, Stachura DL, Hu Y, Banuelos K, Romo-Fewell O, Aroian RV, Traver D.** 2010. Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants. Blood **116:** 3944–3954.
3. **Brunet FG, Crollius HR, Paris M, Aury J-M, Gibert P, Jaillon O, Laudet V, Robinson-Rechavi M.** 2006. Gene loss and evolutionary rates following whole-genome duplication in teleost fishes. Mol Biol Evol **23:**1808–1816.
4. **Canavello PR, Cachat JM, Beeson EC, Laffoon AL, Grimes C, Haymore WAM, Elegante MF, Bartels BK, Hart PC, Elkhayat SI, Tien DH, Mohnot S, Amri H, Kalueff AV.** Measuring endocrine (cortisol) responses of zebrafish to stress, p 135–142. In: Kalueff AV, Cachat JM editors. Zebrafish neurobehavioral protocols. Totowa(NJ): Humana Press.
5. **Chakravarty S, Reddy BR, Sudhakar SR, Saxena S, Das T, Meghah V, Brahmendra Swamy CV, Kumar A, Idris MM.** 2013. Chronic unpredictable stress (CUS)-induced anxiety and related mood disorders in a zebrafish model: altered brain proteome profile implicates mitochondrial dysfunction. PLoS One **8:**e63302.
6. **Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G.** 1992. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. Proc Natl Acad Sci USA **89:**9991–9995.
7. **Davis DJ, Doerr HM, Grzelak AK, Busi SB, Jasarevic E, Ericsson AC, Bryda EC.** 2016. Lactobacillus plantarum attenuates anxiety-related behavior and protects against stress-induced dysbiosis in adult zebrafish. Sci Rep **6:**33726.
8. **Davis DJ, Klug J, Hankins M, Doerr HM, Monticelli SR, Song A, Gillespie CH, Bryda EC.** 2015. Effects of clove oil as a euthanasia agent on blood collection efficiency and serum cortisol levels in *Danio rerio*. J Am Assoc Lab Anim Sci **54:**564–567.
9. **Egan RJ, Bergner CL, Hart PC, Cachat JM, Canavello PR, Elegante MF, Elkhayat SI, Bartels BK, Tien AK, Tien DH, Mohnot S, Beeson E, Glasgow E, Amri H, Zukowska Z, Kalueff AV.** 2009. Understanding behavioral and physiologic phenotypes of stress and anxiety in zebrafish. Behav Brain Res **205:**38–44.
10. **Filby AL, Paull GC, Bartlett EJ, Van Look KJW, Tyler CR.** 2010. Physiological and health consequences of social status in zebrafish (*Danio rerio*). Physiol Behav **101:**576–587.
11. **Gesto M, Hernández J, López-Patiño MA, Soengas JL, Míguez JM.** 2015. Is gill cortisol concentration a good acute stress indicator in fish? A study in rainbow trout and zebrafish. Comp Biochem Physiol A Mol Integr Physiol **188:**65–69.
12. **Ghisleni G, Capiotti KM, Da Silva RS, Oses JP, Piato AL, Soares V, Bogo MR, Bonan CD.** 2012. The role of CRH in behavioral responses to acute restraint stress in zebrafish. Prog Neuropsychopharmacol Biol Psychiatry **36:**176–182.
13. **Gray C, Loynes CA, Whyte MK, Crossman DC, Renshaw SA, Chico TJ.** 2011. Simultaneous intravital imaging of macrophage and neutrophil behaviour during inflammation using a novel transgenic zebrafish. Thromb Haemost **105:**811–819.
14. **Grunwald DJ, Eisen JS.** 2002. Headwaters of the zebrafish—emergence of a new model vertebrate. Nat Rev Genet **3:**717–724.
15. **Institute for Laboratory Animal Research**. 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
16. **Jagadeeswaran P, Sheehan JP, Craig FE, Troyer D**. 1999. Identification and characterization of zebrafish thrombocytes. Br J Haematol **107**:731–738.
17. **Mendelsohn J, Multer MM, Bernheim JL.** 1977. Inhibition of human lymphocyte stimulation by steroid hormones: cytokinetic mechanisms. Clin Exp Immunol **27:**127–134.
18. **Meyer A, Van de Peer Y.** 2005. From 2R to 3R: evidence for a fish-specific genome duplication (FSGD). BioEssays **27:**937–945.
19. **Murtha JM, Qi W, Keller ET.** 2003. Hematologic and serum biochemical values for zebrafish (*Danio rerio*). Comp Med **53:**37–41.
20. **Pavlidis M, Digka N, Theodoridi A, Campo A, Barsakis K, Skouradakis G, Samaras A, Tsalafouta A.** 2013. Husbandry of zebrafish, Danio rerio, and the cortisol stress response. Zebrafish **10:**524–531.
21. **Piato ÂL, Capiotti KM, Tamborski AR, Oses JP, Barcellos LJ, Bogo MR, Lara DR, Vianna MR, Bonan CD.** 2011. Unpredictable chronic stress model in zebrafish (*Danio rerio*): behavioral and physiologic responses. Prog Neuropsychopharmacol Biol Psychiatry **35:**561–567.
22. **Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB.** 2006. Whole-body cortisol is an indicator of crowding stress in adult zebrafish, *Danio rerio*. Aquaculture **258:**565–574.
23. **Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB.** 2009. Whole-body cortisol response of zebrafish to acute net handling stress. Aquaculture **297:**157–162.
24. **Schwartzman RA, Cidlowski JA.** 1994. Glucocorticoid-induced apoptosis of lymphoid cells. Int Arch Allergy Immunol **105:**347–354.
25. **Shrimpton JM, Randall DJ.** 1994. Downregulation of corticosteroid receptors in gills of coho salmon due to stress and cortisol treatment. Am J Physiol **267:**R432–R438.
26. **Stachura DL, Traver D.** 2011. Cellular dissection of zebrafish hematopoiesis. Methods Cell Biol **101:**75–110.
27. **Steenbergen PJ, Richardson MK, Champagne DL.** 2011. The use of the zebrafish model in stress research. Prog Neuropsychopharmacol Biol Psychiatry **35:**1432–1451.
28. **Stockham SL, Scott MA.** 2008. Fundamentals of veterinary clinical pathology. Ames (IA): Blackwell.
29. **Stolte EH, de Mazon AF, Leon-Koosterziel KM, Jesiak M, Bury NR, Sturm A, Savelkoul HFJ, van Kemenade BMLV, Flik G.** 2008. Corticosteroid receptors involved in stress regulation in common carp, *Cyprinus carpio*. J Endocrinol **198:**403–417.
30. **Verburg-Van Kemenade BML, Nowak B, Engelsma MY, Weyts FAA.** 1999. Differential effects of cortisol on apoptosis and proliferation of carp B-lymphocytes from head kidney, spleen and blood. Fish Shellfish Immunol **9:**405–415.
31. **Wendelaar Bonga SE.** 1997. The stress response in fish. Physiol Rev **77:**591–625.
32. **Weyts FAA, Flik G, Kemenade BMLV-v.** 1998. Cortisol inhibits apoptosis in carp neutrophilic granulocytes. Dev Comp Immunol **22:**563–572.
33. **Weyts FAA, Flik G, Rombout JHWM, Kemenade BMLV-v.** 1998. Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of the common carp, *Cyprinus carpio L*. Dev Comp Immunol **22:**551–562.
34. **Wittamer V, Bertrand JY, Gutschow PW, Traver D.** 2011. Characterization of the mononuclear phagocyte system in zebrafish. Blood **117:**7126–7135.
35. **Wojtaszek J, Dziewulska-Szwajkowska D, Lozinska-Gabska M, Adamowicz A, Dzugaj A.** 2002. Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio L.*): cortisol effect on the carp blood. Gen Comp Endocrinol **125:**176–183.
36. **Zang L, Shimada Y, Nishimura Y, Tanaka T, Nishimura N.** 2013. A novel, reliable method for repeated blood collection from aquarium fish. Zebrafish **10:**425–432.

http://prime-pdf-watermark.prime-prod.pubfactory.com/ | 2025-02-28