RESEARCH ARTICLE **(Open Access)**

Effects of stressors on hematological and immunological response in the fresh water crucian carp fish, *Carassius carassius*

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Abstract

Stress is an event that most animals experience and that, induces a number of responses involving all three regulatory systems, neural, endocrine and immune. Fish cultures are especially at risk to the adverse effects of stress. Blood chemistry and hematological measurements can provide valuable physiological indices that may offer critical feedback on different stressors. Blood samples were collected from the caudal vein of *Carassius carassius* after subjected to stressors and parameters such as plasma cortisol and glucose levels were estimated. Also, immunological response through neutrophil/lymphocyte ratio were evaluated. The responses of *C. carassius* to stress were characterized by rapid and transient significant increases in glucose, hemoglobin, hematocrit, as well as an equally dramatic but delayed increase in cortisol levels. High ratio of neutrophils to lymphocytes (N: L) in blood fish were found, which reliably is related with high glucocorticoid levels. Our results strongly indicate the close relationship between stress hormones and neutrophil/lymphocyte ratio, concluding that N: L ratio and its relation with glucocorticoid hormones can provide a reliable method to study responses of fish to stress.

Key words: stress, plasma cortisol, glucose, immunological response, neutrophil, lymphocyte.

1. Introduction

Stress is defined as ‘‘the nonspecific response of the body to any demand made upon it’’ [39]. The response to stress is considered an adaptive mechanism that allows the fish to cope with real or perceived stressors in order to maintain its normal or homeostatic state [7]. Stress can be considered as a state of threatened homeostasis that is re-established by a complex suite of adaptive responses [9] . The stress response applies to a wide range of physiological mechanisms, including gene and protein changes, metabolism, energetics, immune, endocrine, neural and even behavioral changes that will first try to overcome that situation and then compensate for the imbalances produced by either the stressor or the consequences generated by the first array of responses. With these reactions the animal tries to avoid dangerous situations and the risk to life and body integrity, and subsequently to cope with the allostatic load produced by the stressor and reintegrate the balance throughout physiological systems in order to regain homeostasis [43]. Physiological responses of fish to environmental stressors have been grouped broadly as primary, secondary and tertiary [7]. Primary responses, which involve the initial neuroendocrine responses, include the release of catecholamines from chromaffin tissue [35, 37], and the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis culminating in the release of corticosteroid hormones into circulation [15, 26]. Secondary responses include changes in plasma and tissue ion and metabolite levels, hematological features, and heat-shock or stress proteins (HSPs), all of which relate to physiological adjustments such as in metabolism, respiration, acid-base status, hydromineral balance, immune function and cellular responses [27, 20, 26]. Tertiary responses include aspects of whole-animal performance such as changes in growth, condition, overall resistance to disease, metabolic scope for activity, behavior, and ultimately survival [46]. Some plasma chemicals may be useful tools to evaluate the health and/or stress condition of the fishes [8, 45]. Because stress has been reported to

elevate plasma cortisol [33, 47] and glucose levels [40, 10], many researchers consider that fishes undergoing stressful situations exhibit plasmatic increases of cortisol and glucose [3, 4]. Another common trait of the stress response is that this metabolic reorganization may affect the efficiency of other functions, amongst them the immune system. Lymphocytes, monocytes and neutrophils numbers are known to change according to the physiological condition of the fish, exposed in stressful conditions, cortisol induced or during handling and transport [11]. In particular, some mechanisms of the defence repertoire may be delayed or reduced, thus transiently compromising immune defence and resistance to pathogens. The result is that the stressed animal may experience immune suppression [43].

2. Material and Methods

2.1 Fish Length and weight measurement

C. carassius individuals were caught from Seferani Lake and their length (cm) was measured from the anterior-most point of the mouth, to the posterior-most region of the caudal peduncle (18.7±2.4 cm). Weight (g) was measured by using an electronic balance (183.9±24.5 g).

2.2 Anesthesia and Blood collection

Blood samples were taken from anesthetized animals. Fish were anesthetized with a 0.75% aminobenzoic acid ethyl ester (MS-222) solution, pH adjusted to 7.7 with $NaHCO_2$. Blood was withdrawn into a heparinized syringe via caudal vein puncture.

2.3 Hematological examination

Blood films prepared from the blood samples were stained according to Giemmsa Romanowski method, and were used to calculate WBC cell. Evaluation of blood cells and leukocyte morphology were done under the light microscope, through an micrometer ocular x1000.

2.4 Biochemical measurements

Blood glucose concentration were measured spectrophotometrically by glucose oxidase enzymatic method.

To measure blood cortisol concentration, blood samples were taken from the caudal peduncle using heparinized syringes to obtain plasma after centrifugation at 10,000 x g for 5 min, maintained on ice until determination of cortisol concentrations. Ninety-six well plates for cortisol ELISA (DRG Diagnostics, Frauenbergstrasse, Germany) were used. For this assay only 30 wells were used and the fish plasma samples were analysed in duplicate. For the assay, 20 µl of each of cortisol human plasma standard solution and fish plasma sample were added in duplicate to the plate. Subsequently, 200 µl of enzyme conjugated to horseradish peroxidase (DRG Diagnostics, Frauenbergstrasse, Germany) was added into each well. Finally, the wells were gently mixed on a plate mixer at a 200 beats.min-1 for 10 min and incubated for 1 h at room temperature. The well contents were briskly eliminated to avoid any residual content. The solution of each well was removed by washing the plate three times with 400 µl of PBS and shaking out the content onto absorbent paper with the aim of removing residual drops that could affect the accuracy and precision of the assay. Subsequently, 100 µl of TMB (tetramethylbenzidine) enzyme substrate (DRG Diagnostics, Frauenberg Strasse, Germany) was added to each well and incubated for 15 min at room temperature. The enzymatic reaction was visualized by the color change and was stopped by addition of 100 µl of 0.5 M phosphoric acid (H2PO3). The intensity of color is inversely proportional to the concentration of cortisol in the samples. Absorbance was read in a spectrophotometer at 450 nm on a microtiter plate reader within 10 min after addition of stop solution.

2.5 Statistical analysis

The significance of the differences between the group means was assessed by t-test ($P<0.05$). Results are expressed as mean ± SD.

3. Results and Discussion

3.1 Glucose evaluation

Blood glucose concentration measured in stressed fish individuals showed a significant increase comparing with the normal, unmanipulated individuals. The blood glucose values are shown in the table 1.

Table 1. Values of glucose concentration measured in fish *C. carassius.*

Glucose concentration	*Glu. (mg/dl)*
Normal group	75±10
Stressed group	366*±96

*significance for $p<0.05^*$

3.2 Cortisol evaluation

The values of blood cortisol measured in normal and stressed fish individuals are shown in Table 2.

Table 2. Values of cortisol concentration in *C. carassius* fish

Cotisol concentration	*Cortisol concentration (ng/ml)*
Normal group	45±1.4
Stressed group	124.6±42.7*

Significant for p<0.05

As expected, the plasma cortisol levels in experiment fishes were significant, ($p<0.05^*$) compared with control group (Table 2).

3.3 Leukocyte profile

Our Findings shown that there are five basic white cell types in fish: neutrophil/heterophil, eosinophils, basophils, lymphocytes and monocytes [2]. The relative proportions of each WBC type, usually obtained by light microscope examination of 100 leukocytes in a stained blood smear, are the components of the leukocyte profile. Table 3 shows percentage of different leukocytes screened during hematological examination of *C. carassius* blood smears of 30 individuals.

Table 3. Percentage of different leukocytes in hematological examination of *C. carassius*

Cell Types	*Neutro/Heterophil*	*Lymphocyte*	*Monocyte*	*Eosinophil*	*Basophil*
% of cells	73.6	18.5	7.2	0.3	0.4

From these values we found an increased number of neutrophil/heterophils and a reduction of lymphocyte number. Neutrophil/Lymphocyte ratio were evaluated approximately N/L=3.98. This high value compared with normal ones, shows a suppression of immunity, especially of specific immunity related with lymphocytes [43].

Related with characteristics of different types of leukocytes we can note that Neutrophils/Heterophils are predominantly rounded, their cytoplasm containing neutrophilic fine granulations. Their nucleus can be rod-shaped, occasionally segmented and, generally, eccentric, it's nuclear chromatin being mildly compact, lacking a visible nucleolus. Lymphocyte are found small, medium or large with round to irregular nucleus. Sometimes azurophilic granules or vacuoles in the light blu cytoplasm. Immunocytes are activated lymphocytes with ample, dense blue cytoplasm. Monocyte are large cells with unlobed or lobed nuclei and much grey-blu cytoplasm, vacuoles and fine azurophilic granules may be present in cytoplasm. Macrophages are transformed monocytes which have digested debris. Eosinophiles are usually pale, with spherical to rod shaped granules, with nuclei usually unlobed, and blu cytoplasm. Basophils are found round deep blue to purple granules which often mask outlines of unlobed nucleus [2, 1]. Lymphocytes, monocytes, eosinophils, basophils, neutrophils/heterophils that we found in *C. carassius*, (Fig 1) presented similar morphological features to leukocytes as those reported by [2].

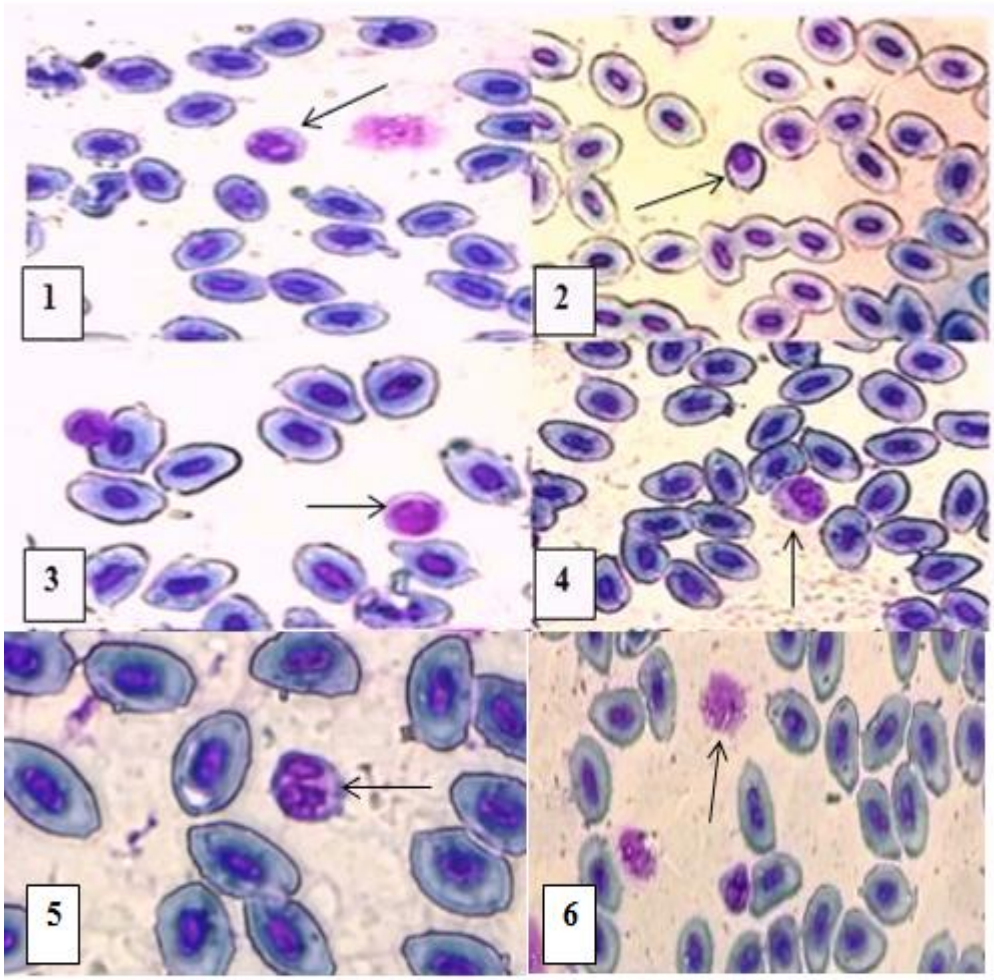

Figure 1. *Carassius carassius* blood cells stained with May Grünwald-Giemsa-Wright. (1) Neutrophil (2) heterophil, (3) lymphocyte, (4) monocyte, (5) eosinophil, and (6) basophil.

When fish are exposed to a stressor, the physiological stress response is initiated by the recognition of a real or perceived threat by the central nervous system (CNS). The sympathetic nerve fibers, which innervate the chromaffin cells, stimulate the release of catecholamines via cholinergic receptors [37]. The chromaffin tissue (adrenal medulla homologue) is located mainly in the anterior region of the kidney in teleostean fishes [37]. Because catecholamines, predominantly epinephrine in teleostean fishes, are stored in the chromaffin cells, their release is rapid and the circulating levels of these hormones increase immediately with stress [25, 35, 37]. Cortisol is the principal glucocorticoid secreted by the interrenal tissue (steroidogenic cells) located in the head-kidney of teleost fish [21]. This hormone is released by the activation of the hypothalamus-pituitary-interrenal axis (HPI axis) [26]. When an organism undergoes stress conditions, the hypothalamus releases corticotropin-releasing factor (CRF) toward blood circulation. This polypeptide further stimulates secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland [18] which finally activates the release of cortisol by the interrenal tissue [26]. Cortisol activates glycogenolysis and gluconeogenesis processes in fish; but also causes that chromaffin cells increase the release of catecholamines which further increase glycogenolysis and modulate cardiovascular and respiratory function [36, 37]. This whole process increases the substrate levels (glucose) to produce enough energy according with the demand [28]. Glucose is a carbohydrate that has a major role in the bioenergetics of animals, being transformed to

chemical energy (ATP), which in turn can be expressed as mechanical energy [24]. The intensity of response is not always caused by a specific stressor in any experiment, instead it may be modulated or affected by different factors that are not considered as direct stressors [17]. Factors that affect/modulate the response may be from intrinsic nature when some factors depend basically on the genotype or phenotype of the organism and from extrinsic nature when response is affected by external factors [28]. Heritability is considered as a modulator with progeny groups of high response and low response showing a similar intensity of cortisol secretion as their ancestors [29]. [31] identified sexual maturity as a factor related with the intensity of response in fishes. Extrinsic factors may affect a variety of biochemical functions within the fish organism such as cortisol biosynthesis and release rates. Environmental color is reported to have an effect on cortisol secretion [44]. In some species the magnitude of the stress response varies with respect of a previous thermal acclimation or acclimatization [42, 41, 22]. Nutritional status [30, 32] is another factor that may affect the response. Several pollutants can stress the fish, activating alarm reactions producing a primary and a secondary response [6]. In Atlantic salmon (*Salmo salar*), cortisol and glucose levels increased after being exposed to high aluminum concentrations [49]. [38] argued that one of the most frequent responses in fish blood to specific chemical intoxication is cortisolemia. In the other hand when a stress response develops it may be assumed that the outcome will depend on the intensity of the stressor and its duration. In this way, recent work demonstrated that stress responses can suppress or enhance certain pathways of the immune response [14, 12, 13]. Work on white cell distribution has shown that stress induce changes in cell numbers and traffic patterns. Since effective immune protection requires recruitment of leukocytes at the affected sites, substantial differences in the leukocyte distribution in different body compartments are observed [48]. Regarding activation, acute stress results in an increase in circulating leukocyte numbers. This is related to the activation of the sympathetic nervous system and release of catecholamines. Blood cells, including both erythrocytes and leukocytes are mobilized as part of the acute response. The changes in blood leukocyte numbers are characterized by a significant reduction in the numbers and percentages of lymphocytes and monocytes and by an increase in the numbers and percentages of neutrophils [43]. Neutrophils and lymphocytes appear to be readily quantifiable in fish, and the same leukocyte responses to stress and to exogenous glucocorticoid treatment (neutrophilia and lymphopenia) can be measured. [16, 5, 19] provide excellent reviews on these responses. In general, acute stress induces both neutrophilia and lymphopenia in fish [34], although sometimes only lymphopenia is reported [23], and these stress-induced changes have been shown repeatedly to be related to elevated glucocorticoids. Neutrophilia, lymphopenia and increased N:L ratios are apparent after treatment with either cortisol or hydrocortisone [16, 48].

4. Conclusions

Knowledge and understanding of what constitutes stress in fish has increased immensely in the past few decades, notably in the area of physiological mechanisms and responses that lead to changes in metabolism and growth, immune functions, reproductive capacity, and normal behavior. The changes observed in the blood metabolites and blood cells of the fish, *Carassius carassius* in the present study indicate that the fish were responding to the direct effects of the stress factors. The analysis of variation of the cortisol and glucose parameters confirms that their alterations are good biomarkers for field assessment, in particular in urban lake areas that are naturally subject to a multiplicity of environmental stressful factors. It must be emphasized that leukocyte profile especially Neutrophil/Lymphocyte ratio is able to evaluate the effects and the responses to acute exposure to different stressors. In conclusion the present study showed that cortisol, glucose and leukocyte profile are all useful biomarkers for

evaluation of general health of fishes exposed in different stressful environmental conditions.

5. References

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