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

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**Abbreviations:** A, adrenaline; ACTH, adrenocorticotropic hormone; AR, adrenergic receptor; BLA, basolateral amygdala; CD, cluster of differentiation; CeA, central nucleus of the amygdala; CNS, central nervous system; CRF, corticotropin-releasing factor; CRH, corticotropin-releasing hormone; DRN, dorsal raphe nucleus; 5-HT, 5-hydroxytryptamine; FoxP3, forkhead box P3; GABA, gamma-aminobutyric acid; GR, glucocorticoid receptor; HPA, hypothalamus-pituitary-adrenal gland; HPI, hypothalamus-pituitary-interrenal cells; IFN, interferon; IgM, immunoglobulin M; IL, interleukin; ITCs, intercalated cell clusters; MAIT, mucosal-associated invariant T cell; MCH, melanin-concentrating hormone; mPFC, medial PFC; NA, noradrenaline; MR, mineralocorticoid receptor; NCC, nonspecific cytotoxic cell; NCCRP1, nonspecific cytotoxic cell receptor protein 1; PFC, prefrontal cortex; PVN, paraventricular nucleus; TNF, tumor necrosis factor; TGF, transforming growth factor; ; Th1, T helper 1 cell; autonomic nervous system (ANS)

**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, recruiting recruits several brain functions to activate the autonomic or limbic systems. Triggering 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 the activation of preoptic nucleus-pituitary gland-interrenal cells in the head kidney, relesing glucocorticoids such as cortisol to the bloodstream. Glucocorticoids cause downregulation of various immune system functions depending on the duration, intensity, and type of chronic stress. As stress persists, most immune functions, with the exception of cytotoxic functions, cease 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 may be the result 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 (Varsamos et al., 2006; Zarate and Bradley, 2003), diet (Costas et al., 2011; Montero et al., 2001), presence of enemies and pathogens (Demers and Bayne, 1997; Saeij et al., 2003; Sunyer and Tort, 1995), 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, have an impact on memory and learning (Buchanan et al., 2006; Roozendaal et al., 2009; Wolf, 2009). The amygdala — in particular, the basolateral amygdala (BLA) — increases its dendritic length and spine density, which results in emotional changes (Holtmaat and Svoboda, 2009; Sousa and Almeida, 2012). Furthermore, stress exaggerates adverse effects such as the shrinking of the thymus, spleen, or other lymphatic organs, changes in leukocyte number and distribution, or the appearance of bleeding or ulcers that increase susceptibility to morbidity and mortality (Harper and Wolf, 2009). Stressors have negative effects on different physiological responses associated with growth, nutrition, reproduction, and immune responses (Campbell et al., 1992; Hoskonen and Pirhonen, 2006; Lefèvre et al., 2008; Olsen et al., 2005; Øverli et al.*,* 2006; Pickering, 1992; Poli et al., 2005; Vargas-Chacoff et al., WendelaarBonga, 1997; 2014; Zarate and Bradley, 2003). Understanding and monitoring the biological mechanisms underlying stress responses in fish may alleviate the harmful effects of stress through selective breeding and changes in management practices, resulting in improved animal welfare and production efficiency.

This review 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 based primarily 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 stimulate autonomic stress responses or limbic circuits, such as the prefrontal cortex (PFC), amygdala, hippocampus, paraventricular nucleus (PVN) of the hypothalamus, and the nucleus accumbens (Russo and Nestler, 2013; Skoluda et al., 2015). The amygdala functions as a command center that processes emotions and sends stress signals to the hypothalamus, while the hypothalamus also 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 responsiveness of this system may impair development, growth, and body composition, potentially leading 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 has been found that the PFC integrates and processes sensory information in mammals (Meaney, 2013; Miskovic and Keil, 2012; Zhuo, 2008) 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 (Cassell et al., 1989; Krettek and Price, 1977; Mcdonald et al., 1996; Porrino et al., 1981), 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 plays a role in mediating mPFC-amygdala interplay (Wellman et al, 2007). Bilateral selective 5-HT depletion in the murine mPFC reduces release of BLA GABA 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) (Moore and Bloom, 1979; Morilak et al, 1987; Pacák et al, 1993), 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 potential mechanism by which the MeA controls the stress-induced activation of the HPA axis. Immobilization stress enhances NA release in the BLA (Bedse et al., 2015; Galvez et al., 1996; Kawahara et al, 2007; Quirarte et al*.,* 1998; Tanaka et al, 1991) and in the central nucleus of the amygdala (CeA) (Khoshboue et al., 2002; Pacák et al., 1993). 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 (Christianson et al., 2010; Funada and Hara, 2001). 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 amygdalae, 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 is a modulator of glutamate and GABA-mediated neurotransmission (Ciranna, 2006). GABAergic transmission in the amygdala is an important pathway for controlling activity, and function, and the flow of information (Cassell et al., 1999; Davis, 1994; Równiak et al., 2017; Woodruff et al., 2006), 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 (Andolina et al., 2013, 2014; Reznikov et al., 2009), while chronic stress decreases GABAergic transmission in the BLA (Reznikov et al., 2009). Animals 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 has been shown to enhance anxiety-like behaviors that are associated with decreased GABAergic inhibition (Rainnie et al., 2004) Because GABA is a predominant co-transmitter in amygdala CRF neurons (Gafford and Ressler, 2015), 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 murine amygdala, particularly the BLA, in shaping an individual mouse’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; McDonald et al., 1999; Turner and Herkenham, 1991). Microdialysis studies have shown that acute restraint stress increases extracellular glutamate levels in rat BLA and CeA complexes (Reaga et al., 2012; Reznikov et al., 2007; Skórzewska et al., 2009), which in turn activates the HPA axis (Gabr et al., 1995; Herman and Cullinan, 1997). Acute restraint stress stimulates the rapid and robust release of glutamate in the BLA and CeA (Reaga et al., 2012; Reznikov et al., 2007; Skórzewska et al., 2009), whereas chronic restraint stress diminishes 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 suitable 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 mouse CeA and virus-mediated overexpression of miR-34 in this area prevent stress-induced anxiety and block the response of CRFR1 to its ligand CRF, suggesting that miR-34 regulates the molecular machinery of the stress response (Andolina et al., 2016; Haramati et al., 2001; Mannironi et al., 2010; 2013; Volk et al., 2014).

In contrast to the significant amount of information that has already been accumulated on stress mechanisms in mammals, which nonetheless has not come close to explaining the detailed molecular processes that occur during stress regulation, information concerning teleosts remains 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; Vargas et al.,2009; Vidal-Gonzalez et al.,2006). Furthermore, the ventral telencephalon has been reported to be functionally homologous to the lateral septum (Goodson and Kingsbury, 2013; Vidal-Gonzalez et al., 2006), which is critical in the regulation of emotional reactivity and goal-oriented behavior (Demski, 2013; Luo et al.,2011; Singewald et al., 2011).

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

In mammals, the HPA axis is modulated by extra-hypothalamic limbic structures, particularly 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, amygdala activity exerts a significant facilitating 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). 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 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 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). The CRF signal is mediated by at least two receptors (CRFR1 and CRFR2). CRFR1 has been reported to mediate HPI axis activation, whereas CRFR2 contributes to 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 the MR and GR in the hypothalamus and pituitary (Bury et al.*,* 2003; Colombe et al., 2000; Sturm et al., 2005). Studies suggest the presence of interactions between HPI and limbic functions in the teleost telencephalon (Alderman and Bernier, 2007; Silva et al., 2015). Moreover, associations found between telencephalic 5-HT and HPI-axis activities (Höglund et al., 2000, 2001; Øverli et al., 2005; Silva et al., 2015; Winberg and Lepage, 1998; Winberg et al., 1997) 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 identified and 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 the GR and RU486 (mifepristone), a specific GR blocker, preventing these cortisol processes (Weyts et al., 1997). In mammals, it has been 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, consequently, 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 (Flik et al., 2006; Wendelaar-Bonga, 1997). 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). An additional 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 (an 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 has been 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 the stress response (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 teleosts through b-AR activation. However, a-AR stimulation leads to the production of antibodies (Flory, 1990; Flory and Bayne, 1991; Narnaware et al., 1994; Roy and Rai, 2008). The adronoceptor 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 is 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 may 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 the autonomic nervous responses in mammals (Fernandez and Acuna-Castillo, 2012), these 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).

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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), 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 then causes the secretion of cortisol from the interrenal cells. Cortisol binds to its receptors in blood cells and, as a result, various processes transpire according to the intensity and duration of stress. Similarly, cortisol in the feedback process 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 responses between different individual carps. Some respond more, and some less, and, consequently, the significance of the results is less robust and the data do not reflect the mechanistic 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 changes in each carp to be ascertained without sacrificing the specimen (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), with the duration of stress differentially changing 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) is upregulated and, at the same time, the activity of regulatory function (TGFb and IL10) is upregulated, probably in order to return pro-inflammatory activity to homeostasis (Banerjee and Leptin, 2014; Shimon-Hophy and Avtalion, 2017). Chronic stress activates the hypothalamus-pituitary-interrenal cell axis and, as a result, 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 change in levels halted and returned to homeostasis (Shimon-Hophy and Avtalion, 2017). TNFa levels did not change during hypoxic stress treatments (Table 1), but TNFa behaved slightly differently in chronic cortisol implants that were fixed on rainbow trout for five days (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; Endsley et al., 2004; Nagata and Golstein, 1995; O’Neill et al., 2020; Trapani and Smyth, 2002;), 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 the 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.**

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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 above results are aggregated 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), s**erum 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 strengthened 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 findings regarding husbandry, confinement, and crowding-induced stresses (Maule et al., 1989; Nagae et al., 1994; Rotllant et al., 1997; Ruane et al., 1999; Varsamos et al., 2006). Presumably, these differences among the results are attributable 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; Mauri et al., 2011; Sunyer and Tort, 1995); however, they are consistent with reported hypoxia and cortisol-induced stress results (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 can is known 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 consistent with others’ findings of a 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 cannot be certain if stress also caused MAIT cell deterioration (Shimon-Hophy et al., 2020), because microscopic pictures of several carp samples revealed both a vast decrease and high levels of these cells; therefore, there is a need to further study these cells. *In vitro* studies confirm the above-mentioned results, revealing 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; Smyth et al*.,* 2001; Tschopp and Nabholz, 1990; Zelinskyy et al., 2004). Moreover, γδT cells are the most numerous cells in carp leukocytes (Fig. 2) and are thought to be the greatest 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 indicates 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 (Elenkov and Chrousos, 1999; Maule et al., 1989; Mauri et al., 2011; Saeij et al., 2003).

**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 aggregated 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 eight 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 three times (according to measurements of the cell area), while during chronic stress responses, the cell volume of γδT cells decreased up to three 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(TCRδ)**  

 **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 continued sustainability 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 enables 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, it remains unclear how these structures cause various pathological disorders, as well as how they cause different responders to respond differently to the same stress stimuli. Previous studies on different stress responses have reported similar changes with respect to 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 of the mechanistic processes of stress regulation is required to reduce fish morbidity and mortality.