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

**Mazal Shimon-Hophy\***1 **and Ramy R. Avtalion**1

1Laboratory of Comparative Immunology and Genetics, The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel

**\*Corresponding author:**

**Mazal Shimon-Hophy**

**Tel: 972-3-5318834, Fax: 972-3-9222202**

**Email: hophymazal@gmail.com**

**Abbreviations:** CNS, central nervous system; PFC, prefrontal cortex; PVN, paraventricular nucleus; CeA, central nucleus of the amygdala; 5-HT, 5-hydroxytryptamine; mPFC, medial 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 necrosis factor; TGF, transforming growth factor; FoxP3, forkhead box P3; NCC, nonspecific cytotoxic cell; MAIT, mucosal-associated invariant T cell; NCCRP1, nonspecific cytotoxic cell receptor protein 1; IgM, immunoglobulin M; CD, cluster of differentiation; Th1, T helper 1 cell; ANS, autonomic nervous system

**Abstract**

Aquaculture conditions expose fish to internal and environmental stressors that increase their susceptibility to morbidity and mortality. The brain accumulates stress signals and processes them according to the intensity, frequency duration, and type of stress, and recruits several brain functions to evoke the autonomic or limbic systems. Activation of the autonomic system causes the rapid release of catecholamines such as adrenaline and noradrenaline into circulation from chromaffin cells in the head kidney. Catecholamines trigger blood cells to release pro-inflammatory and regulatory cytokines to cope with acute stress. Activation of the 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 releases glucocorticoids such as cortisol to the bloodstream. Glucocorticoids cause the downregulation of various immune system functions depending on the duration, intensity, and type of chronic stress. As stress persists, most immune functions, except for cytotoxic functions, halt and return to homeostasis. The deterioration of cytotoxic functions during chronic stress appears to be responsible for increased morbidity and mortality.

**Introduction**

Aquaculture conditions are often exposed to various stressors, which could be consequences of issues such as 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).

Several studies have reported that stressors reduce hippocampal volume (Brown *et al.,* 2015; Gerritsen *et al.,* 2015; Head *et al.,* 2012) and, as a result, impact memory and learning (Buchanan *et al.,* 2006; Roozendaal *et al.,* 2009; Wolf, 2009). The amygdala - in particular, the basolateral amygdala (BLA) - increases the dendritic length and spine density and results in emotional changes (Holtmaat and Svoboda, 2009; Sousa and Almeida, 2012). Furthermore, stress exaggerates adverse effects such as shrinking of the thymus, spleen, or other lymphatic organs, changes in leukocyte number and distribution, or presence 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 may alleviate their harmful 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 influence immune system functions, which are essential to the health, welfare, and production efficiency of common carp. The evaluation of stress’s influence on the immune system will be mainly based on previous studies conducted in our lab.

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

The brain accumulates and processes external and internal stress signals 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 responses 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 as 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 axis to control functions such as breathing, blood pressure, heart rate, and immune response (McEwen, 2006). Excessive or inadequate basal activity and the responsiveness of this system may impair development, growth, and body composition and potentially lead to a host of behavioral and somatic pathological conditions (Chrousos, 2009).

**Prefrontal cortex-amygdala regulation**

The mechanism of stress regulation in teleosts has yet to be elucidated, and information on stress-regulating processes is limited, especially with regard to the upper functions of brain homologs such as the prefrontal cortex (PFC), amygdala, and hippocampus. In contrast, it was revealed that the PFC integrates and processes sensory information in mammals (Zhuo, 2008; Miskovic and Keil, 2012; Meaney, 2013) and regulates information via a complex connectional network with other brain structures (Negrón-Oyarzo *et al.,* 2016). The medial 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 a regulatory function in amygdalar activation during stress response.

Several studies have demonstrated that 5-hydroxytryptamine (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 that 5-HT functions in mediating mPFC-amygdala interplay (Wellman *et al.,* 2007). Bilateral selective 5-HT depletion in the mouse mPFC reduces BLA GABA release that is induced by restraint stress and passive coping in the forced swimming test; this suggests that 5-HT and GABA transmission-mediated PFC/amygdala connectivity is 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; Moore and Bloom, 1979), 5-HT and epigenetic mechanisms (e.g., non-coding RNA).

Administration of α1- or β-adrenergic receptor antagonists directly into the medial amygdala (MeA) mitigates the adrenocorticotropic hormone (ACTH) response to immobilization stress (Ma and Morilak, 2005). This data supports the hypothesis that greater NA release in the MeA, acting primarily through ACTH receptors, facilitates the 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 a potentially mechanism by which the MeA controls 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 nucleus of the 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 tend to develop a greater response (Mitsushima *et al.,* 2006). In rat amygdalas, 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). Several studies have confirmed that 5-HT was a modulator of glutamate and GABA-mediated neurotransmission (Ciranna, 2006). GABAergic transmission in the amygdala is an important pathway that controls the flow of information, activity, and function (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 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, 2014), while chronic stress decreases GABAergic transmission in the BLA (Reznikov *et al.,*2009). Animals that were subjected to repeated stress did not exhibit an 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 that are associated with decreased GABAergic inhibition (Rainnie *et al.,* 2004). GABA is a predominant co-transmitter in amygdala CRF neurons (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 further activation of the HPA axis and CRF release (Davis and Shi, 1999).The impact of stress is also determined by the organism’s ability 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 stress coping style (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 make 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 mouse CeA and 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 stress response (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 also remains far from explaining the detailed molecular processes that occur during stress regulation, the information concerning teleosts is very limited; however, studies have found that while the fish’s telencephalon lacks a cortex, it 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 homologs to mammalian forebrain areas. For example, the dorsomedial and dorsolateral pallium have been characterized as functional homologs 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 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 the hippocampus and the amygdala, in particular (Feldman *et al.,* 1995; Jankord and Herman, 2008). While hippocampal neurons exert an inhibitory effect on the activation of the axis, amygdala activity exerts a significant facilitatory effect (Feldman *et al.,* 1995). The amygdala has two direct efferent connections 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), and (3) an indirect pathway consists of projections from the CeA to the BNST, the efferents of which retroproject to CRH cells in the paraventricular nucleus of the hypothalamus (Sawchenko and Swanson, 1983). In teleosts, the mechanism of stress regulation in the HPI axis is still obscure; however, 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 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 the head kidney’s interrenal cells (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) can bind 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 downstream gene activation depends 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 influences hypothalamic CRF release, where 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). Additionally, the HPI axis is under feedback control by cortisol through MR and GR in the hypothalamus and pituitary (Bury *et al.,* 2003; Colombe *et al.,* 2000; Sturm *et al.,* 2005). Studies suggest 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 activities (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 section of the brain 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 influences the secretion of cytokines from leukocytes, and these cytokines regulate the HPI axis activity in response (Metz *et al.,* 2006). Additionally, cortisol inhibits proliferation and induces apoptosis in lymphocytes of 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 GR agonist, can cause a significant neurotransmission imbalance between glutamate and GABA via upregulation of GABAergic neurons and downregulation of glutamatergic neurons in the amygdala, and as a result, cortisol regulates stress-induced emotions (Wang *et al.,* 2016). The main function of ACTH in fish is the regulation of cortisol production in the head kidney’s interrenal cells (Wendelaar-Bonga, 1997; Flik *et al.,* 2006)). In rainbow trout, mifepristone use reduces stress-induced cortisol secretion by reducing hypothalamic CRH mRNA expression (Alderman *et al.,* 2012). The corticotropic action of CRH can be avoided through the administration of the non-selective antagonist of the CRH receptor (Weld *et al.,* 1987). Another hypothalamic factor is the melanin-concentrating hormone (MCH), a strong inhibitor of CRH-stimulated ACTH secretion (Baker *et al.,* 1985; 1986). Rainbow trout that acclimated to abundant light had higher MCH and ACTH levels 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 goldfish (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 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 α- and β-AR subtypes, while exclusive expression of adrenergic receptors of the β2 subtype was found on T and B lymphocytes (Nance and Sanders, 2007).

In mammals, lymphoid organs are innervated by sympathetic and parasympathetic nerve fibers (Elenkov et al., 2000; Pavlov, 2008) whose activation stimulates or inhibits the immune response. Furthermore, leukocytes express both cholinergic and adrenergic receptors (Kawashima and Fujii, 2003). However, little is known about the fish cholinergic system versus the fish adrenergic system, which is predominant in response to stress (Fig. 1). Catecholamine receptors are present on the 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, α-AR (a-AR) and b-AR, which are present in immune system cells (Roy and Rai, 2008). Catecholamines inhibit the innate and acquired immune response in various species of teleost through b-AR activation. 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 immune organs. During the *in vivo* inflammatory response, b2a-AR expression was upregulated in the peritoneal leukocytes. Additionally, 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 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 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 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).

****

**Fig. 1: Putative regulation of stress in common carp.** Acute stress usually activates the sympathetic neurons in the autonomic nervous system (ANS), which in turn 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 the 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. This causes the pituitary gland to release adrenocorticotropic hormone (ACTH) into the bloodstream, which causes the secretion of cortisol from the interrenal cells. Cortisol binds to its receptors in 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 hypothalamic, hippocampal, and locus coeruleus (LC) activity. Stressor stimuli from various brain areas such as prefrontal cortex-like formation, 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 likely attenuates the negative feedback exerted by glucocorticoids by reducing hippocampal glucocorticoid receptors (GR), thus facilitating HPI axis activation.

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

Studying the effect of stress on the immune system is challenging due to the variable response between carp individuals. 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 the ability to follow 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 influences 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, 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, thus, interrenal cells in the head kidney mainly release 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 the influence of chronic hypoxic stress on 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. However, in the third week, their levels halted and returned to homeostasis (Shimon-Hophy and Avtalion, 2017). TNFa levels do not change during hypoxic stress treatments (Table 1), but in chronic cortisol implants that were fixed on rainbow trout for 5 days, 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 as follows: (1) cytotoxic mediators (Bhat *et al.,* 2017; Nagata and Golstein, 1995; Trapani and Smyth, 2002; O’Neill *et al.,* 2020; Endsley *et al.,* 2004), such as interferon (IFN)-γ2b, Fas ligand (FasL), and NK-lysin and granzyme; (2) IL12 and Tbet, which are responsible for Th1 cell proliferation and maturation, which mediates host defense against intracellular pathogens (Hsieh *et al.,*1993; Szabo *et al.,*2000; Sekiya and Yoshimura, 2016); and (3) IL8, which attracts leukocytes to the infection site (Dixit and Simon, 2012). IL8, which was downregulated during the 22-day chronic stress period, can explain the macrophage/neutrophil/leukocyte mobilization decline in different body compartments, 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 confirmed that nonspecific cytotoxic receptor protein 1 (NCCRP1), which was previously related to a marker of nonspecific cytotoxic cells (NCC) (Evans, 1992) as a variant of NK cells in teleosts, 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 plays in stress processes.

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

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **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 above results are pulled from articles by 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 lines revealed the inhibition of chemotaxis, phagocytosis, and respiratory burst activity in 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 events of juvenile Eurasian perch (*Perca fluviatilis*) (Douxfils *et al.,* 2011), or high stocking density of *Eleginops maclovinus* (Vargas-Chacoff *et al.,* 2014). These results contradicted husbandry, confinement, and 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 either acute or chronic stresses, although its levels fluctuated throughout the chronic stress period (Table 1). These results differ from hemolytic findings (Demers and Bayne, 1997; Sunyer and Tort, 1995; Mauri *et al.,* 2011); however, they agreed with reported hypoxia and cortisol-induced stress (Douxfils *et al.,* 2012; Eslamloo *et al.,* 2014).

Stress-influenced functions revealed the 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, macrophage, 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 the apoptosis of B cells in *Cyprinus carpio* (Verburg-Van Kemenade *et al.,* 1999). However, we are not sure if stress also caused MAIT cell deterioration (Shimon-Hophy *et al.,* 2020) since microscopic pictures of several carp samples revealed both a vast decrease and high levels of these cells; therefore, the cells should be studied further. *In vitro* studies strengthened the above-mentioned results and revealed that cortisol treatments had the following effects: (1) decreased the phagocytosis of head kidney cells from tilapia, common carp, and silver sea bream (*Sparus sarba*) (Law *et al.,* 2001); (2) inhibited the pro-oxidative activity of leukocytes from the head kidneys of golden sea bream (Esteban *et al.,* 2004); (3) inhibited the proliferation of monocyte/macrophage cell lines from rainbow trout (Pagniello *et al.,* 2002); and (4) induced 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 great producers of IFNγ (Chen *et al.,* 2007; Skeen and Ziegler, 1995); however, their cell amounts do not decrease following chronic stress or the decrease in cytotoxic cytokine levels. This means that chronic stress suppresses cytotoxic cytokine metabolism and the proliferation of Th1, macrophages/monocytes, and plasma cells. Consequently, this suppression may explain the increased susceptibility to diseases resulting from chronic stress (Saeij *et al.,* 2003; Mauri *et al.,* 2011; Elenkov and Chrousos, 1999; Maule *et al.,* 1989).

**Table 2: Changes in 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**\*** |
| B-like cells | 8.50±1.69 | 4.28±0.95**\*** | 3.86±1.40 | 1.34±0.37**\*** | 1.38±0.17**\*** |
| Plasma-like 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 a 1-week period; CSW2, chronic stress during a 2-week period; CSW3, chronic stress during a 3-week period; PBL, peripheral blood leukocytes. Results pulled from Shimon-Hophy and Avtalion (2017, 2018).

**Fig. 2: The distribution of leukocyte types in the peripheral blood of common carp 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), and (7) NCCRP1.

The decrease or increase in metabolism was shown in the volume of the cells (Fig. 3). During acute stress responses, when the metabolism of pro-inflammatory and regulatory cytokines was upregulated, cell volume increased up to 3 times (according to measurements of the cell area), while during chronic stress responses, the cell volume of γδT cells decreased up to 3 times following 3-week periods of chronic stress. Figure 3 reinforces the perception that chronic stress mainly impairs the metabolism of cytotoxic cytokines.

**T(TCRε**)   **γδT(TCRγδ)**  

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

**Fig. 3: The difference in the cell volume of T and γδT cells in common carp peripheral blood following stress treatments.** “3W” denotes chronic stress after 22 days. Results were adapted from Shimon-Hophy *et al.* (2020).

**Summary**

The persistence of the aquaculture industry depends on its profitability. Stress is considered to be a major factor contributing to poor health in cultured fish. Studying the influence of stress on the immune system allows us to recommend tools to manage fish sensitivity, morbidity, and mortality in fish ponds.

The mechanisms of processes regulating the immune system during stress have not been fully elucidated in mammals and are 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 or the interactions between neurotransmitters such as NA, 5HT, GABA, and glutamate.

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

Recent studies indicate that the brain accumulates and processes stress signals and activates several brain structures to maintain physiological integrity. The intensity, duration, and type of stress evoke autonomic system or limbic circuits. The autonomic system immediately responds to acute stress and stimulates chromaffin cells in the head kidney to release pro-inflammatory and regulatory cytokines. The limbic structures tend to respond slowly to chronic stress; the limbic homologs of the amygdala and hippocampus accumulate signals from different brain areas to process emotions and the memory of stress, and activate the HPI axis and other body functions such as blood pressure, heart rate, and energy accumulation. The HPI axis stimulates interrenal cells in the head kidney to release glucocorticoid hormones such as cortisol to the bloodstream. Glucocorticoids deteriorate cytotoxic activity, resulting in the downregulation of cytokines involved in cytotoxic activity and the downregulation of cell proliferation as well as cells involved in phagocytosis, antibody production, and Th1. The downregulation of cytotoxic activity is critical for disease resistance and unwanted cell elimination; therefore, further study is required.