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Cortisol in Correlation to Other Indicators of Fish Welfare

Hanna Kalamarz-Kubiak

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Abstract

Cortisol is the major corticosteroid in teleost fish, secreted and released by interrenal cells of the head kidney during activation of the hypothalamic-pituitary-interrenal (HPI) axis. Although cortisol is universally recognized as a key mediator of stress-associated responses, other hormones are also involved in the stress response, e.g., arginine vasotocin (AVT), isotocin (IT), urotensins, dopamine, serotonin or β-endorphin. Cortisol affects AVT and IT secretion from nerve endings in gilthead sea bream (*Sparus aurata)* and round goby (*Neogobius melanostomus*). Moreover, it is pointed out that different mechanisms are involved in the regulation of AVT and IT release from the hypothalamic-pituitary complex in round goby. In the case of AVT, both genomic and nongenomic pathways are mediating the effect of cortisol while in the case of IT, it is only the nongenomic pathway. In turn, urotensin I instead of corticotropin-releasing factor (CRF) may contribute to the regulation of HPI axis and regulate AVT in *Sparus aurata*. In this species, urotensin II together with AVT and IT may control stress response to different salinities. Therefore, AVT, IT and urotensins, and their interactions with cortisol, seem to be significant in response to stress in fish.

Keywords: stress, cortisol, AVT, IT, UI, UII, *in vitro* techniques, fish

1. Introduction

Stress triggers reactions in all living organisms, and fish are no exception to this rule. It is known that fish are exposed to stress, not only in nature but also in aquaculture, fish markets and laboratories. In the past decades, knowledge and understanding of stress in fish has increased, particularly in the field of physiological mechanisms and responses that lead to changes in metabolism, growth, immune function, reproductive capacity and natural behavior. Interestingly, fish have proved to be more sensitive to stressors than many other vertebrates and

responded to stressors at the intensity levels that are often far below those that can be detected by terrestrial animals [1–4]. The stress response in fish has been widely categorized into the primary, secondary and tertiary responses [5–11]. The primary response (the neuroendocrine response) includes the rapid release of stress hormones, catecholamines and corticosteroids, into the circulation [1, 12, 13]. This physiological response to stressors encompasses activation of the brain-sympathetic-chromaffin cell (BSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis [1] (**Figure 1**). During the BSC axis activation, chromaffin cells of the head kidney release catecholamines (adrenaline and noradrenaline) from sympathetic nerve terminals. Catecholamines are controlled by factors released from sympathetic nerve terminals, mainly acetylcholine and angiotensin. The action of catecholamines includes increased hemoglobin oxygen affinity, arterial blood pressure [14] and glucose mobilization from liver and muscles [1]. The activation of HPI axis comprises the corticotropin-releasing factor (CRF) release from the hypothalamus, which in turn stimulates the corticotrophic cells in the anterior pituitary to secrete adrenocorticotropic hormone (ACTH). Following that, the interrenal cells of the head kidney synthesize and release cortisol into the circulatory system. In teleosts, the head kidney a major endocrine, hematopoietic and lymphatic tissue, are the equivalent of the adrenal gland in mammals [1, 12]. The secondary response comprises the various biochemical and physiological effects such as metabolic changes (increased glucose and lactate in blood and decreased tissue glycogen), osmoregulatory disturbance (water/ion balance), changes in hematological features (hematocrit, leukocrit and hemoglobin), cellular changes (increased heat shock or stress protein production) and changes in the immune response (lysozyme activity and antibody production) [13, 15–17]. The tertiary response represents changes in whole-animal performance characteristic (growth, swimming capacity and disease resistance) and modified behavioral patterns (feeding, aggression and reproduction) ("for review [9, 11, 18]").

In fish, cortisol acts as a regulatory factor for a wide range of physiological functions under normal conditions and also to allow for rapid physiological adjustments in the face of exposure to stressors [13]. Cortisol appears to play a pivotal role in the aerobic and anaerobic
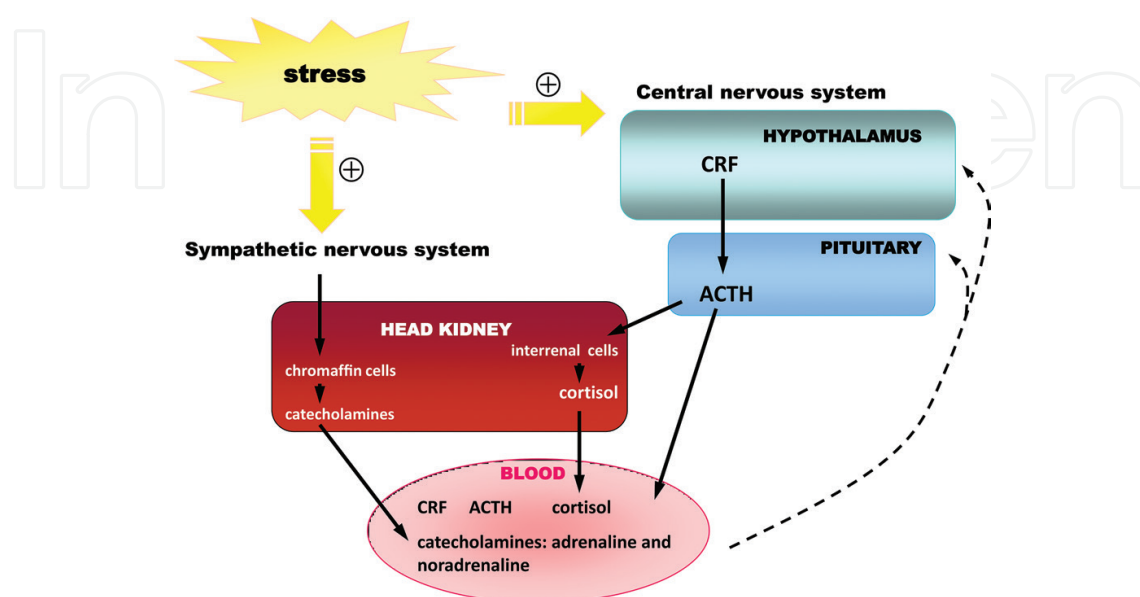

Figure 1. The stimulation of BSC axis and HPI axis in response to stress in fish.

metabolism, stimulating several aspects of intermediary energy metabolism, elevating oxygen uptake, increasing gluconeogenesis and inhibiting synthesis of glycogen synthesis [1, 13, 19–21]. Furthermore, increases in plasma corticosteroids have a wide range of other metabolic effects including increases in protein turnover, regulation of amino acid metabolism, ammonia output and increased lipolysis ("reviewed in [13]"). This hormone also performs an osmoregulatory function in teleosts, being the main hormone for seawater adaptation and ion uptake [22, 23]. Moreover, cortisol may regulate the immune response in fish [1, 13, 24]. Cortisol modulates, among others, the tissue inflammatory response through inhibitory effects on cytokine production [25] and appears to attenuate the cellular heat shock protein response to thermal insult [26, 27]. Corticosteroid hormones may highly participate in the modulation of the reproductive endocrine control in both sexes [18].

It should be noted that cortisol dramatically rises during stress and seems to be a key mediator of stress-associated responses [13, 28]. There is considerable variability in the magnitude of the corticosteroid response among species [9, 29, 30]. Among teleosts, some species exhibit high cortisol concentrations (10^{-7}–10^{-6} M) in response to acute stress [9], while some species reveal low cortisol levels (10^{-9}–10^{-8} M) in response to the same stress [31–33]. Most fish species show their increase in plasma cortisol within about 0.5–1 hour after a stressful disturbance [34, 35], but there are exceptions to the role. In the sea raven (*Hemitripterus americanus*), circulating cortisol takes up to 4 hours to reach its peak level following an acute stressor [36]. Probably, the slow rate of response to the stressor may help conserve energy in a normally inactive, sedentary, benthic marine species having a slow metabolic rate [36]. Corticosteroid responses to stress also vary within species according to the duration or severity of the stressor ("for review: [9]"). What is more, differences in corticosteroid stress responses may exist among strains or stocks within the same fish species [37, 38], their hybrids [39], and between wild and hatchery fish [40]. It should be noted that the variation in stress responses within a single strain or population may indicate genetic determinants [41–43]. Beyond genetic and environmental factors, the developmental stage of the fish can also affect its responsiveness to a stressor ("for review: [9]").

2. How does cortisol interact with other hormones in fish?

Although cortisol is universally recognized as a critical component of the endocrine response to stress, other hormones are also involved in the stress response, e.g., arginine vasotocin (AVT), isotocin (IT), urotensins, dopamine, serotonin or β-endorphin [13, 44–48]. However, other hormones, such as thyroxine, prolactin and somatolactin can also elevate during stress but they have not yet been demonstrated to be useful stress indicators *per se* [49–51]. Our interest has focused on nonapeptides AVT, IT and urotensins, and their interactions with cortisol, in response to stress.

2.1. Arginine vasotocin, isotocin and urotensin I

Nonapeptides, such as AVT and IT, are fish homologs of the mammalian arginine vasopressin (AVP) and oxytocin (OT) [52]. In fish, AVT and IT are synthesized in separate parvo- and magnocellular neurons of the preoptic area (POA), stored in axon terminals in neurohypophysis and released into the circulatory system after proper stimulation [53–55]. Only mature nonapeptides, after dissociation from the noncovalent complex, play an active role as peripheral

hormones and neurotransmitters or neuromodulators in the central nervous system (CNS). The physiological role of AVT involves cardiovascular activity and maintenance of water/ion homeostasis. Both nonapeptides interact with other endocrine systems and control social and reproductive behavior [56–59]. More importantly, there is evidence that AVT and IT are engaged in physiological stress response in fish. Changes in hypothalamic, pituitary and plasma AVT and IT concentrations were found in many fish species subjected to various unfavorable situations such as confinement, disturbance, high density, food deprivation or osmoregulatory stress [33, 47, 60]. Therefore, AVT and IT are important components of stress axis in fish [61]. Moreover, AVT neurons are colocalized with CRF in the preoptic nucleus (NPO) [62, 63], and the expression of AVT and CRF mRNAs increases simultaneously in response to various stressors in many fish spices [56, 64, 65]. *In vitro* studies have shown that independently or in synergy with CRF, AVT stimulates ACTH release from fish pituitary cells [44, 66, 67]. In gilthead sea bream (*Sparus aurata*), unlike other teleosts, CRF is not a releasing factor for ACTH and cortisol, because there are no anatomical connections between CRF perikarya and ACTH cells in the adenohypophysis [68–70]. Therefore, it is possible that urotensin I (UI) instead of CRF regulates AVT and IT release in *S. aurata*.

It has been known that UI is implicated in the regulation of neuroendocrine, autonomic and behavioral responses to stressors in fish [71, 72]. Gene expression of UI was found not only in urophysis but also in the telencephalon-preoptic, hypothalamic, optic tectum-thalamus and posterior brain regions, which indicates the regulatory action of this peptide in CNS [73–75]. The structural similarity of UI with CRF suggests similar hypophysiotropic roles of both hormones in HPI axis in fish [76–78]. It has been established that UI modulates cortisol secretion either directly by acting on steroidogenic cells of an interrenal tissue or indirectly via the hypothalamic-pituitary axis [71, 77, 79, 80]. In many fish species, UI-immunoreactive (UI-ir) fibers from the nucleus lateral tuberalis (NLT) extend to the pituitary where they may interact with AVT and IT nerve terminals [81–84].

The effect of cortisol on AVT has been examined *in vivo* in gilthead sea bream. The application of cortisol implants enhanced the hypothalamic expression of AVT mRNA and subsequently hypophysial AVT content in this species [85]. Although IT studies are very limited, they suggest that IT potentiates ACTH release from fish pituitary cells [44]. The *in vitro* effect of cortisol or UI on AVT and IT secretion in fish has been studied only by Kalamarz-Kubiak et al. [86]. In this study, primary cultures of pituitary cells were prepared by a modification of the method described by Levavi-Sivan et al. [87, 88]. Pituitary cells were cultured with medium supplemented with cortisol (1.4×10^{-8}, 1.4×10^{-7} and 0.4×10^{-6} M) or UI (10^{-12}, 10^{-10} and 10^{-8} M). The doses of cortisol were chosen taking into account different cortisol responses to stress in various fish species [9, 29, 30]. The doses of UI used in the cell culture were determined based on the literature, considering its concentration in different tissues [29, 30, 80, 89, 90]. After 6, 24 and 48 hours, the media were collected and stored at −70°C until AVT and IT analysis. AVT and IT concentrations were determined in incubation media by HPLC with fluorescence and UV detection according to a modified procedure by Kulczykowska [91].

The study performed by Kalamarz-Kubiak et al. [86] demonstrated that AVT and IT secretion from nerve ending of *S. aurata* pituitary was influenced by cortisol and UI. In this study,

cortisol showed a stimulatory action on pituitary cells of *S. aurata* inducing AVT secretion at all doses. Dose-dependent effect of cortisol on AVT secretion has been manifested after 24 hours of cell culture. In mammals, the influence of cortisol on AVP secretion was studied by *in vivo* and *in vitro* methods [92, 93]. In turn, other findings indicate that the expression of AVP in parvocellular neurons of the paraventricular nucleus (PVN) and AVP secretion into the pituitary portal circulation increase under chronic stress in rats [94–97]. It is also shown that stress upregulates the number of AVP receptors in rat anterior pituitary [96]. The results presented by Kalamarz-Kubiak et al. [86] demonstrated that the stimulatory effect of cortisol on AVT secretion from nerve ending of *S. aurata* pituitary diminishes after 48 hours of culture. The most likely explanation for the decline seems to be the depletion of AVT stores without subsequent supplementation of secretory granules from AVT-ergic nerves. However, corticoid receptor (CR) desensitization could be another cause. In mammals, desensitization of CRs is the result of physiological processes, as well as stress, and disease [98–100]. On the other hand, the reduction of AVT secretion after 48 hours of cortisol exposure could be also linked with an increase of aminopeptidase activity responsible for nonapeptide metabolism as it was shown in rats and chickens [101–103]. As in the case of AVT, *in vitro* cortisol action on IT secretion in teleosts was not known. Results presented by Kalamarz-Kubiak et al. [86] showed that cortisol decreased IT secretion from nerve ending of *S. aurata* pituitary. In mammals, cortisol action on OT was investigated by *in vitro* and *in vivo* experiments. It was found that glucocorticoids exert an inhibitory effect on the neurosecretory activity of parvocellular OT-ergic neurons of rats [104]. In rats, the increase in plasma OT levels after intravenous injection of isotonic or hypertonic saline was blocked by dexamethasone [105].

For the reasons mentioned above, it was presumed that UI, instead of CRF, might regulate AVT and IT release in *S. aurata*. In the *in vitro* study presented by Kalamarz-Kubiak et al. [86], the dose-dependent stimulatory effect of UI on AVT secretion from nerve ending of *S. aurata* pituitary was observed after 6 hours of culture. In rats, it has been shown that UI slightly increases the hypothalamus AVP secretion *in vitro*, indicating the probable stimulatory effect of this peptide on AVT production [106]. In turn, the presented *in vitro* results [86] have demonstrated that after 24 hours only the highest dose of UI elevates AVT secretion from *S. aurata* pituitary cells. Moreover, this stimulatory effect of UI completely expires after 48 hours of pituitary cell culture. Since UI is a natural ligand of CRF receptors (CRFRs) [78, 107], the later desensitization of CRFRs may be an explanation of these results. A number of *in vitro* studies demonstrate desensitization of CRFRs [108–111]. Moreover, it is also known that UI increases cortisol secretion [108–111]. Thus, UI may also influence AVT secretion indirectly, stimulating cortisol release. In gilthead sea bream, UI did not affect IT secretion from pituitary cells. Note that the influence of UI on IT or OT secretion had never been investigated before. The opposite response of AVT and IT to UI or cortisol exposure in pituitary cell culture is in accordance with other data showing an independent regulation of nonapeptide secretion [58, 112]. In a summary, the following conclusions were formulated:

- Cortisol affects AVT and IT secretion from nerve endings in *S. aurata* pituitary.

- Cortisol stimulates AVT secretion in a dose-dependent manner and inhibits IT secretion in *S. aurata* pituitary cell culture.

- UI stimulates AVT secretion but does not influence IT secretion from nerve endings in *S. aurata* pituitary.

- UI instead of CRF may contribute to the regulation of HPI axis and regulate AVT secretion.

- AVT and IT are essential components of stress response in fish.

2.2. Urotensin II

At the beginning of this chapter, it was noticed that besides cortisol, urotensins are also involved in the response to stress in fish. UI action has already been discussed. In turn, urotensin II (UII), a cyclic peptide originally isolated from the urophysis of the goby (*Gillichthys mirabilis*) [113], appears to be involved in the control of osmoregulatory and metabolic functions and also in the cardiovascular and gastrointestinal activities, and immune response in teleosts [114–118]. In the European flounder (*Platichthys flesus*), urophysial UII content rose over the 24 hours following a transfer from seawater to fresh water, whereas plasma UII content and UII receptor expression in kidney and gill decreased, implying downregulation of the UII system [115, 119]. It should be noted that in fish, hormonal regulation of water and ion homeostasis requires participation and interaction of many endocrine systems at the various functional levels of the organism [58]. In teleosts, also AVT and IT seem to be involved in the maintenance of water and ion homeostasis [57, 58]. What is more, there is also evidence of the role of AVT and IT in response to different osmotic stimuli [47, 60]. It has been observed that the synthesis of AVT and IT and their release from the neurohypophysis are sensitive to changes in water salinity. In teleosts, an acute change in water salinity results in altered pro-AVT and pro-IT mRNA expression in hypothalamic neurons [120–122] and in the altered content of AVT and IT in the pituitary [119, 122, 123]. It should be emphasized that the potential relationship between AVT and other hormonal systems such as UII contributing to the osmoregulation in fish has been suggested before [119, 124, 125]. As already mentioned, AVT and IT are synthesized in the POA and transported to the neurohypophysis for storage and release into the vascular system via axon terminals. UII has been identified in teleost and nonteleost fish not only in the urophysis but also in the CNS [126–129]. Moreover, UII and UII receptor mRNA expression has been detected in all brain regions of European flounder, including the telencephalon-preoptic region, hypothalamus and pituitary [115]. These results indicate the probable site of interaction between the UII and AVT/IT systems within the POA, hypothalamus and pituitary. In the European flounder, it was found that both UII and AVT are engaged in the hyper- and hypo-osmotic stress In the European flounder [119, 124, 125]. However, to the best of our knowledge, the influence of UII on AVT and IT secretion in teleosts has been studied only by Kalamarz-Kubiak and coworkers [130]. The aim of this study was to determine whether AVT and IT release from nerve endings is affected by UII in the pituitary of gilthead sea bream. Three-year-old gilthead sea bream of both sexes were used for *in vitro* study. Primary cultures of pituitary cells were prepared by a modification of the method described by Levavi-Sivan et al. [87, 88]. Pituitary cells were cultured with medium supplemented with UII (10^{-12}, 10^{-10} and 10^{-8} M). The doses of UII used in this *in vitro* study were determined based on the literature, considering its concentration in different fish tissues [30, 125, 131]. After 6, 24 and 48 hours of incubation, the media were collected and stored at −70°C until HPLC analysis of AVT and IT. The results of this *in vitro* study indicate that UII

inhibits AVT secretion in pituitary cell culture. It has been shown that AVT is an antidiuretic hormone reducing urine production in fish [132, 133]. Thus, by inhibiting AVT secretion, UII may have a diuretic effect. Furthermore, it is known that UII administrated *in vivo* increases renal blood flow and glomerular filtration rate and consequently enhances diuresis and natriuresis in the rat [134, 135]. This mammalian paradigm could be helpful in the interpretation of fish data. The *in vitro* study in *S. aurata* indicated that UII's strong inhibitory action on AVT release from nerve endings in the pituitary is independent of tested doses and exposure time. What is more, after 24 hours of incubation, AVT inhibition was lower and persisted to the end of culture. This disinhibition of AVT secretion after a long time of incubation may indicate the desensitization of UII receptors as it was proved in human cell lines [136, 137]. In contrast to AVT, UII significantly increased IT release from nerve endings after 24 hours of culture. This stimulatory effect of UII appeared to be independent of tested doses. In mammals, UII is a naturally occurring somatostatin analog sharing some functional similarities with somatostatin [113, 138]. The results in fish are consistent with data in mammals that show that the intracerebroventricular somatostatin infusion significantly increases plasma OT secretion in virgin and pregnant rats [139]. Moreover, the opposite response of AVT and IT to UII exposure in pituitary cell culture showed an independent regulation of nonapeptide secretion. This idea was documented previously in rainbow trout (*Oncorhynchus mykiss*) [47, 112, 140].

From those results, the following conclusions were formulated:

- UII affects AVT and IT release from nerve endings in the pituitary of gilthead sea bream.

- UII inhibits AVT release and stimulates release of IT in *S. aurata* pituitary cell culture.

- UII together with AVT and IT may control response to different salinities in fish.

The hormonal interactions between UII and AVT and IT are presented in **Figure 2**.

3. What is the mechanism of cortisol action in fish?

It has been established that cortisol has both a corticosteroid and a mineralocorticoid function in fish [1]. An involvement of both classes of corticoid receptors (CRs), mineralocorticoid (MRs) and glucocorticoid (GRs), was widely demonstrated during adaptation to different salinities and osmoregulatory stress [141–144], fish reproduction [145, 146] and expression of social behavior [147–149]. It is worth noting that both MRs and GRs were engaged in tilapia's response to handling stress [150] and expressed in rainbow trout organs with slow-release cortisol implants [151].

Glucocorticoid and mineralocorticoid receptors are involved in the genomic and nongenomic mechanisms of cortisol action in fish [149, 152, 153]. Corticosteroid-intracellular receptor complex binds to the nuclear glucocorticoid response elements (GRE) to modulate transcription and protein synthesis (genomic pathway) [13, 25, 154]. The nongenomic effect is mediated through either nonspecific physicochemical interaction with the plasma membrane [155] or specific membrane receptors such as the G protein–coupled receptor (GPCR) [156] or the
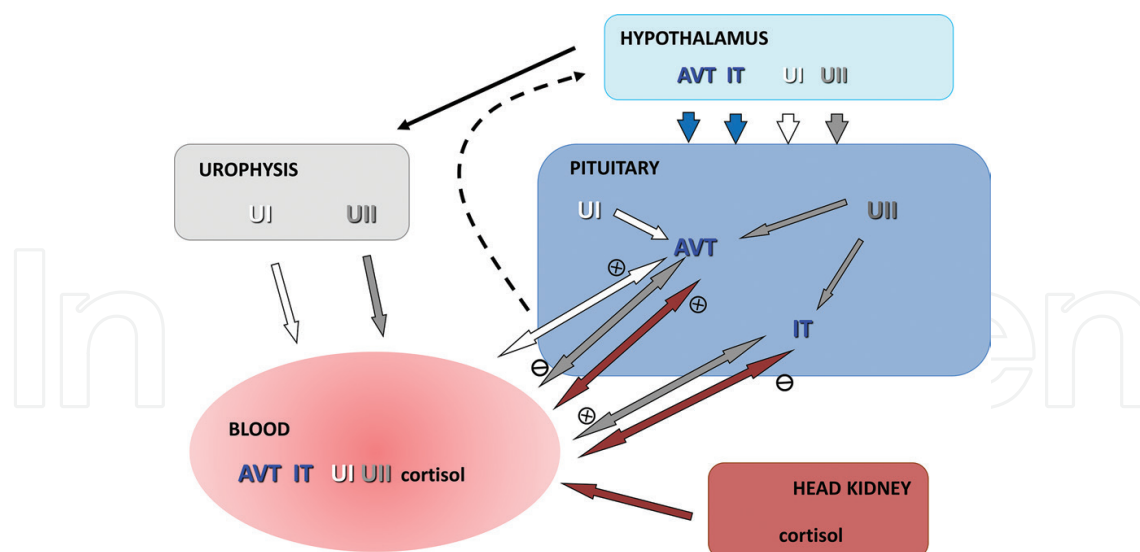
Figure 2. The effect of cortisol, urotensin I and urotensin II on arginine vasotocin and isotocin secretion in gilthead sea bream.

plasma membrane-bound form of GR (mGRs) (nongenomic pathway) [157]. ("Nongenomic steroid action is presented in accordance with Mannheim classification [155].")

3.1. What method can investigate the mechanism of action of cortisol?

Recently, there is growing concern about effects of farming and environmental pollution on fish well-being; thus, there is the need for new tests to study the endocrine responses in fish [158]. Furthermore, fish are increasingly being used as substitutes for mammalian model organisms in fundamental research and as a research model for chemical testing. Hence, research must remain focused on the discovery of new alternative techniques or on an adaptation of methods established for mammalian models for use as fish models [159].

The mechanism presented in this section requires a method that allows monitoring the dynamic hormone secretion and registering even small and short-term fluctuations in their release. Only perfusion culture method allows detailed examination of changes in the release of hormones while ensuring optimal culture conditions. Kalamarz-Kubiak et al. [160] developed a new procedure for the unique gradient perfusion technique (3D) of brain and pituitary explants collected from three-spined stickleback (*Gasterosteus aculeatus*) and round goby (*Neogobius melanostomus*). So far, organ perfusion methods have not been often used in fish for lack of suitable techniques. Simple organ perfusion systems were applied in pituitary [161–165] and pineal gland [166–168] studies. However, an innovative system for organ perfusion (MINUCELLS and MINUTISSUE Vertriebs GmbH, Germany), proposed by Minuth in early 1990s, gives more options for this kind of technique. This gradient perfusion technique meets the requirements for studies of nervous tissues, blood-brain barrier, retina and blood-retina, regeneration of blood vessels, skin renewal, bone and muscular tissue in mammals [169]. Thus, Kalamarz-Kubiak et al. [160] presented the first application of the MINUCELLS and MINUTISSUE tissue engineering technique for perfusion of fish brain tissues. In this

study, tissues were placed on the membrane between rings of tissue carriers inside the gradient container. A specific construction of this container facilitated the uniform supply of medium to the luminal and basal sides to avoid the dead space. The methods of medium transport into the gradient container were tested using three perfusion sets. *Set 1* and *set 2* allow the supply of one medium from the top without aeration or with aeration, respectively. *Set 3* allows the supply of one or two aerated media from the top and bottom, simultaneously. Moreover, *set 1* was used to determine the time required to achieve a stable basal level of AVT and IT release during tissue explant perfusion. The stable basal level of AVT and IT release was achieved between 60 and 80 minutes of perfusion for both fish species. *Set 2* equipped with gas exchange module was aerated by an air pump (0.3% CO_2) or a mixture of 95% O_2 and 5% CO_2 at a pressure of 127.51 mmHg. The results indicated that only usage of a mixture of 95% O_2 and 5% CO_2 provided the proper conditions for perfusion and tissue reactivity in the medium supplemented with high K^+ concentration (60 mM KCl) (**Figure 3**). In order to optimize the conditions of perfusion, the various pressure of gas mixture (127.51, 255.02 and 315.03 mmHg at the outlet of the gas bottle) was tested. The gas pressure of 127.51 mmHg provides optimal conditions for perfusion in the *set 2* with one gas exchange module. To ensure the same pressure conditions in *set 3*, with two gas exchange modules, higher pressure of 315.03 mmHg at the outlet of the gas bottle must be applied. Concentrations of AVT and IT in the media collected after perfusion were determined by HPLC with fluorescence and UV detection according to the modified procedure by Gozdowska et al. [170]. Although the presented procedure has been elaborated for studies of AVT and IT in fish explants, after only minor modification, if any, it can serve many other purposes. From those results, the following conclusions were drawn and the recommendations were formulated:

- *Set 1* is preferable only for short-term research.

- *Set 2*, where the medium is aerated with a mixture of 95% O_2 and 5% CO_2 at a pressure of 127.51 mmHg, is recommended for long-term studies.

- *Set 3* is also preferable for long-term studies but requires aeration with a mixture of 95% O_2 and 5% CO_2 at a pressure of 315.03 mmHg.

- *Sets 1* and *2* allow the supply of only one type of medium at the same time to the gradient perfusion container. *Set 3* allows the transport of two different media from the top and bottom to the perfusion container at the same time.

The schemes of sets used for gradient perfusion and graphs of AVT and IT release during tests of those sets are presented in **Figure 3**.

3.2. How does cortisol affect the release of AVT and IT and what kind of pathway, genomic or nongenomic, is involved in this regulation?

In teleost, two different GR coding genes (GR1 and GR2) and one MR gene were found [171, 172]. The expression of GR1, GR2 and MR genes, as well as the immunoreactivity of GRs (GRs-ir), was noted in most of the magno- and parvocellular neurons of the preoptic nucleus
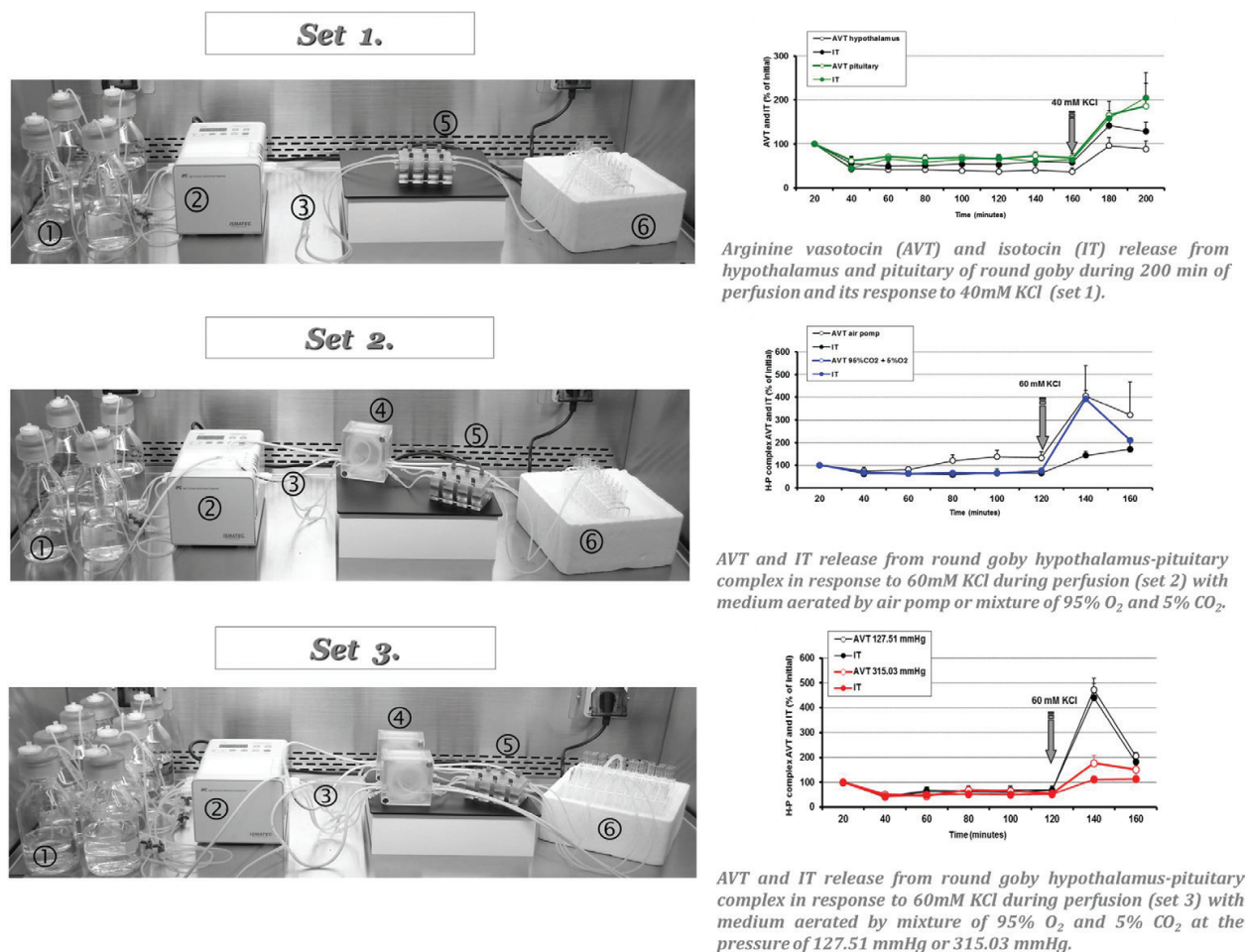
Arginine vasotocin (AVT) and isotocin (IT) release from hypothalamus and pituitary of round goby during 200 min of perfusion and its response to 40mM KCl (set 1).

AVT and IT release from round goby hypothalamus-pituitary complex in response to 60mM KCl during perfusion (set 2) with medium aerated by air pomp or mixture of 95% O_2 and 5% CO_2.

AVT and IT release from round goby hypothalamus-pituitary complex in response to 60mM KCl during perfusion (set 3) with medium aerated by mixture of 95% O_2 and 5% CO_2 at the pressure of 127.51 mmHg or 315.03 mmHg.

Figure 3. The schemes of culture sets used for gradient perfusion. Components of perfusion culture set: (1) storage medium bottles, (2) peristaltic pumps, (3) connecting fittings, (4) gas exchange modules, (5) gradient culture container, (6) sampling vials. The release of arginine vasotocin and isotocin during tests of these sets (graphs).

(NPO), known for synthesizing AVT, IT and CRF, in tilapia (*Oreochromis mossambicus*), rainbow trout and common carp (*Cyprinus carpio*) [173–175]. In the pituitary, GR1, GR2 and MR mRNA expression and GRs-ir have localized in *pars distalis* and *pars intermedia* where AVT-ergic fibers give their projections [173–175].

As it was mentioned earlier, AVT and IT are engaged in physiological stress response and seem to be important components of stress axis in fish [33, 47, 61]. In gilthead sea bream, the application of cortisol implants in this species enhanced the hypothalamic expression of provasotocin mRNA and pituitary AVT content [85]. What is more, an *in vitro* study indicated that cortisol affects AVT and IT release from the *nerve terminalis* in *S. aurata* pituitary [86]. However, to the best of the authors' knowledge, the mechanism of cortisol action on AVT and IT release in teleosts has been studied only by Kalamarz-Kubiak and coworkers [176]. This *in vitro* perfusion study was performed to determine which class of receptors, GRs or MRs, participated in cortisol regulation of AVT and IT release from the hypothalamic-pituitary (H-P) complex of round goby (*Neogobius melanostomus*). Moreover, this *in vitro* study allowed to determine which pathways, genomic or nongenomic, are engaged in the aforementioned process. Adult round gobies of both sexes were used in this in vitro study.

Hypothalamic-pituitary explants were perfused using *set 2* of gradient perfusion technique (for details see Section 3.2). The explants were perfused with medium supplemented with different treatments (cortisol, mifepristone [RU486], spironolactone [C03DA01] and actinomycin D). Mifepristone is a glucocorticoid receptor antagonist, which affects a wide range of physiological and behavioral traits (metabolism, reproduction, osmotic stress, vocalizations and aggression in fish) [13, 177]. Spironolactone is a mineralocorticoid receptor antagonist, which blocks the ion uptake in osmoregulation [142, 152] and reduces aggression during social interaction [149, 178]. Actinomycin D is a transcription inhibitor, which binds DNA at the transcription initiation complex and prevents elongation by RNA polymerase [179–181]. Cortisol was tested at three doses (1.4×10^{-7} M, 2.8×10^{-7} M and 0.4×10^{-6} M). Cortisol doses were selected based on our previous experiments and literature [9, 86, 182–185]. The doses of inhibitors were selected on the basis of available data [186–190]. Finally, cortisol at 0.4×10^{-6} M dose in combination with RU486 (0.3×10^{-6} M) or C03DA01 (0.36×10^{-6} M) or actinomycin D (1×10^{-7} M) was used in experiments. Concentrations of AVT and IT in the media collected after perfusion were determined by HPLC with fluorescence and UV detection according to the modified procedure by Gozdowska et al. [170]. In this study, cortisol showed a dose-dependent stimulatory effect on AVT release from H-P explants similar to the one presented previously in pituitary cells of *S. aurata*. In rats, corticosterone also affected AVP release from hypothalamic slices containing paraventricular and supraoptic nuclei in a dose-dependent manner [191]. The results presented by Kalamarz-Kubiak et al. [176] indicate that cortisol, most likely acting through GRs, stimulates the release of AVT from the H-P complex of round goby. It has been suggested that cortisol preferentially binds to GR2 in teleosts, in response to low or mild stress, and to both GR2 and GR1 in response to extreme stress [192, 193]. Therefore, it is probable that both isoforms of GRs are engaged in cortisol action on AVT release from the H-P complex of round goby [176]. However, a biphasic AVT response may depict an initial release of mature AVT from the pool stored in the secretory granules, followed by the release of newly matured AVT molecules just after their dissociation from the noncovalent complex. Cortisol may exert biphasic effects on the release of inflammatory mediators, e.g., the plasma macrophage migration inhibitory factor and the tumor necrosis factor-α, interleukin-6 and acute-phase proteins in vertebrates, including fish [194, 195]. The results of presented *in vitro* study indicate that cortisol affects AVT release through GRs via genomic and nongenomic pathways in round goby. The biphasic response of AVT to cortisol was hindered by both the GR antagonist RU486 and the transcription inhibitor actinomycin D [176]. In the marine medaka (*Oryzias dancena*), RU486 blocked the transcriptional activity of both GR isoforms in response to cortisol action [193]. However, RU486 blocks some rapid, nongenomic effects of cortisol mediated via plasma membrane receptors in fish [181, 196, 197]. Probable mGRs are engaged in the first phase of the biphasic AVT response to cortisol in *Neogobius melanostomus*. Alternatively, cortisol may demonstrate nongenomic action through specific membrane receptors such as the GPCRs or without receptor engagement through the nonspecific action that alters the plasma membrane's physicochemical properties, as it has been shown in mammals [155] and fish [153, 180]. It is worth noting that in higher vertebrates and fish, the mechanism of corticosteroid action may integrate nongenomic and genomic pathways [25, 156, 198]. For instance, in rodents, such integration between nongenomic and genomic mechanisms has been shown in the neurons of the amygdala, hippocampus and cortex in response to stress and the administration of corticosterone ("for a review: [198]").

In results presented by Kalamarz-Kubiak et al. [176], the stimulation of IT secretion by cortisol appeared within 20 minutes and persisted for the next 100 minutes, similarly as in the case of AVT, but did not disclose a biphasic character. The nongenomic, stimulatory effect of cortisol *in vivo* on Na$^+$ -K$^+$ and Ca^{2+} -ATPase activity in gills of tilapia occurred after 30 minutes and persisted for 120 minutes. [180]. Similar observations, i.e., fast and long-lasting effects of corticosteroids *in vitro* on the excitability of different brain areas, were noted in rodents ("for a review: [198]"). In round goby, cortisol probably influenced IT release by GRs via the nongenomic pathway because cortisol action was inhibited by RU486, but not by actinomycin D. In contrast to the data in round goby, *in vitro* study of pituitary cells in *S. aurata* showed that cortisol decreased the IT release from nerve endings [86]. It should be noted that gilthead sea breams approached the reproductive season, while round gobies were out of their spawning season. Therefore, the IT responses to cortisol may be dependent on their physiological status and/or differ in various species.

In fish, the cortisol effects are mediated through both the GRs but also through MRs [1]. However, the *in vitro* study suggests that cortisol effect on AVT and IT release from the H-P complex in round goby is not mediated by MRs because the MRs' antagonist, C03DA01, does not hinder AVT and IT release caused by cortisol.
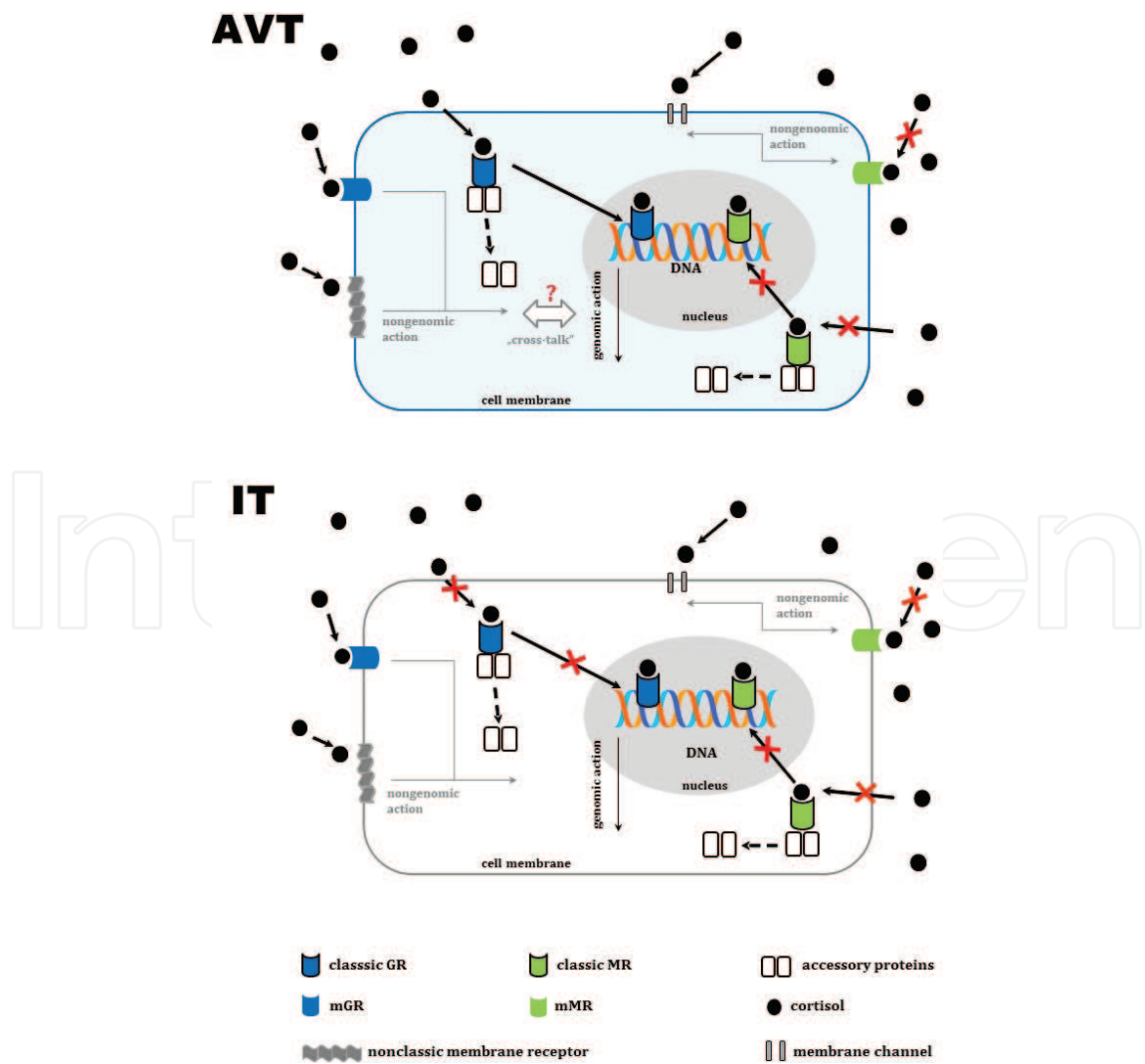

Figure 4. The mechanism of cortisol action on arginine vasotocin and isotocin release in round goby.

Outside the scope of this study, an opposite effect, i.e., the stimulation of cortisol secretion by AVT, should also be considered. There is evidence that AVT neurons innervate corticotrophic cells in green molly (*Poecilia latipinna*) pituitary [199] and that AVT synergizes with CRH/CRF (corticotrophin-releasing hormone/factor) to promote ACTH secretion from the pituitary in rainbow trout [66]. Consequently, AVT can stimulate cortisol release, and thus relationships between AVT and cortisol may be more complicated.

From those data, the following conclusions were formulated:

- Cortisol affects AVT and IT secretion from the H-P complex in round goby.

- Cortisol stimulates the release of both nonapeptides. However, the effect of cortisol on AVT release is dose-dependent.

- Cortisol has biphasic effects on the release of AVT, while this effect on IT is monophasic.

- GRs but not MRs are involved in cortisol regulation of AVT and IT release.

- In the case of AVT, both genomic and nongenomic pathways mediate the effect of cortisol.

- In the case of IT, only the nongenomic pathway mediates the effect of cortisol.

The mechanism of cortisol action on AVT and IT release in round goby are presented in **Figure 4**.

4. Summary

The purpose of this chapter was to gain new knowledge on the involvement of cortisol and other indicators of fish welfare in the regulation of stress response in fish. The basis of the subject was to assume that both nonapeptides and urotensins are essential components of stress response in fish. So far, nobody has attempted to check if there is a functional relationship between cortisol and both nonapeptides and urotensins using *in vitro* technique of cell culture and gradient perfusion. For the first time, MINUCELLS and MINUTISSUE tissue engineering technique (3D) has been applied for the gradient perfusion of fish brain and pituitary by Kalamarz-Kubiak et al. [160]. Although the presented procedure has been elaborated for studies of AVT and IT in fish explants, after only minor modification, if any, it can serve many other purposes. It has been confirmed that AVT and IT are essential components of stress response in fish. Presented results showed an independent regulation of nonapeptide secretion. Cortisol affects AVT and IT secretion from nerve endings in gilthead sea bream and round goby. Therefore, the cortisol effect may be different in various species and/or dependent on their physiological status. *S. aurata* is a very interesting species for this type of research. In gilthead sea bream, unlike other teleosts, CRF is not a releasing factor for ACTH, because there are no anatomical connections between CRF perikarya and ACTH cells. It has been investigated that urotensin I instead of CRF may contribute to the regulation of HPI axis and regulate AVT. In turn, urotensin II together with AVT and IT may control response to different salinities in fish. The results confirm that urotensins together with nonapeptides are involved in the regulation of stress response in fish. Here, the first feasible mechanism of cortisol action on AVT and IT release from the H-P complex has been presented in round goby.

The different mechanisms have been pointed out, where GRs are involved, whereas MRs are not. In the case of AVT, both genomic and nongenomic pathways mediate the effect of cortisol. In the case of IT, only the nongenomic pathway mediates the effect of cortisol. Therefore, AVT and IT seem to be good candidates for welfare indicators. Probably, the examination of cortisol in relation to other welfare indicators in the regulation of stress response will allow the separation of (physiological) stress from (psychological) distress, the separation of chronic stress from acclimation and the interactions between feelings, mood and behavior.

In conclusion, it is worth to quote the statement of Victoria Braithwaite [200], about the pain and stress in fish, for The Los Angeles Times dated October 8, 2006: "Their brains are not as different from ours as we once thought. Although less anatomically complex than our own brain, the function of two of their forebrain areas is very similar to the mammalian amygdala and hippocampus – areas associated with emotion, learning and memory. If these regions are damaged in fish, their learning and emotional capacities are impaired; they can no longer find their way through mazes, and they lose their sense of fear".

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