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Humoral and Cellular Effects of Stress-An Extensive Model System

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Abstract: The influence of stress on the immune system of the common carp (*Cyprinus carpio*) was studied by measuring leukocytes levels using flow cytometry and mRNA immune components by real time qPCR. Acute and chronic oxidative stresses were generated by different regimes of exposure of carp to environmental air. In acute stress, induced by single air exposure, the pro-inflammatory cytokines (IL1β, IL6 and TNFα) and the down-regulatory ones (IL10 and TGFβ) showed significant simultaneous elevations (515, 147, 373, 300 and 198% respectively). Following chronic stress (multiple air exposures) however, a drastic decline of 80%, in macrophages/monocytes, B-cells likes and plasma-cells like, occurred in peripheral blood. No statistical changes in IL6 and TNFα, as well as in IgM and C3s mRNA levels could be shown during this experiment. CD4 mRNA decreased up to 6% in the 2nd week of chronic stress and elevated only to 55% at the 3rd week Vs a temporal decline of up to 22% in CD8a mRNA at the 2nd week. The regulatory cytokines (IL10, FoxP3 and TGFβ) as well as the pro-inflammatory ones (IL1β and IL17) decreased significantly up to 0.06, 0.2, 5, 6 and 4% respectively, at the second week before being restored to normal at the 3rd week. Moreover, a persistent decrease, up to null levels, in the cytokines IFNγ2b, IL12b and IL8 was also revealed. These downregulations were suggested as a result of the impaired Th1 and/or cytotoxic cell function and, to a certain degree, the leukocytes mobilization. The above findings show that in contrast to the detrimental effects of chronic stress, in which cells and functions of acquired immunity were partially or completely impaired, the acute stress was found rather beneficial and in line with the known ephemeral "fight and flight" response.

Keywords: Chronic Stress, Acute Stress, Fish, Leukocyte, Cytokines

Introduction

Fish under intensive culture conditions are often exposed to a variety of acute and chronic stressors. These include: Elevated rearing densities (Vazzana *et al*., 2002), suboptimal water quality, decreased dissolved oxygen and elevated carbon dioxide (CO_2) levels (Franco *et al*., 2009; Lefèvre *et al*., 2008), thermal fluctuations (Zarate and Bradley, 2003; Avtalion, 1969; 1981; Avtalion *et al*., 1976; Varsamos *et al*., 2006), diet (Montero *et al*., 1999; Montero *et al*., 2001; Costas *et al*., 2011), presence of enemies and pathogens (Demers and Bayne, 1997; Sunyer and Tort, 1995; Saeij *et al*., 2003), transportation and sorting, handling and confinement stresses (Costas *et al*., 2011; Harmon 2009; Maule and Schreck, 1991; Noga *et al*., 1999). Stressors have negative impacts on different physiological responses associated with growth, nutrition, reproduction and immune responses (Lefèvre *et al*., 2008; Zarate and Bradley, 2003; Øverli *et al*., 2006; WendelaarBonga, 1997; Campbell *et al*., 1992; Poli *et al*., 2005; Pickering 1992; Olsen *et al*., 2005; Hoskonen and Pirhonen, 2006; Vargas-Chacoff *et al*., 2001). Understanding and monitoring the biological mechanisms underlying stress responses in fish may alleviate their negative effects through selective breeding and changes in management practices, resulting in improved animal welfare and production efficiency. This might also

provide further understanding of stress mechanisms involved in higher vertebrates.

Stressors were reported to exaggerate adverse effects like sensitivity to illness, autoimmunity, shrinking of the thymus and spleen or other lymphatic organs, changes in the number and distribution of white blood cells, or appearance of bleeding or ulcers (Harper and Wolf, 2009). Stress increases immunosuppressive pathways and increases proinflammatory cytokines (Tort *et al*., 1996; Douxfils *et al*., 2011; Milla *et al*., 2010; Talbot *et al*., 2009; Petrovsky, 2001). These stress effects impact both the innate and adaptive immune system (Øverli *et al*., 2006; Mommsen *et al*., 1999), mainly following considerable decrease in lymphocyte numbers (Engelsma *et al*., 2003), plasma IgM concentration (Nagae *et al*., 1994), a selective suppression in phagocytosis and complement activities in head kidney and blood. As a consequence, an increase in susceptibility to infection occurs in teleost fish (Pickering, 1984; Law *et al*., 2001; Small and Bilodeau, 2005; Mauri *et al*., 2011).

The effect of stress depends on the duration and intensity of the stressor. Mild and/or acute stressors enhance immune responses while, severe or long-term stressors can be immunosuppressive (Demers and Bayne, 1997; Sunyer and Tort, 1995; Harris and Bird, 2000; Raberg *et al*., 1998). In handling acute stress, an increase in C3, lysozymes (Demers and Bayne, 1997; Sunyer and Tort, 1995) and leukocytes (Maule and Schreck, 1991) were reported in head kidney. In chronic stress however, there is a decrease in C3 and lysozyme levels (Sunyer and Tort, 1995) as well as in immune cell numbers and functions (Verburg-Van Kemenade *et al*., 2009). It was assumed that this dual response depends on the intensity and duration of the stressor and that these processes are controlled by different hormonal and neuronal paths (Tort, 2011; Nardocci *et al*., 2014).

The stress mechanism has been mostly studied in higher vertebrates and much less in fish. In mammals, immune and inflammatory responses are followed by the activation of the stress hormones that systemically inhibit the T-helper-1(Th1) pro-inflammatory responses but potentiate a Th2 shift which is followed by down-regulation of some cytokines involved in cellular immunity (TNF-α, IFN-γ, IL-2, IL-12) as well as production of cytokines belonging to other Th-cell subsets (IL-4, IL-10, IL-13, TGF-β) (Elenkov and Chrousos, 1999). Furthermore, it has been shown that stress induces changes in cell numbers and in their traffic patterns. Substantial differences in the leukocyte distribution in different body compartments have been observed in carp (Wojtaszek *et al*., 2002).

It was stated by these authors that such a situation may lead to ineffective immune protection due to decreased leukocyte recruitment at the affected sites. The activation of leukocytes is related to the activation of the sympathetic nervous system and to the release of catecholamines (Tort, 2011; Dhabhar, 2002). Blood cells, including both erythrocytes and leukocytes, are mobilized as part of the acute stress 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 (Dhabhar, 2002).

Several studies in fish, support stress mechanism as reported in mammals (Wojtaszek *et al*., 2002; Dhabhar, 2002; Cortés *et al*., 2013), while the participating immune cells and humoral processes are still vague. Therefore, in the present work we describe the influence of air exposure acute and chronic stresses (Melamed *et al*., 1999; Dror *et al*., 2006) on different immune components of spleen, blood, kidney and head kidney in the common carp. Hence, we studied the participation of most of the known components in the fish immune system in acute and chronic stresses by examining changes in the levels of: (1) Immune cell groups of small and large lymphocytes, Polymorphonuclear (PMN) cells and monocytes/macrophages during stress treatments; (2) CD4 and CD8a cells which represent the majority of cells involved in immune processes (Todaa *et al*., 2011; Annunziato and Romagnani, 2009; Wan and Flavell, 2009); (3) IgM and the complement C3s (a fish variant of mammal C3) which are considered as significant agents of the innate immunity (Nakao *et al*., 2000; Brattgjerd and Evensen, 1996; Kaattari and Irwin, 1985); (4) The pro-inflammatory cytokines IL1b, IL6 and TNFa (Secombes and Fletcher, 1992); (5) the inflammatory cytokines related to Th1 cells (IFNγ2b and IL12b) and Th17 cells (IL17) (Du *et al*., 2014; Zou *et al*., 2005; Wang *et al*., 2014); (6) IL10, TGFβ and FoxP3 (Wei *et al*., 2013; Wang *et al*., 2010; Kohli *et al*., 2003) regulatory cells cytokines; (7) The chemoattractant CXCL8 that acts similarly to the mammalian IL8 in mobilizing macrophages/neutrophils/leukocytes to the target area (Van der Aa *et al*., 2012).

Materials and Methods

Animals

Common carp (150±30 gr.) were obtained from a local fish farm (Mishmar Hasharon, Israel). The fish were acclimatized to laboratory conditions for at least one month before experiments. Fish were maintained in containers (105×105×80 cm) with air bobbling and recirculating fresh water at 24±2°C, in a 12 h. light/12 h. Dark cycle and fed a commercial diet once a day. Two weeks before the experiment, the fish were kept into net cages (75×28×48 cm), 2 fish in each one. The

cages were maintained in water tanks (350×300×100 cm), equipped with a biological filter and continuous flow of water and air.

Acute Stress

A group of 8 fish was exposed for 10 min. to the air and then immersed for 30 min. in water, after three cycles of exposure/immersion, the fish were left for 24 h in the water (Melamed *et al*., 1999; Dror *et al*., 2006) and then anaesthetized by immersion in 0.01% benzocaine/water. Their spleens were collected into liquid Nitrogen for RNA extraction. In order to minimize handling stress, all stress treatments were done into the net cages i.e., the net with the fish was exposed to the air and immersed into the water.

Chronic Stress

The fish were similarly treated as in the acute stress group, but the exposures to the air and immersions, as above, were repeated three times a week for three weeks. Twenty four hours following the last air exposure, performed at the 9th, 16th and 23th days, groups of 8 fish each were anaesthetized by immersion in a 0.01% benzocaine solution and their spleens were collected into liquid Nitrogen for RNA extraction.

Gene Expression Quantification

Total RNA was extracted from each spleen using 1ml TRI reagent according to the manufacturer's instructions (Geneall Biotechnology, Seoul, Korea). RNA quantification was carried out using a NanoDrop ND-2000c spectrophotometer (Thermo Scientific). Total RNA quality was monitored by running samples on a 1.3% agarose gel. Adequate samples were used for complementary DNA (cDNA) synthesis which was carried out with the FastQuant RT Kit (with gDNase) (Tiangen, Beijing, China) and served as a negative control for quantitative PCR (qPCR). Part of the cDNA was used for a standard curve in each qPCR experiment and the rest of the material was diluted to 100 ng μL^{-1}. qPCR amplification was carried out in 20 µL reaction volume containing 5 µL of diluted cDNA (500 ng) used as a template for qPCR cytokine quantification, 10 µL FastFire qPCR PreMix (Syber Green) (Tiangen, Beijing, China) and 5 µL primer, resulting in a final concentration of 0.1 µM.

All immune component samples were run in triplicate while standards (standard curve of each cytokine and of the RNA negative control following gDNase) in duplicate in the CFX96 (Bio Rad) following the manufacturer's conditions, as follows: Initial denaturation for 1 min, 95°C, followed by 40 cycles of 5 sec denaturation at 95°C and 15 sec for annealing/extension at 59°C to 62°C (Table 1). The melting curve in each experiment was used to examine qPCR and primer quality. Results of qPCR experiment were accepted if: (1) There was no contamination of dimmers or other material; (2) the efficiency of the qPCR reaction was 90 to 109%, (3) the R line of the reaction was 0.98 to 1.

PCR Qualification

Amplification was performed in a 20 µL of a reaction volume containing 10 µL GoTaq Green Master Mix (Promega, Madison, WI, USA), 5 µL primer (in a final concentration of 0.1 µM) and 5 µL diluted cDNA (500 ng). This solution was used as a template to synthesize immune components in each stress treatment. Samples were run in the UNO II (Biometric) as follows: Initial denaturation for 5 min at 95°C, followed by 30 cycles of 30 sec denaturation in 95°C, 30 sec annealing at 60°C and 30 sec. extension at 72°C, ending with 72°C for 10 min. Samples were loaded on an 1.3% agarose gel and visualized by a MiniLumi Imaging System (DNR Bio Imaging Systems).

Primer Design

Primers were designed by the NCBI tool and purchased from Integrated DNA Technologies, Leuven, Belgium (IDT). Each primer was analyzed by an IDT Oligo Analyzer. Running conditions of each primer were analyzed and only those which showed negligible dimmer, high PCR efficiency (90-109%) and $R \geq 0.98$. were used (Table 1).

Data Analysis

All experiments were analyzed by the CFX96 (Bio-Rad) software. Ratio production of immune components between stress conditions to control was expressed as fold changes. Cq was normalized to gene reference 40S rRNA and analyzed according to the Pfaffl and Livak method (Pfaffl, 2001; Livak and Schmittgen, 2008) by correcting the efficiency of each primer at stress relative to control.

Cell Separation

About 1 mL blood was removed from the caudal vein of each fish by a heparinized syringe and diluted in 9 mL Dulbecco's Modified Eagle Medium (DMEM) solution (Biological Industries, Israel). The spleens, kidneys and head kidneys of six fish from each treatment were removed following anesthesia from the groups of control, unstressed, acutely stressed and chronically stressed fish after one, two and three weeks. Organs were minced through a net with a 10 mL syringe piston into DMEM solution. Leukocytes were separated on Ficoll-Paque™ plus (GE Healthcare). After three washes, cells were used for Flow Cytometry (FACS) and for May-Grunwald/Gimsa/right staining and identification.

Table 1. Primers for immune components and their annealing/extension temperature in the PCR and RT-qPCR reaction

Cytokine		Product Lenth	AET*	Reverse	Forward
IL1β	AB010701.1	98	62	TGGCAACTCATGGATTGTGGA	GATTTGTCAGAAGCATTCGAGAC
IL6	AY102632.1	96	60	AGCTGGCTGCAAGTTTCGT	AGCTAAATTCAGAATGATCCTCGCT
IL8	AB470924.1	137	60	TTGGCTCTTGAGGTTCCTCTTTT	TTATTCCTGCTGGACCAATTTGC
IL10	AB110780.1	94	60	ACCTTTTTCCTTCATCTTTTCATACGA	GGATATGCGGAAATGTAGGAATTAC
IL12b	AJ628699.	102	60	GCAGCGATACCTCAAAGCTG	AAGCTGTCCTTCAGTTGGCA
IL17A/F2	HM231140.1	82	60	GGC AGT GAG TTCAGT CTC GTA	CGC AGG TCATCTTTGAAGCCCA
INFγ2b	JX181980.1	120	62	TGTGCCAGTTTTTCTTTTGTAGC	AGACATAAAGGAACCTGAGCAGAA
TNFα	AJ311801.2	111	60	TGTAGCTGCCGTAGGACTCAG	ACAGCCAGGTGTCTTTCCAC
C3s	AB016213.1	94	60	CTGACTGCCCACCACTTCTA	CAAACCTGGAATGCCCTTCG
IgM	AB004105.1	101	62	CAGCAAGCCAAGACACAAACA	CGTATTAGCACCCCCAGAGC
FoxP3	AB741577.1	71	59	TGACTTCCCCACACTGTTACCAT	TACAGGCTATGCAGCTACACC
TGFb	AF136947.1	120	60	CACAGTTATCCGCCATCTTC	CACGCTTTATTCCCAACCA
CD4	DQ400124.1	111	60	CAGGGATGGACAGAGAAGAT	GCACACTAGGACATCAACATAG
CD8a	EU251078.1	96	59	GTTGCTGGATCAGGTTCTC	GACAGACAGTGGTTTCTACAC
40S	AB012087	83	60	TCCTTCAACAGCGAGAACCC	TGGCGGACATACAGAACGAGAG

AET*, annealing/extension temperature

FACS

Cells were incubated in PBS solution containing monoclonal mouse anti carp IgG (produced in our lab), 0.1% sodium azide and 2% Bovine Serum Albumin (BSA) (Sigma) for 30 min at 4°C, were washed twice and incubated in PBS with FITC-goat anti mouse IgG (Sigma) for 30 min at 4°C, were washed twice and kept in a PBS solution containing 0.1% sodium azide, 2% BSA and 0.6% paraformaldehyde, at 4°C. Cell analysis was performed on a flow cytometer, FACSCalibur (Becton Dickinson) equipped with a 488 nm cooled argon-ion laser. Green fluorescence was collected through a 520-530 nm bandpass filter. About 30,000 cells within the gated region were identified. Results were analyzed by the FlowJo software (FlowJo, LLC, Ashland, Or, USA).

Cell Staining

Slides were stained as follows: (1) Fixed for 3 min in methanol and dried by air; (2) Immersed for 20 min in diluted May-Grunwald solution (Sigma) (1:1 in methanol), then were washed for 1 min in phosphate buffer pH 6.3, 0.01 M (PB) and dried by air; (3) Immersed for 30 min in diluted Giemsa stain (Sigma) 1:3 in PB and then were washed for 6 min in PB and dried by air; (4) Immersed in Wright stain (Sigma) 200 mg/40 mL methanol for 20 min and then washed for 15 min in PB. Cells were observed and counted by axioimager.Z1 microscope (Zeiss).

Follow-up of Blood Leukocyte Profile in Stressed Fish

We used a group of 4 fish to follow changes in their individual peripheral blood leukocyte profiles throughout the stress treatments. Therefore, blood control samples (1 mL each) were taken from the caudal vein of each fish, with heparinized syringe, before stress treatments. Two weeks later, the fish were treated for acute stress, as detailed above and 24 h later, blood samples were taken, as above, from each fish. Two weeks later, the fish were treated for chronic stress during 3 weeks, as detailed above and blood samples were taken at the end of each week. Leukocytes from each blood sample were then separated on a Ficoll gradient and used for FACS evaluation and for cell staining as detailed above.

FACS-Determination of Cell Groups

Blood samples of 3 fish were taken as above and leukocytes were separated on Ficoll gradient. Leukocytes sorted by FACS ARIA III (BD Bioscience) to 4 main FACS gated groups (Fig. 1). Cell sample of each leukocytes group was transferred to slides by cytocentrifugation (Elliot-Shandon, Recyclab), stained as above for microscopy and FACS identification of each gated group.

Identification of Macrophages/Neutrophils

Leukocytes were incubated in a solution of 200 µL PBS containing 2% Hepes, 0.2% BSA, 10^7 FITC-*Staphylococcus albus* and 50 µL carp inactivated serum, for 1 h at 28°C. The reaction was stopped by adding cold PBS. Fluorescence of phagocytosing cells in the analyzed gates in flow cytometry was examined by FACSCalibur (Becton Dickinson) and ImageStream (Merck Millipore).

Statistical Analysis

The acute stress results were tested for significance by F and T tests and those of chronic stress were analyzed by a one way ANOVA followed by Bonferroni and Tamhane Post Hoc Tests.

Results

Cell sorting and staining of the different cell groups showed that: Cells of group A consisted mostly in small lymphocytes; of group B in medium and large lymphocytes; of group C in PMN cells and of group D in macrophages/monocytes (Fig. 1). Neutrophils and macrophages were identified by phagocytosis of marked *Staphylococcus albus*, flow cytometry and cell staining. B-cell like and plasma-cell like were identified by staining with FITC-bounded monoclonal mouse anti carp IgG and fluorescence examination using FACSCalibur and ImageStream. Leukocyte levels showed high variability between individuals which disguised stress influence (Fig. 2). Consequently, the influence of stress was also studied by following changes in peripheral blood leukocyte levels in 4 carp throughout different stress treatments (Table 2). In acute stress, only the follow up of peripheral blood leukocytes levels showed a significant decrease in small lymphocytes and B-like cells (~10%, ~50% respectively) ($p \leq 0.05$) (Table 2). In chronic stress, by sampling 8 carps, leukocyte levels throughout stress treatments did not show significant changes in ANOVA test in the kidney and the head kidney except a decrease of 15% of B-cell like at the 3rd week of chronic stress in the kidney. On the other hand, in the spleen and the blood, macrophages/monocytes decreased significantly up to 50% in the blood by one way ANOVA test and in the spleen by trend test. Moreover, B-cell-like and plasma-cell like decreased significantly, as evaluated by trend test, up to 50% in the blood ($p \leq 0.05$) (Fig 2). Moreover, trend test indicated that PMN cells levels rose slightly and permanently during weeks 1-3 of chronic stress ($R = 0.998$) in head kidney (Fig. 2). On the other hand, by following changes in leukocyte profiles of peripheral bloods of 4 individuals throughout stress treatments resulted in a drastic decline of 70-80% of macrophages ($p \leq 0.05$, in one way ANOVA) (table 2). In a similar way, B-like lymphocytes and plasma-like cell levels, decreased significantly by 80% in the blood (Table 2) at week 2 and 3 of chronic stress ($p \leq 0.05$, in one way ANOVA test).

Results of immune components revealed that in acute stress, the level of the proinflammatory cytokines, IL1β, IL6 and TNFα, showed a significant increase of 515%, 147%, 373% Vs control, respectively ($p \leq 0.05$), as well as that of the down-regulatory ones, IL10 and TGFβ, that showed a significant increase by 300 and 198%, respectively (Table 3 and Fig. 3, first and second wells). The level of the other components mRNAs: IL8, IgM, IFNγ2b, FoxP3, C3s and the cell markers CD4 and CD8α, showed no significant changes, except a slight insignificant decrease in C3s mRNA (Table 3 and Fig. 3, well 2).

In chronic stress, cytokine mRNA levels of IL6, TNFα, C3s and IgM showed no significant changes compared to the control throughout the whole treatments (Fig. 4A and 4D) in spite of some fluctuations were seen especially in C3s levels (Fig. 3). IL1β, TGFβ and CD8a mRNA decreased respectively to 6%, 5% and 22% levels of control at the second week, after 7 regimes of stress. At the third week, however, after 10 regimes of stress, they returned to control levels (Fig. 4A, 4E and 4C), except IL1β which increased by 2.7 times above the control level ($p \leq 0.05$) at returning to homeostasis. IL12b, IFNγ2b mRNA decreased dramatically to zero levels throughout the whole chronic stress time and their levels did not recover even after three weeks of stress (Fig. 4B). IL8 and CD4 mRNA alike IL12b and IFNγ2b considerably decreased at the first week up to 1 and 6% and rose to 25%, 55% levels of the control, respectively, at the third week (Fig. 4A and 4C). IL10 and FoxP3 mRNA decreased sharply ($p \leq 0.05$) in the second week to the level of 0.06 and 0.2% of the control, respectively. In the third week of chronic stress, after 10 regimes of air exposure, their mRNA amounts returned to control levels (Fig 4E). It is noteworthy that the evaluation of IL17mRNA was quantified by PCR instead of real time qPCR because of difficulties in selecting proper primers. IL17 mRNA decreased following acute stress to 4% and at the first week of chronic stress to almost zero levels. However, its levels rose from the second week, reaching control levels in the third week of chronic stress (Fig. 3).

Table 2. Leukocyte percentages in common carp peripheral blood following stress treatments

	Small lymphocyte	Large lymphocyte	Monocytes/ Macrophage	PMN cells	B cells like	Plasma cell like
ctrl	70.07±3.03	14.98±1.77	1.05±0.09	3.35±0.89	8.50±1.69	4.86±2.52
as	62.85±3.95*	13.36±2.13	1.01±0.32	8.84±2.49	4.28±0.95*	3.76±0.76
csw1	67.73±4.22	14.43±2.2	0.30±0.06*	3.27±1.27	3.86±1.40	2.54±0.70
csw2	71.25±5.90	11.32±2.47	0.26±0.09*	1.90±0.81	1.34±0.37*	1.64±0.42*
csw3	68.67±6.85	9.93±3.16	0.21±0.05*	4.77±0.69	1.38±0.17*	0.93±0.25*

*,$p \leq 0.05$ in one way ANOVA. Each result was the mean of 4 followed individual fish ± SEM. The results represented changes in the percent of leukocytes following five different stress treatments. The cell percentage was calculated from 30000 cells identified by FACSCalibur in the gated area and analyzed by the FlowJo software. Cell type was identified by cell sorting, binding of monoclonal mouse anti carp IgG to leukocytes, phagocytosing FITC-*Staphylococcus albus* and by cell staining. ctrl, control; as, acute stress; csw1, one week of chronic stress; csw2, two weeks of chronic stress; csw3, three weeks of chronic stress.

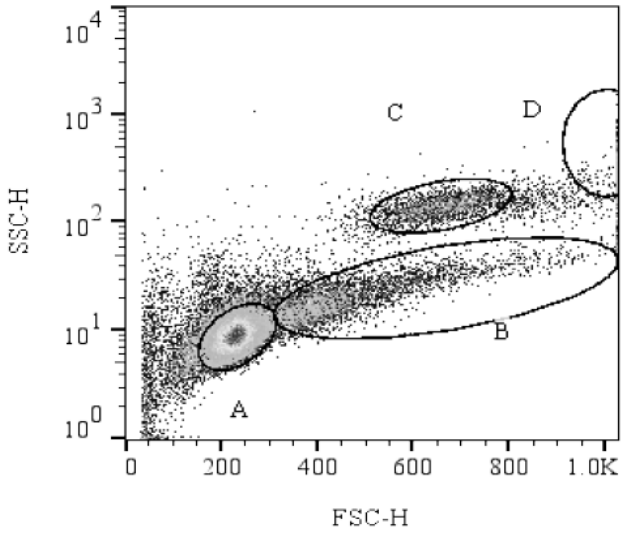

Fig. 1. Four main leukocytes groups A, B, C and D were sorted by FACS and then the sorted samples were cytocentrifuged on slides, fixed by methanol and stained with MayGrunwald/Giemsa/Wright stains. Cell classification was performed by microscopy (x100)

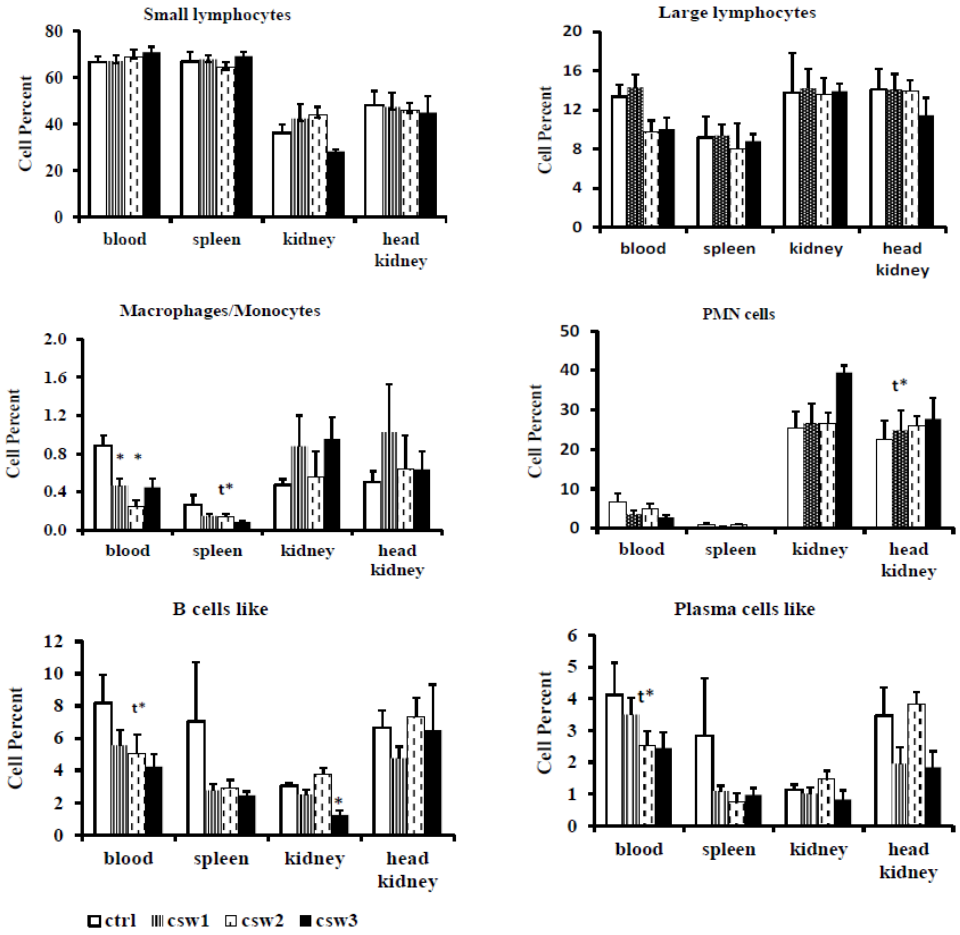

Fig. 2. Percent of leukocytes in carp lymphatic organs following chronic stress treatments: *, one way ANOVA p≤0.05; t*, a significant trend of elevation/decrease of cells ≤ 0.05. The results represent the mean of 6-8 carp ± SEM. Carp were treated in chronic stress along three weeks. At each week leukocytes from lymphatic organs were separated. Cell percent levels were calculated from 30000 cells identified at the gated area by FACSCalibur and analyzed by the FlowJo software. ctrl, control; csw1, one week of chronic stress; csw2, two weeks of chronic stress; csw3, three weeks of stress

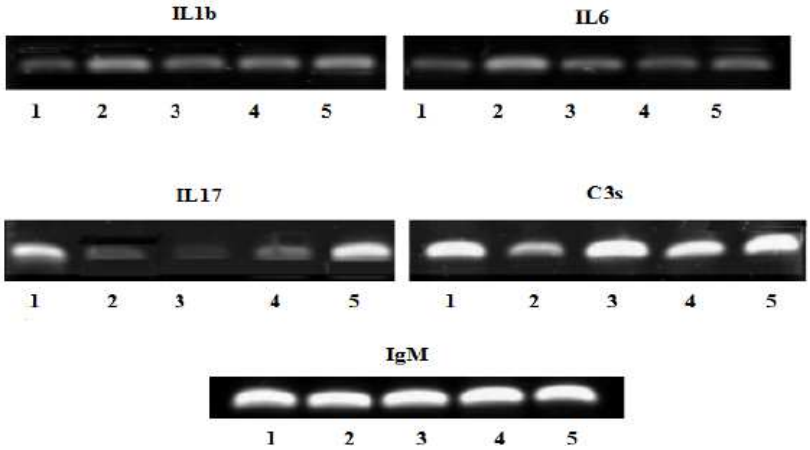

Fig. 3. Comparative cytokine levels in common carp spleen following different stress treatments. Cytokines were produced from mixed 500 ng cDNA of 8 fish by PCR amplification and loaded on 1.3% agarose gel with TBE running solution. 1, control; 2, acute stress; 3, one week of chronic stress; 4, two weeks of chronic stress; 5, three weeks of chronic stress

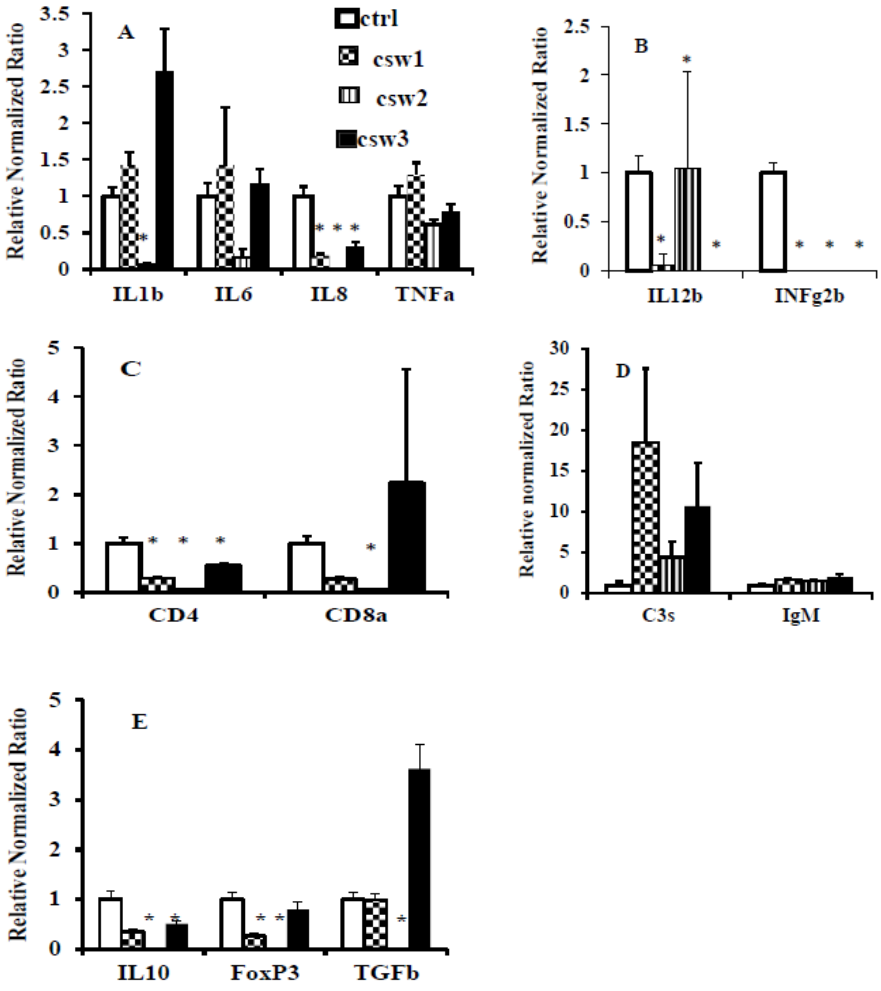

Fig. 4. Immune component levels during chronic stress. *, $p<0.05$ in one way ANOVA. Each result was a mean of 8 carp spleens ± SEM, Component levels were measured by qPCR after three air exposure regimes per week. Results were normalized to 40S rRNA and the component ratio to the control was calculated by the ΔΔCq method. ctrl, control fish; csw1, one week of chronic stress; csw2, two weeks of chronic stress; csw3, three weeks of chronic stress

Table 3. Normalized ratio of carp spleen cytokines following acute stress induction

	IL1b	IL6	IL8	IL10	INFγ2b	TNFa	C3s	IgM	FoxP3	TGFβ	CD4	CD8a
ctrl	1±0.12	1±0.18	1±0.13	1±0.15	1±0.12	1±0.14	1±0.8	1±0.12	1±0.14	1±0.14	1±0.12	1±0.15
as	5.15±0.67*	1.47±0.28*	0.81±0.08	3.01±0.34*	1.4±0.19	3.73±0.27*	0.79±0.21	1.35±0.16	2.51±0.73	1.98±0.21*	0.84±0.1	1.3±0.18

*, T test significance, p≤0.05. Each result represents a mean of 8 carp ± SEM. Acute stress was induced by a single regime of air exposure. Spleen cytokine levels were evaluated 24 h. after air exposure by qPCR amplification. Results were normalized to 40S rRNA and the ratio calculated by the ΔΔCq method. ctrl, control; as, acute stress

Discussion

In the present study, we followed changes in leukocytes profiles of blood and lymphatic organs and measured levels of components representing the main functions of the immune system during acute and chronic stresses in order to further elucidate the involved cellular and molecular mechanisms. The spleen was used mainly to follow changes in immune constituents in the humoral system because preliminary experiments (data not shown) revealed that the cytokines level profile in the spleen was similar to that of the blood and provided more material for gene expression experiments than the blood. In addition, the leukocyte spread of spleen and peripheral blood displayed similar patterns, while that of kidney and head kidney were different. Whereas, the lymphocytes levels were higher than those of PMN cells in the peripheral blood and the spleen, the levels of both populations were almost similar in the kidney and the head kidney (Fig. 2).

In acute stress, our results are in agreement with previous studies (Barker *et al*., 1991; Banerjee and Leptin, 2014) showing significant increased levels of pro-inflammatory cytokines (IL1β, IL6 and TNFα), as well as of down-regulatory ones (IL10 and TGFβ) (Table 3). It is possible that the joint elevation of these regulatory cytokines with that of the pro-inflammatory ones was involved in the shortened time of the pro-inflammatory response and the restoring of homeostasis. The pro-inflammatory cytokines are known to be involved in "fight or flight" response (Tort, 2011) in order to overcome ephemeral stressors.

The effect of stress on fish was widely variable between individuals. Consequently, the leukocyte averages in lymphatic organs were ambiguous (Fig. 2). Therefore, a follow up of changes in leukocyte levels of peripheral blood throughout stress treatments (Table 2) elicited the question of the sampling size, or of the research tool. As a result, following changes in leukocyte levels of peripheral blood were considered as a significant parameter.

In acute stress, the leukocytes levels showed significant changes only by following levels in peripheral blood. Lymphocytes decreased 10% possibly due to the significant decrease of up to 50% in the B-cell like (Table 2).

In chronic stress, CD4 and CD8a mRNA levels decreased up to the second week (p≤0.05), but while CD4 mRNA remained depressed towards the third week, CD8a mRNA returned to homeostasis (Fig. 4C). This may explain the drastic decrease of 80% of macrophages, B-cell like and plasma-cell like in peripheral blood (Table 2). These results were in agreement with the decrease in leukocyte numbers in *Oncorhynchus mykiss* (Cristea *et al*., 2012), suppression of phagocytic and lymphocyte proliferative activities in *Platichthys flesus* and *Solea senegalensis* (Pulsford *et al*., 1995) and apoptosis of B cells in *Cyprinus carpio* (Verburg-Van Kemenade *et al*., 1999). Nevertheless, IL12b and IFNγ2b mRNA products (Fig. 4B) decreased to null throughout 22 days and their levels did not recover even after the third week of chronic stress. It was possible that this dramatic decrease was a result of the deleterious functions of producing IFNγ2b and IL12b mRNA in CD4 cells, especially following the impairment of Th1 cells (Wojtaszek *et al*., 2002; Cristea *et al*., 2012). Production in Th1 cells alone can't explain the zero levels of IFNγ2b. Therefore, it is suggested that additional impaired cell types like NK cells, might be involved because of the partial decline in CD8a mRNA. The sharp decrease in IFNγ2b production, macrophages levels, B-cell like and plasma-cell like amounts might explain the increased susceptibility to diseases occurring in chronic stress (Saeij *et al*., 2003; Small and Bilodeau, 2005; Mauri *et al*., 2011; Elenkov and Chrousos, 1999; Maule *et al*., 1989). Moreover, the improvement of CD4 mRNA amounts from 6 to 55% of control levels, which occurred between second and third weeks of chronic stress (Fig. 4C) may explain the recovery of inflammatory and regulatory functions at that time.

The IL17 PCR results (produced in Th17 cells) (Fig 3) suggest that its levels decline in acute and chronic stresses but increase towards the third week as seen in the case of CD4 elevation (Fig. 4C). This finding might reveal a recovery of the inflammatory functions in progressing chronic stress.

IL1β (p≤0.05) and IL6 (p≤0.06) mRNA ratios which decreased during the second week of chronic stress, reached homeostasis at the third week, whereas, TNFα mRNA (Fig. 4A) remained stable along three weeks of stress. This result was slightly different from that reported for cortisol induced chronic stress in rainbow trout (Cortés *et al*., 2013), which showed that TNFα and IL1β increased after 5 days. That occurred in our study in the acute stress but not in the chronic stress. This

contradiction might be due to differences in experimental conditions, i.e., the use of cortisol implants versus repeated air exposure. The unchanged levels of TNFα and minor temporarily changes in IL1β and IL6 levels throughout the chronic stress (Fig. 4A), even though there was a drastic decrease in macrophages/monocytes, B-cells like, plasma-cells like (Table 2) and supposedly Th1 and NK cells, might point on additional stable pro-inflammatory resources. Moreover, the chemoattractant IL8 which was down-regulated along 22 days of the chronic stress (Fig. 4A) and did not relieve after the third week may explain the macrophage/neutrophil/leukocyte mobilization decline in different compartments of the body as shown by others (Wojtaszek *et al*., 2002).

FoxP3, known to be produced by regulatory cells (CD4 cells) decreased towards the second week before being elevated to homeostasis levels at the third week as also seen by the moderate elevation of CD4 mRNA (Fig. 4C and 4E).

Other regulatory cytokines, IL10 and TGFβ behaved in different ways throughout the stress period. While, IL10, was down-regulated throughout the three weeks of chronic stress and slightly rose at the third week ($p \leq 0.09$), TGFβ showed considerable changes only at the second week of chronic stress and increased at the third week to homeostasis levels (Fig. 4E). This may indicate that regulatory functions may have different resources that respond in different ways to stress. In general, regulatory functions were indeed influenced by chronic stress but only for a while and eventually almost returned to homeostasis.

The increase of TGFβ together with IL6 mRNA at the third week may also explain the recovery of Th17 cells as indicated by the followed up-regulation of IL17 (Fig. 3) and CD4 mRNA (Fig 4C). IgM mRNA levels neither changed in acute stress nor in chronic stress in our experimental conditions (Table 3, Fig 3 and 4D). This result was in contradiction with husbandry, confinement or crowding induced stresses findings (Varsamos *et al*., 2006; Nagae *et al*., 1994; Maule *et al*., 1989; Rotllant *et al*., 1997; Ruane *et al*., 1999) and to the decrease in B-cell likes and plasma-cell likes (Table 2 and trend in Fig. 2), but in agreement with other studies (Douxfils *et al*., 2011; Cuesta *et al*., 2004; Vargas-Chacoff *et al*., 2014). These discrepancies were also shown in our lab as a result of pollution and temperature stress (not yet published) and it might be due to a presence of inhibitor controlling IgM humoral activity. Similarly, C3s mRNA showed no significant changes in both acute and chronic stresses, although its levels fluctuated throughout the chronic stress period (Fig. 3 and 4D). These results differ from the hemolytic findings of previous reports (Demers and Bayne, 1997; Sunyer and Tort, 1995; Mauri *et al*., 2011), but were in agreement with the reported hypoxia and cortisol induced stress (Douxfils *et al*., 2012; Eslamloo *et al*., 2014). However, one cannot disregard that the measured plasma ACH50 which reported above (Demers and Bayne, 1997; Sunyer and Tort, 1995; Mauri *et al*., 2011) is based on the sum of the protein cascade in the complement activity and not solely on the C3s mRNA production. Therefore, it is likely that during stressful events complement protein variants, stress intensity and its duration, individual variations and the presence of inhibitors, represented a possible cause of these disagreements and fluctuations in C3s levels.

The involvement of some immune components in acute and chronic stresses, as discussed above, emphasizes which of these functions need further clarifications as follows: (1) The unchanged production in IgM and C3s mRNA levels in our study is not in agreement with the reported decrease in their activity in the blood (Varsamos *et al*., 2006; Nagae *et al*., 1994; Maule *et al*., 1989; Rotllant *et al*., 1997; Ruane *et al*., 1999; Demers and Bayne, 1997; Sunyer and Tort, 1995; Mauri *et al*., 2011). This discrepancy elicited the necessity to further clarify the existence of inhibitory functions during chronic stress. (2) Were the zero levels in IFNγ2b and IL12b throughout chronic stress, due only to the impairment in monocytes, NK/Th1 and B cells? (3) The unchanged levels of TNFa and almost unchanged levels of IL1b and IL6, even after a decrease up to 80% in monocytes/macrophages levels, might enable the verification of the involved cellular mechanisms in the production of these cytokines during chronic stress. (4) We need to clarify the meaning of a persistent increase of PMN cell levels in the head kidney in trend tests ($p \leq 0.05$) (Fig. 2).

Conclusion

Based on the above findings, it can be concluded that:

- The decrease up to null in mRNA levels of IFNγ2b and IL12b throughout chronic stress is probably due to the presence of an additional impaired population of cells, producing these cytokines, besides probable Th1 and/or NK cells
- The cells that were the most affected by chronic stress were macrophages, B-cell likes, plasma-cell likes and to a certain extent, the Th1 cells and subtypes of the NK cells/cytotoxic cells. This decline in these cells might explain the susceptibly to diseases during chronic stress
- The decrease in IL8 mRNA levels during chronic stress undoubtedly reduced leukocytes mobilization. As a result, the leukocyte recruitment at the affected sites might be injured
- The increase in pro-inflammatory and regulatory cytokines seems to counter balance temporary stresses but these cytokines stay almost unchanged throughout chronic stress

- The levels of some constituents of innate immunity such as C3s and IgM mRNA were unchanged following acute and chronic stresses

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Author's Contributions

Mazal Shimon-Hophy: Planed and make all experiments, summarized the results and wrote the manuscript.

Ramy R. Avtalion: The idea of learning and ocmparing the effects of acute and chronic stresses in crap (Cyprinus carpio), an aninmal model currently used in our laboratory.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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