**The influence of stress on the mechanism regulating the cytotoxic function in common carp (Cyprinus carpio)**

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**Abreviations:** CNS-central nervous system, PFC-prefrontal cortex, PVN-paraventricular nucleus, CeA-central Amygdala, 5-HT-5-hydroxytryptamine, mPFC-median PFC, BLA-basolateral Amygdala, GABA-gamma aminobutyric acid, ITCs-intercalated cell clusters, DRN-dorsal raphe nucleus, HPA-hypothalamus-pituitary-adrenal gland, CRF-corticotropin releasing factor, HPI-hypothalamus-pituitary-interrenal cells, CRH-corticotropin releasing hormone, ACTH-adrenocorticotropic hormone, GR-glucocorticoid receptor, MR-mineralocorticoid receptor, MCH-melanin concentrating hormone, A- adrenaline, NA-noradrenaline, AR-adrenergic receptor, IL-interleukin, IFN-interferon, TNF-tumor necrotic factor, TGF-transforming growth factor, FoxP3-forkhead box P3, NCC-none specific cytotoxic cell, MAIT-mucosal associated invariant T cell, NCCRP1-none specific cytotoxic receptor protein 1, IgM-immunoglobulin M, CD-cluster of differentiation, Th1-T helper 1 cell,

**Abstract**

Aquaculture conditions expose fish to internal and environmental stressors that increase susceptibility to morbidity and mortality. The brain accumulates stress signals processes them according to the intensity, frequency duration and type of stress and recruits several brain functions to evoke autonomic or limbic system. Activation of the autonomic system causes to release rapidly catecholamines such as adrenaline and noradrenaline into circulation from chromaffin cells in the head kidney. Catecholamines activate blood cells to release proinflammatory and regulatory cytokines to cope with the situation of acute stress. Activation of limbic axis involves the dorsolateral and dorsomedial pallium to process emotions, memory, behavior and activation of preoptic nucleus-pituitary gland-interrenal cells in the head kidney to release glucocorticoids such as cortisol to the bloodstream. Glucocorticoids cause to downregulation of various functions in the immune system depending on the duration, intensity and type of chronic stress. As the stress continues, most of the immune functions overcome and return to homeostasis except the cytotoxic functions. The deterioration of the cytotoxic functions during chronic stress is apparently responsible to the increase in morbidity and mortality.

**Introduction**

Aquaculture conditions are often exposed to various stressors. Stressors can be a consequence of elevated rearing densities (Vazzana *et al*., 2002), suboptimal water quality, decreased dissolved oxygen and elevated carbon dioxide (CO2) levels (Franco *et al*., 2009; Lefèvre *et al*., 2008), thermal fluctuations (Zarate and Bradley, 2003; Varsamos *et al*., 2006), diet (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, and handling and confinement stresses (Costas *et al*., 2011; Harmon 2009; Maule and Schreck, 1991; Noga *et al*., 1999).

Stressors were reported to reduce hippocampal volume (Brown et al., 2015; Gerritsen et al., 2015; Head et al., 2012) and, as a result, to impact memory and learning (Buchanan et al., 2006; Roozendaal, et al., 2009; Wolf, 2009). The amygdala - in particular, the basolateral amygdala (BLA) - increases dendritic length and spine density and, as a result, there are changes in the emotions (Holtmaat and Svoboda, 2009; Sousa and Almeida, 2012). Furthermore, stress exaggerates adverse effects like shrinking of the thymus and spleen or other lymphatic organs, changes in the number and distribution of leukocytes, or appearance of bleeding or ulcers that increase susceptibility to morbidity and mortality (Harper and Wolf, 2009). 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*., 2014). Understanding and monitoring the biological mechanisms underlying stress responses in fish can alleviate their negative effects through selective breeding and changes in management practices, resulting in improved animal welfare and production efficiency.

In this review, we will summarize the processes that regulate stressors and affect the functions of the immune system, which is essential for the health, welfare, and production efficiency of the common carp. The influence on the immune system will be mainly based on what is studied in our lab.

**The central nervous system (CNS) regulation of stress**

The brain accumulates external and internal signals of stress, processes them, and recruits several neuronal circuits to maintain physiological integrity (Ulrich-Lai and Herman, 2009). The intensity, frequency, duration, and type of stress will evoke autonomic stress response or limbic circuits, such as the prefrontal cortex (PFC), amygdala, hippocampus, paraventricular nucleus (PVN) of the hypothalamus, and the nucleus accumbens (Skoluda et al., 2015, Russo and Nestler, 2013). The amygdala functions like a command center that processes emotions and sends stress signals to the hypothalamus, while the hypothalamus works as a command center that communicates through other parts of the body, such as the autonomic nervous system and the hypothalamus-pituitary-adrenal/head kidney axis to control functions such as breathing, blood pressure, heart beat, and the immune system (McEwen, 2006). Excessive or inadequate basal activity and responsiveness of this system might impair development, growth, and body composition, and lead to a host of behavioral and somatic pathological conditions (Chrousos, 2009).

**Prefrontal cortex-amygdala regulation**

The mechanism of stress regulation in teleost is still obscure and lacks much information on stress-regulating processes, especially in the upper functions of the brain homologues as the prefrontal cortex (PFC), amygdala and hippocampus. In contrast, in mammals were found that the PFC in the brain integrate and process sensory information (Zhuo, 2008; Miskovic and Keil, 2012; Meaney, 2013) and regulate it via a complex connectional network with other brain structures (Negrón-Oyarzo et al., 2016). The median PFC (mPFC) and amygdala have reciprocal anatomical interconnections (Krettek and Price, 1977; Porrino et al, 1981; Mcdonald et al, 1996; Cassell et al, 1989), and the former appears to have regulatory function in amygdalar activation during the stress response. Several studies demonstrates that 5-hydroxy tryptamine (5-HT) neurotransmission in the mPFC constitutes a potential mechanism through which the mPFC regulates amygdala-mediated arousal in response to stressful events (Fisher et al, 2009). Studies on 5-HT transporters have also proposed 5-HT to function in mediating mPFC-amygdala interplay. (Wellman et al, 2007). Bilateral selective 5-HT depletion in the mPFC in mice decreases the BLA GABA release that is induced by restraint stress and passive coping in the forced swimming test, implicating 5-HT and GABA transmission-mediated PFC/amygdala connectivity as a critical neural mechanism of stress-induced behavior (Andolina et al, 2013, 2014). Stress exposure increases the release of amygdala neurotransmitters including glutamate, GABA, noradrenaline (NA) (Morilak et al, 1987; Pacák et al, 1993 and Moore and Bloom 1979), 5-HT and epigenetic mechanisms, including noncoding RNA. Administration of α1- or β-adrenergic receptor antagonists directly into the median amygdala (MeA) mitigates the adrenocorticotropic hormone (ACTH) response to immobilization stress (Ma and Morilak 2005). These data support the hypothesis that greater release of NA in the MeA, acting primarily through ACTH receptors, facilitates activation of the hypothalamus-pituitary-adrenal gland (HPA) axis in response to acute stress (Ma and Morilak 2005). Stress-induced noradrenergic activity in the MeA, through projections to the bed nucleus of the stria terminalis (BNST) and preoptic area is one possible mechanism by which the MeA modulates the stress-induced activation of the HPA axis. Immobilization stress enhances NA release in the BLA (Kawahara et al, 2007; Galvez et al, 1996; Quirarte et al 1998; Tanaka et al, 1991; Bedse et al 2015) and in the central amygdala (CeA) (Pacák et al, 1993; Khoshboue et al, 2002). The amygdala receives dense projections from the dorsal raphe nucleus (DRN) (Ma et al, 1991), and psychological stress activates ascending serotonergic neurons from the DRN to the BLA (Funada and Hara, 2001; Christianson et al., 2010). In rats, restraint stress significantly elevates extracellular 5-HT levels in the BLA in both genders, but females develop a greater response (Mitsushima et al, 2006). The Amygdala in rats, stressful stimuli enhance the release of 5-HT in the CeA (Adell et al, 1997), and serotoninergic receptor stimulation in the CeA is sufficient and necessary for stress-induced activation of the HPA axis (Feldman et al, 1998; 2000). Agonist-induced stimulation of 5-HT1A receptors in the CeA stimulates the HPA axis (Feldman et al, 2000), whereas depletion of 5-HT in CeA or infusion of 5-HT2 receptor antagonists blocks its excitatory effects on the HPA axis (Feldman et al, 1998; 2000). In several studies, 5-HT was found to be a modulator of glutamate and GABA-mediated neurotransmission (Ciranna, 2006). GABAergic transmission in the amygdala is an important pathway by which the flow of information, activity, and function can be controlled (Cassell et al, 1999; Davis et al, 1994; Woodruff et al, 2006; Równiak et al., 2017), and considerable evidence has shown that this neurotransmitter in the amygdala is critical in mediating several aspects of the stress response. Studies in rats have demonstrated that acute restraint stress increases GABA efflux in the BLA (Reznikov et al, 2009; Andolina et al, 2013 and 2014), while chronic stress decreases GABAergic transmission in the BLA (Reznikov et al 2009). Animals that were subjected to repeated stress showed no acute stress-induced rise in GABA release in the BLA and did not experience any effects on GABA outflow in the CeA (Reznikov et al, 2009). Repeated stimulation of corticotropin-releasing factor (CRF) receptors in the BLA enhances anxiety-like behaviors, which are associated with decreased GABAergic inhibition (Rainnie et al, 2004). GABA is the predominant co-transmitter in CRF neurons of amygdala (Gafford and Ressler, 2015). Consequently, excessive stress-induced CRF might induce a depression of local GABAergic inhibition and the resultant hyper excitability of the amygdala. CRF neurons in the CeA can directly project to the PVN or – via indirect GABAergic projections – to the bed nucleus of the stria terminalis (BNST), which contributes to the further activation of the HPA axis and CRF release (Davis and Shi, 1999).The impact of stress is also determined by the ability of the organism to cope with its situation (Ursin and Olff, 1995). Several reports have highlighted the function of GABAergic transmission in the mouse amygdala, particularly the BLA, in shaping an individual’s coping style to stress (Andolina et al, 2013, 2014). The amygdala receives glutamatergic afferents from several areas of the brain, including cortical and thalamic regions (LeDoux et al, 1990; Turner and Herkenham, 1991 and McDonald et al, 1999). Microdialysis studies have shown that acute restraint stress increases extracellular glutamate levels in rat BLA and CeA complexes (Reznikov et al, 2007; Skórzewska et al, 2009 and Reaga et al, 2012), which in turn activates the HPA axis (Gabr et al, 1995; Herman and Cullinan, 1997) Acute restraint stress elicits the quick and robust release of glutamate in the BLA and CeA (Reznikov et al, 2007; Skórzewska et al, 2009; Reaga et al, 2012), whereas chronic restraint stress diminished glutamate levels (Grillo et al., 2015). In the brain, miRs are critical in modulating many neurobiological processes, including changes in neuronal morphology and neurotransmitter homeostasis. The ability of miRs to selectively and reversibly silence mRNAs and their involvement in neuronal plasticity and neurotransmitter release render miRNAs well suited as fine-tuning regulators of the complex and extensive molecular network that drives stress responses (Leung and Sharp, 2010). Acute stress upregulates miR-34 in the CeA of mice and that virus-mediated overexpression of miR-34 in this area prevents stress-induced anxiety and blocks the response of CRFR1 to its ligand CRF, suggesting that miR-34 regulates the molecular machinery of the response to stress (Haramati et al, 2001; Andolina et al, 2016; Mannironi et al, 2010; 2013; Volk et al, 2014).

In contrast to the amount of information accumulated in the study of stress mechanisms in mammals which is also still far from explaining the detailed molecular processes that occur during stress regulation, in teleost the information is very poor but known that the fish’s telencephalon lacks a cortex but possesses telencephalon cortical-like functions, as reported in several fish species (Silva et al 2015). The fish’s telencephalon contains several distinct neuronal populations that have been characterized as functional homologues to mammalian forebrain areas. For example, the dorsomedial and dorsolateral pallium have been characterized as functional homologues to the mammalian BLA and hippocampus, respectively, and are implicated in stimulus salience, memory, and learning (Goodson and Kingsbury 2013; Vidal-Gonzalez et al 2006; Vargas et al 2009). Furthermore, the ventral part of the telencephalon was reported to be functionally homologous to the lateral septum (Goodson and Kingsbury, 2013; Vidal-Gonzalez et al., 2006), which is very important in the regulation of emotional reactivity and goal-oriented behavior (Luo et al 2011; Singewald et al., 2011; Demski, 2013).

**Hypothalamus-pituitary-interrenal (HPI) axis regulation of stress**

In mammals, the HPA axis is modulated by extra hypothalamic limbic structures and in particular the hippocampus and the amygdala (Feldman et al, 1995; Jankord and Herman, 2008). While hippocampal neurons exert an inhibitory effect on the activation of the axis, the activity of the amygdala exerts a significant facilitatory effect (Feldman et al, 1995). The amygdala has two direct and one indirect efferent connection with the hypothalamus: (1) the stria terminalis directly connects the amygdala with the preoptic area in the hypothalamus. (2) The ventral pathway directly connects the CeA and BLA with the hypothalamus (Gray et al, 1989). (3) An indirect pathway consists of projections from the CeA to the BNST the efferents of which retro project to CRH cells in the paraventricular nucleus of the hypothalamus (Sawchenko and Swanson, 1983). In teleost the mechanism of stress regulation in the HPI axis is still obscure but when stress signals are perceived, the hypothalamic region of the nucleus preopticus responds by releasing corticotropin-releasing hormone (CRH) into the pituitary. This signal is received by the CRH receptor subtype 1 (CRH-R1) on pituitary corticotropes from the pars distalis. The binding of CRH with its receptor stimulates adrenocorticotropic hormone (ACTH) release into the circulation (Huising et al., 2004; Metz et al., 2004). ACTH stimulates the production and release of the main corticosteroid cortisol from interrenal cells of the head kidney (Flik et al., 2006) (Fig. 1).

Cortisol exerts its effect on target cells by binding to the cytosolic glucocorticoid receptor (GR) (Thornton 2001). The cortisol-GR complex translocates into the nucleus, where it binds to responsive glucocorticoid elements and modifies gene expression (Stolte et al., 2006). As in mammals, both the GR and the mineralocorticoid receptor (MR) are capable of binding cortisol (Bridgham et al., 2006). In contrast to mammals, fish have duplicate GR genes (GR1 and GR2) that are translated into functional proteins (Stolte et al., 2006). GR1 also exists in two variants: GR1a and GR1b (Ducouret et al., 1995; Stolte et al., 2008a). Thus, there are four receptors capable of binding cortisol in fish: GR1a, GR1b, GR2, and MR. However, their ability to induce activation of downstream genes is dependent on the cortisol concentration (Stolte et al., 2008b). CRF signal is mediated by at least two receptors (CRFR1 and CRFR2). CRFR1 has been reported to mediate HPI axis activation, whereas CRFR2 takes part in the expression of several behavioral and physiological reactions in response to stress (Backström and Winberg, 2013; Flik et al., 2006). Moreover, similar to mammals, 5-HT in teleosts affects hypothalamic CRF release, where the 5-HT receptor type 1A (5-HT1A) plays a central role in the regulation of the HPI axis (Dinan, 1996; Winberg et al., 1997; Höglund et al., 2001; Medeiros et al., 2010). In addition, the HPI axis is under feedback control by cortisol through MR and GR in the hypothalamus and the pituitary (Bury et al., 2003; Colombe et al., 2000; Sturm et al., 2005). Studies indicate interactions between HPI and limbic functions in the teleost telencephalon (Alderman and Bernier, 2007; Silva et al., 2015). Moreover, associations between telencephalic 5-HT and HPI-axis activity (Höglund et al., 2000, 2001; Øverli et al., 2005; Silva et al., 2015; Winberg et al., 1997; Winberg and Lepage, 1998) support similar involvement of this brain part in HPI-axis regulation, as observed in mammals (De Kloet et al., 2005).

Corticosteroids regulate multiple aspects of immune defenses in mammals and influence the secretion of pro- and anti-inflammatory cytokines (Elenkov and Chrousos, 2006). Similarly, cortisol receptors have been described in fish immune cells, and cortisol affects the immune response in common carp (*Cyprinus carpio*) (Stolte et al., 2008a, b), rainbow trout (*Oncorhynchus kisutch*), and gilthead sea bream (*Sparus aurata*) (Acerete et al., 2007). Cortisol affects the secretion of cytokines from leukocytes and, in response, these cytokines regulate the HPI axis activity (Metz et al., 2006). In addition, cortisol inhibits proliferation and induces apoptosis of lymphocytes from the blood, head kidney, spleen, and thymus (Saha et al., 2003). This process is dependent on GR and RU486 (mifepristone), a specific GR blocker, prevents these cortisol processes (Weyts et al., 1997). In mammals, it was reported that chronic or acute administration of dexamethasone, a potent agonist of GR, can significantly cause a neurotransmission imbalance between glutamate and GABA via upregulation of GABAergic neurons and downregulation of glutamatergic neurons in amygdala and as results cortisol regulates stress-induced emotions (Wang et al., 2016). The main function of ACTH in fish is the regulation of cortisol production in the interrenal cells of the head kidney (Wendelaar-Bonga, 1997; Flik et al., 2006)). In rainbow trout, the use of mifepristone decreases stress-induced cortisol secretion by reducing hypothalamic CRH mRNA expression (Alderman et al., 2012). The corticotropic action of CRH can be avoided by the administration of the non-selective antagonist of the CRH receptor (Weld et al., 1987). Another hypothalamic factor is the melanin-concentrating hormone (MCH), which is a strong inhibitor of CRH-stimulated ACTH secretion (Baker et al., 1985; 1986). Rainbow trout acclimated to abundant light had higher levels of MCH and ACTH and lower cortisol levels in plasma, unlike fish acclimated to a dark environment (Baker and Rance, 1981; Gilham et al., 1985). MCH is a peptide that mediates color changes in teleost fish (antagonist of the alpha-melanocyte-stimulating hormone a-MSH) (Kawauchi et al., 1983), and its plasma levels are modified under stress conditions. However, hypothalamic MCH regulates food intake and energy balance in mammals (Qu et al., 1996) and gold fish (Matsuda et al., 2006). However, the effect of MCH is significantly lower than the effect of CRH on food intake and energy balance in fish under stress conditions.

**Autonomic nervous system regulation of stress**

In mammals and teleost fish, immune organs are innervated by sympathetic neurons. In fish, sympathetic innervation of lymphoid tissue was found in the spleen of coho salmon, where nerve fibers are associated with the vasculature and melanomacrophage centers (Flory, 1989). Moreover, immune cells express receptors for stress hormones and neurotransmitters, including adrenergic receptors (ARs). Mammalian innate immune cells express both a- and b-AR subtypes, while exclusive expression of adrenergic receptors of the b2 subtype was found on T and B lymphocytes (Nance and Sanders, 2007).

In mammals, lymphoid organs are innervated by both sympathetic and parasympathetic nerve fibers (Elenkov et al., 2000; Pavlov, 2008) whose activation stimulate or inhibit the immune response. Furthermore, leukocytes express both cholinergic and adrenergic receptors (Kawashima and Fujii, 2003). However, little is known about the cholinergic system in fish versus the adrenergic system, which is predominant in response to stress (Fig. 1). Catecholamine receptors are present on immune cells of teleost fish (Roy and Rai, 2008), and many lymphoid tissues receive sympathetic innervation. For example, in coho or silver salmon (*Onchorhynchus kisutch*), the spleen is highly innervated by adrenergic fibers in the vasculature and parenchyma (Flory, 1989). Several radio-ligand binding experiments have demonstrated the presence of β adrenergic receptors (b-AR) in the anterior kidney, spleen, and peritoneal leukocytes of goldfish (*Carassius auratus*) (Jozefowski and Plytycz, 1998), and in the head kidney and spleen leukocytes of the American catfish (*Ictalurus punctatus*) (Finkenbine et al., 2002). The influence of sympathetic innervations on the immune system of teleost fish is exerted through the binding of adrenaline (epinephrine) and NA (norepinephrine) to their functional adrenoceptors, a-AR and b-AR, which are present in the cells of the immune system (Roy and Rai, 2008). Catecholamines inhibit the innate and acquired immune response in various species of teleost through the activation of b-AR. However, a-AR stimulation leads to the production of antibodies (Roy and Rai, 2008; Flory, 1990; Flory and Bayne, 1991; Narnaware et al., 1994). b2a-AR mRNA is constitutively expressed in the brain, especially in the preoptic nucleus (homologous to the mammalian hypothalamus) and in immune organs. During the in vivo inflammatory response, b2a-AR expression was upregulated in the peritoneal leukocytes. In addition, adrenaline inhibits the expression of pro-inflammatory cytokines, chemokines, and their receptors in fish phagocytes cultured in vitro (Chadzinska et al., 2012). Adrenaline might influence the inflammatory response via direct regulation of leukocyte migration and/or apoptosis during zymosan-induced peritoneal inflammation in the common carp (Kepka et al., 2013). Similar to mammals (Fernandez and Acuna-Castillo, 2012), autonomic nervous responses in fish can be influenced by the immune system through the cytokines produced by glial cells (e.g., astrocytes) in the CNS, which modulates neuroendocrine responses. The autonomic nervous response can also be altered by peripheral signals that gain access to the CNS through the circumventricular organs, which are structures without blood-brain barriers (Quan and Banks, 2007). Conversely, catecholamine secretion from teleost chromaffin cells in the head kidney is regulated by a host of cholinergic and non-cholinergic pathways that ensure sufficient redundancy and flexibility in the secretion process to permit synchronized responses to a myriad of stressors (Perry and Capaldo, 2011).

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**Fig. 1: Putative regulation of stress in common carp.** Acute stress usually activates the sympathetic neurons in the autonomic nervous system (ANS) and this activates the chromaffin cells of the head kidney to release catecholamines, such as adrenaline (A) and noradrenaline (NA). Catecholamines bind to their receptors in the blood cells and promote production of specific cytokines. Chronic stress activates the axis of hypothalamus-pituitary-interrenal cells of the head kidney (HPI) and promotes the release of corticotropin-releasing hormone (CRH) from the hypothalamus that causes the pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream, which in turn causes the secretion of cortisol from the interrenal cells. Cortisol binds to its receptors in the blood cells and, as a result, various processes occur that are typical of the intensity and duration of stress. Likewise, cortisol in the process of feedback regulates the hypothalamic, hippocampal and locus coeruleus (LC) activity. Stressors stimuli from various brain areas such as prefrontal cortex-like, LC and dorsal raphe nucleus (DRN) stimulate the amygdala to elicit the proper activation of the HPI axis and different body functions. The amygdala facilitates the release of NA, corticotropin-releasing factor (CRF) and 5-hydroxytryptamine (5-HT) from the hypothalamus. The amygdala attenuates the negative feedback exerted by glucocorticoids probably by reducing hippocampal glucocorticoid receptors (GR) and thus facilitating the activation of the HPI axis.

**The influence of stress on the immune system**

Studying the effect of stress on the immune system is challenging because of the variable response between one carp and another. Some respond more and some less and consequently, the significance of the results weakens and does not represent the real effect on the immune system. Therefore, monitoring changes in cytokine and leukocyte levels in peripheral blood throughout stress treatments was preferred over sampling their levels in the spleen, kidney, head kidney, and liver. Monitoring the blood enables following changes in each carp without killing it (Shimon-Hophy and Avtalion, 2017). A systematic study revealed which function of the carp immune system was most affected by hypoxic stress and how the duration of stress affects the expression of these functions (Table 1). The duration of stress differentially changes the activity of different functions in the carp immune system. Acute stress enhances the fast pathway that activates the sympathetic nervous system to release catecholamines, such as adrenaline and noradrenaline, from chromaffin cells in the head kidney, and the released catecholamines bind to their receptors in leukocytes (Bernier et al., 1999; Montpetit and Perry, 2002). As a result, the pro-inflammatory function (IL1b, IL6, and TNFa) was upregulated and, at the same time, the activity of regulatory function (TGFb and IL10) was upregulated as well, probably to return pro-inflammatory activity to homeostasis (Shimon-Hophy and Avtalion, 2017; Barker *et al*., 1991; Banerjee and Leptin, 2014). Chronic stress activates the hypothalamus-pituitary-interrenal cell axis and, as a result, interrenal cells in the head kidney release mainly cortisol (Flik et al., 2006). The cortisol binds to its receptors in leukocytes and promotes different processes in the leukocytes (Thornton 2001; Stolte et al., 2006).

Monitoring that influence in hypoxic chronic stress on the immune activity in the common carp peripheral blood leukocytes revealed downregulation of regulatory (IL10, TGFb, FoxP3), pro-inflammatory (IL1β, IL6) and inflammatory (IL17) functions until the second week of chronic stress but in the third week, their levels overcome and returned to homeostasis (Shimon-Hophy and Avtalion 2017). TNFa levels do not change during hypoxic stress treatments (Table 1), but in chronic cortisol implant for 5 days in rainbow trout TNFa behaved slightly different (Cortés et al., 2013. The chronic cortisol treatment showed results similar to that in acute hypoxic stress. In contrast, the main impaired functions even after 22 days of chronic stress (Shimon-Hophy and Avtalion, 2017, 2018) were: 1. cytotoxic mediators (Bhat et al., 2017; Nagata and Golstein, 1995; Trapani and Smyth, 2002; O'Neill et al., 2020 and Endsley et al., 2004), such as interferon (IFN)γ2b, Fas ligand (FasL) and NK-lysin and granzyme; 2. IL12 and Tbet, the responsible for the proliferation and maturation of Th1 cells that mediate host defense against intracellular pathogens (Hsieh et al 1993; Szabo et al 2000; Sekiya and Yoshimura, 2016); and 3. IL8, the attractant of leukocytes to the infection site (Dixit and Simon, 2012). IL8, which was downregulated during 22 days of chronic stress can explain the macrophage/neutrophil/leukocyte mobilization decline in different compartments of the body, as shown by Wojtaszek et al. (2002) and others.

In contrast to the sharp decrease in the level of cytotoxic cytokines following chronic stress, it has been proved that the non-specific cytotoxic receptor protein 1 (NCCRP1), which was previously related to a marker of non-specific cytotoxic cells (NCC) (Evans, 1992) as a variant of NK cells in teleost, is not a marker of any cell type but is abundant in γδT, mucosal-associated invariant T (MAIT), T carp lymphocytes, and even in thrombocytes (Shimon-Hophy et al 2020). Further study will clarify what role it played in the stress process.

**Table 1: Changes in the levels of mRNA components that represent different functions in the immune system of common carp following stress.**

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| --- | --- | --- | --- | --- | --- |
| **Cytokines** | **Con** | **AS** | **CSW1** | **CSW2** | **CSW3** |
| **IL1b** | **1±0.12** | **5.15±0.67‎‎\*‎** | **1.42±0.17** | **0.06±0.02\*** | **2.69±0.60\*** |
| **IL6** | **1±0.18** | **1.47±0.28‎‎\*‎** | **1.43±0.79** | **0.16±0.11** | **1.16±0.20** |
| **TNFa** | **1±0.14** | **3.73±0.27‎‎\*‎** | **1.29±0.17** | **0.61±0.06** | **0.79±0.10** |
| **IFNg2b** | **1±0.12** | **1.4±0.19** | **0.00±0.00\*** | **0.00±0.00\*** | **0.00±0.00\*** |
| **C3s** | **1±0.8‎** | **0.79±0.21** | **18.44±9.11** | **4.43±1.91** | **10.66±5.36** |
| **IgM** | **1±0.12‎** | **1.35±0.16** | **1.67±0.22** | **1.51±0.10** | **1.99±0.30** |
| **IL10** | **1±0.15** | **3.01±0.34‎‎\*‎** | **0.35±0.07\*** | **0.0006±0.0001\*** | **0.51±0.07** |
| **FoxP3** | **1±0.14‎** | **2.51±0.73** | **0.27±0.04\*** | **0.0021±0.0004\*** | **0.80±0.14** |
| **TGFb** | **1±0.14‎** | **1.98±0.21‎‎\*‎** | **0.99±0.13** | **0.0027±0.0004\*** | **3.63±0.48** |
| **IL8** | **1±0.1 3** | **0.81±0.0‎‎8‎** | **0.18±0.03\*** | **0.0016±0.0003\*** | **0.30±0.07\*** |
| **CD95** | **1.00±0.28** | **1.14±0.17** | **1.78±0.4** | **3.38±0.87\*** | **2.34±0.45\*** |
| **FasL** | **1.00±0.17** | **1.00±0.19** | **0.83±0.18** | **0.47±0.1\*** | **0.23±0.08\*** |
| **granzyme** | **1.00±0.39** | **0.45±0.11** | **0.81±0.28** | **0.50±0.14** | **0.26±0.06\*** |
| **NKlysin** | **1.00±0.61** | **0.26±0.07\*** | **0.35±0.12** | **0.25±0.06\*** | **0.30±0.07\*** |
| **NILT1** | **1.00±0.81** | **1.77±0.57** | **1.50±0.50** | **1.39±0.63** | **0.56±0.26** |
| **NILT2** | **1.00±0.31** | **1.56±0.71\*** | **1.32±0.59** | **1.78±0.60** | **0.77±0.23** |
| **IL12b** | **1.00±0.18** |  | **0.06±0.12\*** | **1.04±0.99\*** | **0.00003±0.00006\*** |
| **Tbet** | **1.00±0.41** | **0.90±0.27** | **0.52±0.11** | **0.72±0.18** | **0.29±0.13\*** |
| **STAT4** | **1.00±0.63** | **3.16±0.45\*** | **1.03±0.31** | **1.51±0.55\*** | **0.75±0.28** |
| **CXCR3** | **1.00±0.38** | **0.83±0.21\*** | **0.84±0.34** | **0.80±0.23** | **0.44±0.32** |

**The results are from the articles Shimon-Hophy and Avtalion, (2017, 2018).**

**\* - p≤0.05, Con- control, AS- acute stress, CSW1- chronic stress after 8 days, CSW2- chronic stress after 15 days, CSW3- chronic stress after 22 days**

Chronic administration of cortisol (simulating chronic stress) decreased the relative expression of IFNa-1, heat shock proteins 70 (HSP70) and 90 (HSP90), **serum amyloid A protein** (SAA), and glucocorticoid receptors in *Salmo salar* (Engelsma et al., 2003). Macrophage cell line revealed inhibition of chemotaxis, phagocytosis, and respiratory burst activity in a goldfish (Wang and Belosevic, 1995). These chronic administrations of cortisol strengthen the downregulation of cytotoxic functions by chronic stress (Table 1).

Innate function (immunoglobulin M (IgM) and complement C3s mRNA) (Table 1) was not significantly affected during acute or chronic hypoxic-stress treatments (Shimon-Hophy and Avtalion 2017), chronic confinement stress of juvenile Eurasian perch (*Perca fluviatilis*) (Douxfils *et al*., 2011) and high stocking density of *Eleginops maclovinus* (Vargas-Chacoff *et al*., 2014). These results were 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), Presumably these differences between the results are due to the presence of modulators that regulate IgM humoral activity (Cuesta et al., 2004). Similarly, C3s mRNA showed no significant changes in both acute and chronic stresses, although its levels fluctuated throughout the chronic stress period (Table 1). These results differ from the hemolytic findings (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).

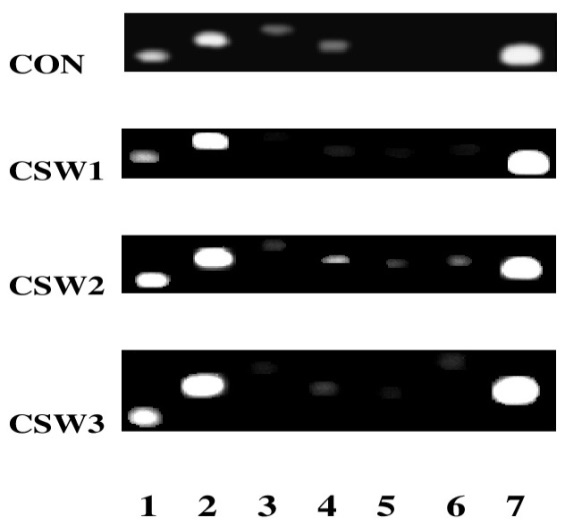
Stress-influenced functions revealed deterioration of cytotoxic activity and cytokines regulating Th1 proliferation (Table 1) but what about the other leukocytes? Studies of leukocyte levels by FACS and by mRNA levels of cell markers revealed a decrease in the levels of B, plasma, macrophages, and CD4 (Th1) cells (Table 2 and Fig. 2) (Shimon-Hophy and Avtalion, 2017). These results are in agreement with the decrease in leukocyte numbers in *Oncorhynchus mykiss* (Cristea *et al*., 2012), the 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). We are not sure if stress also caused deterioration of MAIT cells (Shimon-Hophy et al., 2020) because the microscopic picture of several carps revealed a vast decrease and in others – high levels; therefore, these cells should be further studied. In vitro studies strengthened the above mentioned and showed that cortisol treatments: 1. decrease the phagocytosis of head kidney cells from tilapia, common carp, and silver sea bream (*Sparus sarba*) (Law et al., 2001); 2. inhibit the pro-oxidative activity of leukocytes from head kidney in golden sea bream (Esteban et al., 2004); 3. inhibit the proliferation of a monocyte/macrophage cell line from rainbow trout (Pagniello et al., 2002); and 4. induce programmed cell death (apoptosis) of macrophages from silver sea bream and Atlantic salmon (*Salmo salar*) (Fast et al., 2008).

CD8, NK, and γδT cells (Table 2 and Fig. 2) did not show any decrease corresponding to that of cytotoxic cytokines although they are known for the high production of IFNγ, FasL, granzyme, and NK lysin (Hayday, 2000; Tschopp and Nabholz, 1990; Zelinskyy et al., 2004; Smyth et al., 2001). Moreover γδT cells are the most numerous cells in carp leukocytes (Fig. 2), and are supposed to be the great producer of IFNγ (Chen et al., 2007; Skeen and Ziegler, 1995), but their cell amounts do not decrease following chronic stress and following the decrease in cytotoxic cytokine levels. This means that chronic stress suppresses the metabolism of cytotoxic cytokines and proliferation of Th1, macrophages/monocytes and plasma cells. Consequently, this suppression might explain the increased susceptibility to diseases occurring in chronic stress (Saeij *et al*., 2003; Mauri *et al*., 2011; Elenkov and Chrousos, 1999; Maule *et al*., 1989).

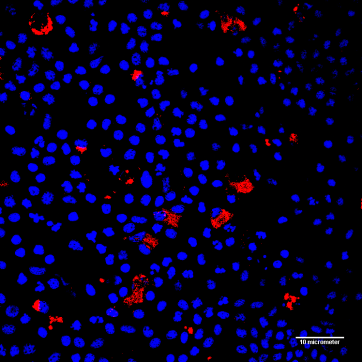
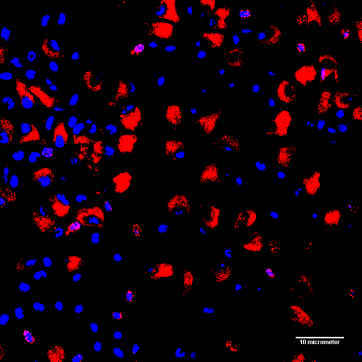
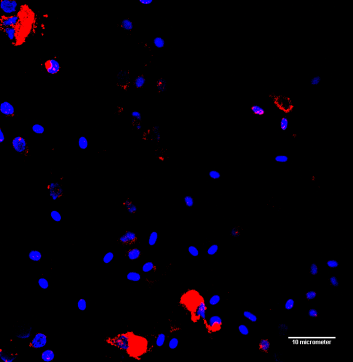
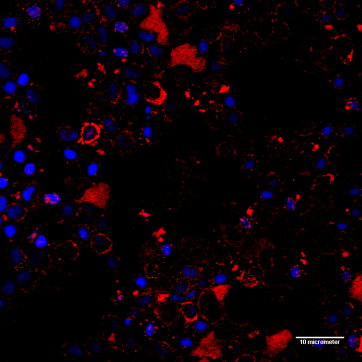
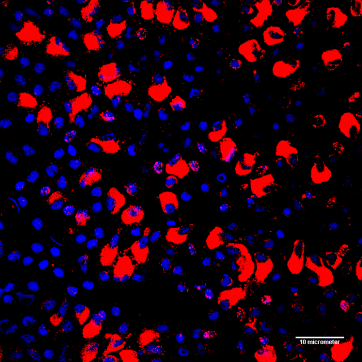
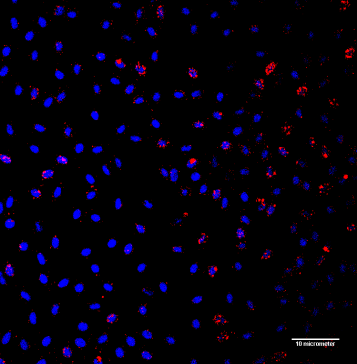
Table 2: Changes in the cell types following stress treatments in peripheral blood leukocytes of common carp.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | Cell type | Con | AS | CSW1 | CSW2 | CSW3 |
| Relative normalized Ratio of mRNA levels | CD4 | 1.00±0.41 | 0.92±0.37 | 0.37±0.12\* | 0.38±0.11\* | 0.14±0.06\* |
| CD8a | 1.00±0.44 | 1.16±0.56 | 0.85±0.16 | 1.18±0.48 | 0.38±0.14 |
| T (TCRε) | 1.00±0.23 | 0.52±0.06\* | 0.38±0.06\* | 0.49±0.09\* | 0.41±0.06\* |
| γδT(TCRγδ) (TCRγδ) | 1.00±0.13 | 1.29±0.35 | 1.70±0.40\* | 1.29±0.23 | 0.67±0.22 |
| Cell percent in PBL | Monocytes/ macrophages | 1.05± 0.09 | 1.01 ± 0.32 | 0.30 ± 0.06**\*** | 0.26 ± 0.09**\*** | 0.21 ± 0.05**\*** |
| Like B cells | 8.50±1.69 | 4.28 ± 0.95**\*** | 3.86 ± 1.40 | 1.34 ± 0.37**\*** | 1.38 ± 0.17**\*** |
| Like Plasma cells | 4.86±2.52 | 3.76 ± 0.76 | 2.54 ± 0.70 | 1.64 ± 0.42**\*** | * 1. 0.25**\*** |

\*P ≤ 0.05, Con – control, AS – acute stress, CSW1 – chronic stress during 1 week, CSW2 – chronic stress during 2 weeks, CSW3 – chronic stress during 3 weeks, PBL – peripheral blood leukocytes. Results from Shimon-Hophy and Avtalion (2017, 2018).

**Fig. 2: The distribution of leukocytes' types in common carp peripheral blood following stress treatments.** Cell markers were produced from mixed 1000 ng cDNA of 8 fish by PCR amplification and loaded on 1.3% agarose gel with TBE (Tris/Borate/EDTA) running solution**.** 1- T cell (CD3-TCRε), 2-γδT cells (TCRγδ), 3-CD4, 4-CD8, 5-NK cells (CD56), 6- macrophages/monocytes (CD209), 7-NCCRP1

The decrease or increase in metabolism was shown in the volume of the cells (Fig. 3). In acute stress, when metabolism of pro-inflammatory and regulatory cytokines was upregulated, cell volume increased up to 3 times (by measuring cell area), while in chronic stress, cell volume of γδT cells decreased up to 3 times following 3 weeks of chronic stress. This picture reinforces the perception that chronic stress impairs mainly the metabolism of cytotoxic cytokines

**T(TCRε**)   **γδT(TCRγδ)**  

**Control Acute stress Chronic stress (3w)**

Fig. 3: The difference in cell volume of common carp peripheral blood T and γδT cells following stress treatments. 3W- chronic stress after 22 days

Results from Shimon-Hophy et al (2020).

**Summary**

The continued existence of the aquaculture industry depends on its profitability. Stress is considered a primary factor contributing to impaired health in cultured fish. Studying the influence of stress on the immune system can suggest tools to handle sensitivity to the morbidity and mortality of fish in fish ponds.

The processes regulating the immune system during stress are not clear enough in mammals and even more unclear in fish. Little is known about the specific etiological pathways that lead from a triggering stressor to the development of a specific pathological phenotype as well as about the interactions between the nuirotransmittors as NA, 5HT, GABA, glutamate and others

Despite the clear involvement of brain structures such as amygdala, hippocampus and HPI axis, it remains unknown how these structures cause a variety of pathological disorders and how they cause different responders to respond differently to the same stimulus of stress. Moreover, studies on different stress responses have reported similar alterations with regard to neurotransmitter activity, neuroplastic changes, and alterations in amygdalar and HPI function, suggesting that these properties are common and that the phenotypic specificity is rooted in upstream mechanisms.

So far studies indicate that the brain accumulates stress signals, process them and activate several brain structures to maintain physiological integrity. The intensity, duration and type of stress evoke autonomic system or limbic circuits. The autonomic system responds immediately to acute stress and stimulates the chromaffin cells in the head kidney to release mainly proinflamatory and regulatory cytokines. The limbic structures respond slowly mainly to chronic stress, the limbic homologues of amygdala and hippocampus accumulate signals from different brain areas process emotions, memory of stress and activate HPI axis and other body functions as blood pressure, heart beat, energy accumulation etc.. HPI axis promotes interrenal cells in the head kidney to release glucocorticoid hormone such as cortisol to the bloodstream. Glucocorticoids deteriorate the cytotoxic activity- downregulation of cytokines involved in the cytotoxic activity and downregulation of cell proliferation, of cells involved in phagocytosis, antibody production and Th1. Downregulation of the cytotoxic activity is critical for disease resistance and unwanted cells elimination therefore further study is needed.