Journal of FISH BIOLOGY

fsbi An International Society for Fish Biology

Journal of Fish Biology (2014) **84**, 448–458
doi:10.1111/jfb.12297, available online at wileyonlinelibrary.com

In vitro effect of cortisol and urotensin I on arginine vasotocin and isotocin secretion from pituitary cells of gilthead sea bream *Sparus aurata*

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(Received 2 March 2013, Accepted 13 November 2013)

This study aimed at determining whether *in vitro* secretion of two neuropeptides, arginine vasotocin (AVT) and isotocin (IT), from pituitary cells of gilthead sea bream *Sparus aurata* was affected by cortisol and urotensin (UI). Pituitary cells were exposed to $1{\cdot}4 \times 10^{-8}$, $1{\cdot}4 \times 10^{-7}$ and $0{\cdot}4 \times 10^{-6}$ M cortisol and 10^{-12}, 10^{-10} and 10^{-8} M UI for 6, 24 and 48 h, respectively. AVT and IT contents were determined in the culture media by high-performance liquid chromatography (HPLC). An increase in AVT secretion and a decrease in IT secretion were observed at all cortisol doses. UI increased AVT secretion after 6 h of incubation at all doses. After 24 h, however, only the highest dose of UI still displayed an effect. IT secretion was not influenced by UI. It was thus demonstrated that cortisol does influence AVT and IT secretion from *S. aurata* pituitary cells, while UI regulates AVT secretion, as a component of hypothalamic–pituitary–interrenal (HPI) axis in this species.

Key words: fish nonapeptides; primary hypophysis cell cultures; stress hormones.

INTRODUCTION

Arginine vasotocin (AVT) and isotocin (IT) belong to a family of neuropeptides, closely related to the mammalian homologues arginine vasopressin (AVP) and oxytocin (OT) (Acher, 1993). In fishes, AVT and IT are synthesized separately in the parvocellular and magnocellular neurons of the preoptic area (POA), stored in axon terminals in neurohypophysis and are released from there into the vascular system (Van den Dungen *et al.*, 1982; Holmqvist & Ekström, 1995; Saito *et al.*, 2004). After dissociation from non-covalent complex, mature nonapeptides act as peripheral hormones and active neurotransmitters or neuromodulators in the central nervous system (CNS). AVT plays a key role in cardiovascular control and maintenance of water and ionic homeostasis. It also interacts with other endocrine systems and controls reproductive and social behaviour (Balment *et al.*, 2006; McCormick & Bradshaw, 2006; Kulczykowska, 2007; Goodson, 2008).

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There is also evidence for a role of AVT and IT in physiological stress responses in fishes. Changes in hypothalamic, pituitary and plasma AVT and IT concentrations were found in many fish species in response to stressors such as confinement, disturbance, high density, food deprivation or salinity (Kulczykowska *et al.*, 2001; Mancera *et al.*, 2008). This suggests that AVT and IT are important components of stress axis in fishes (Kulczykowska, 2008). AVT neurons are co-localized with corticotropin-releasing factor (CRF) in the preoptic nucleus (NPO) of the white sucker *Catostomus commersonii* (Lacépède 1803) and eels [European eel *Anguilla anguilla* (L. 1758), American eel *Anguilla rostrata* (LeSueur 1817) and Japanese eel *Anguilla japonica* Temminck & Schlegel 1846] (Yulis & Lederis, 1987; Olivereau *et al.*, 1988). The expression of AVT and CRF mRNAs increases simultaneously in response to various stressors in rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) and European flounder *Platichthys flesus* (L. 1758) (Ando *et al.*, 1999; Gilchriest *et al.*, 2000; Balment *et al.*, 2006). *In vitro* studies have shown that, independently or in synergy with CRF, AVT stimulates adrenocorticotropic hormone (ACTH) release from goldfish *Carassius auratus* (L. 1758) pituitary cells (Fryer *et al.*, 1985) and from perfused *O. mykiss* pituitaries (Baker *et al.*, 1996; Pierson *et al.*, 1996).

The effect of cortisol on AVT has been examined only *in vivo* in gilthead sea bream *Sparus aurata* L. 1758 (Román-Padilla *et al.*, 2011). The application of cortisol implants in *S. aurata* enhanced the hypothalamic expression of AVT mRNA and subsequently hypophysial AVT content. Although IT studies are very limited, they suggest that IT potentiates ACTH release from *C. auratus* pituitary cells (Fryer *et al.*, 1985). The *in vitro* effect of cortisol on AVT and IT secretion in teleosts has never been studied before.

In teleosts, urotensin I (UI) is implicated in the regulation of neuroendocrine, autonomic and behavioural responses to stressors (Lovejoy & Balment, 1999; Flik *et al.*, 2006). 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 regulatory action of this peptide in the CNS (Bernier *et al.*, 1999; Lu *et al.*, 2004; Alderman & Bernier, 2007). The structural similarity of UI with CRF suggests similar hypophysiotropic roles of both hormones in the hypothalamic–pituitary–interrenal (HPI) axis in fish (Ichikawa *et al.*, 1982; Fryer *et al.*, 1983; Lederis *et al.*, 1994). It has been established that UI modulates cortisol secretion either directly by acting on steroidogenic cells of interrenal tissue or indirectly *via* the hypothalamic–pituitary axis (Fryer *et al.*, 1983, 1984; Arnold-Reed & Balment, 1994; Lovejoy & Balment, 1999). In *C. commersonii*, *C. auratus* and West African lungfish *Protopterus annectens* (Owen 1839), UI-immunoreactive (UI-ir) fibres from the nucleus lateral tuberalis (NLT) extend to the pituitary where they may interact with AVT and IT nerve terminals (Yulis *et al.*, 1986; McMaster & Lederis, 1988; Fryer, 1989; Mathieu *et al.*, 1999).

In *S. 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 (Quesada *et al.*, 1988; Mancera & Fernandez-Lebrez, 1995; Duarte *et al.*, 2001). In *S. aurata*, UI instead of CRF may regulate AVT and IT release.

The aim of this study was to determine whether AVT and IT secretion from pituitary cells was affected by cortisol and UI in *S. aurata*.

MATERIALS AND METHODS

FISH

Three year-old *S. aurata* of both sexes were held at the National Center for Mariculture, Eilat, Israel. Fish were kept in a 5 m^3 circular tank supplied with aeration at ambient seawater temperature (20–26° C), salinity of 40 ± 0·5 (mean ± S.E.), under natural light conditions (11L:13D). Fish were fed with commercial *S. aurata* diet containing 45% protein. At the time of experiments (October), with the reproductive season approaching, 49 fish (560 ± 24 g, mean ± S.E.) were euthanized with an overdose of bicarbonate-buffered MS-222 (0·02%; Sigma-Aldrich; www.sigmaaldrich.com), and after decapitation, the pituitary glands were immediately collected.

CULTURE OF DISPERSED PITUITARY CELLS

Primary cultures of pituitary cells were prepared by a modification of the method described by Levavi-Sivan *et al.* (1995, 2004). *Sparus aurata* pituitaries ($n = 49$) were pooled into the basic medium (pH 7·4) containing Medium199 (Biological Industries; www.bioind.com) supplemented with 0·3% bovine serum albumin (BSA;Sigma-Aldrich), 10 μM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and 1% Pen–Strep–Nystatin solution (Biological Industries). The glands were cut into small fragments using a razor blade and dispersed by trypsinization for 30 min at room temperature in trypsin–EDTA solution (0·25% trypsin, 25% glucose and 250 mM EDTA) (Biological Industries). Trypsinization was halted by addition of foetal calf serum (FCS) (Biological Industries). After counting and determination of viability using trypan blue exclusion method (Freshney, 1986), dispersed cells were plated on 24 multi-well plates (Greiner Bio-One; www.greinerbioone.com) at a density of $1{\cdot}105 \times 10^6$ cells per well in 1·5 ml growth medium [Medium199, 10% FCS, 10mM HEPES and 1% penecillin-streptomycin-Nystatin (Pen-Strep-Nystatin) solution]. The cells were cultured at 28° C under 5% CO_2 for 3 days. After that time, growth medium was replaced with harvest medium (Medium199 supplemented with 0·1% BSA, 10 μM HEPES and 1% Pen–Strep–Nystatin solution) and cortisol ($1{\cdot}4 \times 10^{-8}$, $1{\cdot}4 \times 10^{-7}$ and $0{\cdot}4 \times 10^{-6}$ M) (Sigma-Aldrich) or UI (10^{-12}, 10^{-10} and 10^{-8} M) (*C. commersonii* sequence; Bachem; www.bachem.com) was added. Each plate consisted of a cell culture separately supplemented with cortisol or UI and a cell culture without treatments as control. The plates were incubated at 28° C under 5% CO_2 for 6, 24 and 48 h. Six replicas were done for each cortisol or UI concentration and control. After 6, 24 and 48 h, subsequent plates were removed from the incubator and the media were collected and stored at −70° C until high-performance liquid chromatography (HPLC) analysis.

AVT AND IT ANALYSIS

AVT and IT concentrations were determined in incubation media by HPLC with fluorescence and UV detection preceded by solid-phase extraction (SPE) according to a modified procedure by Kulczykowska (1995) that allows for measuring both neurohormones in one sample. SPE was carried out on Strata-X (30 mg ml^{-1}) columns (Phenomenex, www.phenomenex.com). Before SPE, refrozen media were acidified with 1 M HCl to pH 3–4. Extraction procedure for perfusion media was as follows: samples were loaded on conditioned columns [1 ml of 100% (v/v) methanol (J. T. Baker; www.jtbaker.nl) and then 1 ml of H_2O] and then 2 μl × 300 μl of H_2O and 2 μl × 300 μl of 0·1% (v/v) trifluoroacetic acid (TFA) (J. T. Baker) in 5% (v/v) acetonitrile (J. T. Baker) were passed through the columns to wash out impurities. Hormones were eluted with 2 μl × 600 μl of 80% (v/v) acetonitrile. Eluates were evaporated to dryness using TurboVap LV Evaporator (Caliper Life Sciences; www.caliperls.com). Dried samples were then reconstituted with 50 μl of 0·1% TFA in 15% acetonitrile in H_2O and directly injected to HPLC system (1200 series Quaternary HPLC system, Agilent Technology; www.agilent.com) with fluorescence detector and diode array detector. The chromatographic separations of peptides were carried out on

ZORBAX Eclipse XDB-C18 (4·6 mm × 150 mm, 5 μm) (Agilent Technology) using linear gradient system: 20–40% mobile phase B [0·1% TFA in acetonitrile:H_2O (3:1)] in mobile phase A (0·1% TFA in H_2O) for 15 min. The column temperature was 20° C and flow rate was 0·7 ml min^{-1}. Fluorescence detection was performed at 312 nm with excitation at 280 nm and UV detection at 215 nm. AVT and IT standards (Bachem) were used for standard curve preparation. Recovery of AVT and IT was in the 89–93% range. The detection limit was defined as 100 fmol ml^{-1}. Intra-day repeatability expressed as relative S.D. (RSD) was in the 2–4·5 and 5·3–8·2% range for AVT and IT, respectively; inter-day repeatability was in the 2·5–5·5 and 5·5–8·5% range for AVT and IT, respectively.

STATISTICAL ANALYSIS

AVT and IT contents are presented as a per cent of the control. For multiple comparisons, two-way analysis of variance (ANOVA) was used. *Post hoc* comparisons were made with the Newman–Keuls test. Significance was considered at $P < 0{\cdot}05$. Statistical analysis was performed using STATISTICA 7.1 programme (www.statsoft.com).

RESULTS

THE INFLUENCE OF CORTISOL ON AVT AND IT SECRETION

Cortisol at all doses significantly increased AVT release after 6, 24 and 48 h of culture. Moreover, AVT secretion after 24 h exposure to $1{\cdot}4 \times 10^{-7}$ and $1{\cdot}4 \times 10^{-6}$ M of cortisol was significantly higher ($P < 0{\cdot}001$) compared to that induced by $1{\cdot}4 \times 10^{-8}$ M of cortisol [Fig. 1(a)]. In contrast to AVT, IT secretion was significantly decreased by cortisol at all tested doses ($P < 0{\cdot}001$) after 6, 24 and 48 h of exposure. IT secretion after 6 h exposure to $1{\cdot}4 \times 10^{-6}$ M of cortisol was significantly lower ($P < 0{\cdot}01$) compared to that caused by $0{\cdot}4 \times 10^{-7}$ M of cortisol [Fig. 1(b)].

THE INFLUENCE OF UI ON AVT AND IT SECRETION

UI at all doses significantly increased AVT secretion ($P < 0{\cdot}05$ for 10^{-12} and 10^{-10} M or $P < 0{\cdot}001$ for 10^{-8} M) after 6 h of culture. After 24 h, only 10^{-8} M of UI significantly ($P < 0{\cdot}05$) elevated AVT secretion [Fig. 2(a)]. UI did not significantly influence IT release from dispersed pituitary cells. IT secretion measured after 6 h exposure to 10^{-12} M of UI was significantly lower compared with that caused by two other doses of UI ($P < 0{\cdot}05$ for 10^{-10} M and $P < 0{\cdot}01$ for 10^{-8} M) [Fig. 2(b)].

DISCUSSION

This study demonstrates for the first time that AVT and IT secretion from *S. aurata* pituitary cells was influenced by cortisol and UI. The response of AVT and IT to different stress stimuli and the presence of arginine vasotocinergic innervation of corticotrophic cells in the pituitary strongly suggest that both neuropeptides can be important components of the stress axis in fish (Yulis & Lederis, 1987; Olivereau *et al.*, 1988; Duarte *et al.*, 2001; Kulczykowska, 2007). In this study, cortisol showed a stimulatory action on pituitary cells of *S. aurata* inducing AVT secretion at all doses. The doses of cortisol were chosen taking into account different cortisol responses to stress in various fish species (Arnold-Reed & Balment, 1989; Kelsall

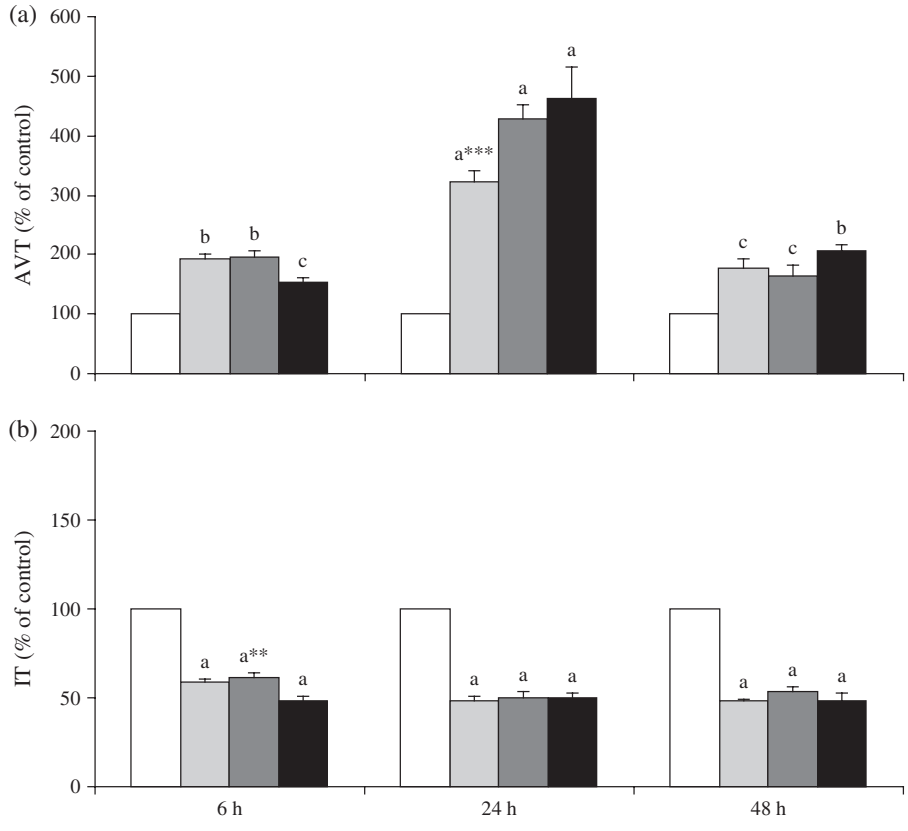

FIG. 1. (a) Arginine vasotocin (AVT) and (b) isotocin (IT) secretion by primary cultures of dispersed pituitary cells from *Sparus aurata* in response to cortisol [$1{\cdot}4 \times 10^{-8}$(□), $1{\cdot}4 \times 10^{-7}$ (■) and $0{\cdot}4 \times 10^{-6}$ M (■)] for 6, 24 and 48 h of incubation. Values are mean ± S.E. AVT and IT values are expressed as % of control (□). Lowercase letters above bars indicate values significantly different *v*. control within time point of incubation; **a**, $P < 0{\cdot}001$; **b**, $P < 0{\cdot}01$; **c**, $P < 0{\cdot}05$. Asterisks above bars indicate values that are significantly different between applied cortisol doses within time point of incubation; ***$P < 0{\cdot}001$ and **$P < 0{\cdot}01$.

& Balment, 1998; Barton, 2002). Among teleosts, some species exhibit high cortisol concentrations (10^{-7}–10^{-6} M) in response to acute stress (Barton, 2002) while some species reveal low cortisol levels (10^{-9}–10^{-8} M) in response to the same stress (Alderman *et al.*, 2008; Bernier, *et al.*, 2008; Mancera *et al.*, 2008). Also, in rats *Rattus norvegicus*, a wide range of corticosterone doses, *i.e.* between 10^{-9} and 10^{-4} M, was found to affect AVP release from the hypothalamic slices containing paraventricular (PVN) and supraoptic nuclei (SON) (Liu *et al.*, 1995). In studies presented here, the dose-dependent effect of cortisol on AVT secretion has been manifested after 24 h of cell culture. *In vitro* cortisol action on AVT secretion in teleosts was not studied before. *In vivo* studies on *S. aurata* have shown an increase in hypophysial AVT content after 12 and 24 h post-implantation of cortisol (Román-Padilla *et al.*, 2011). In mammals, influence of cortisol on AVP secretion was studied by *in vivo* and *in vitro* methods. In zebu steers *Bos indicus*, stimulatory *in vivo* effect of cortisol on plasma AVP content was noted in response to water deprivation (Parker *et al.*,

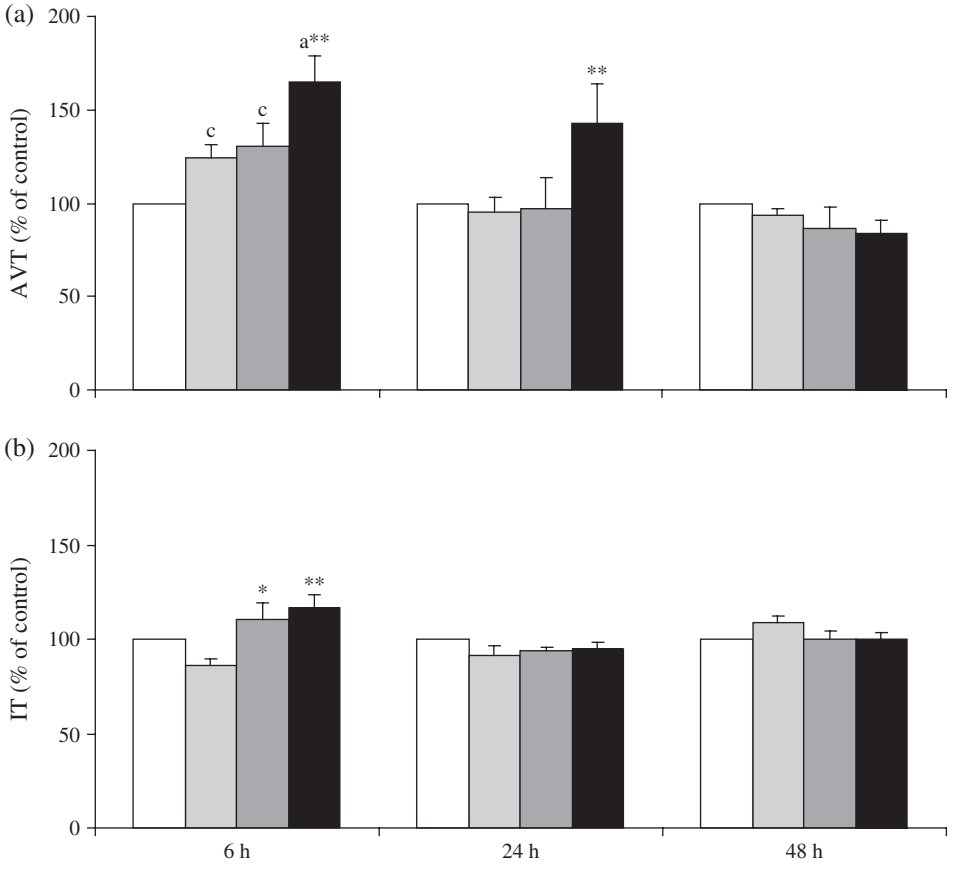

FIG. 2. (a) Arginine vasotocin (AVT) and (b) isotocin (IT) secretion by primary cultures of dispersed pituitary cells from *Sparus aurata* in response to urotensin (UI) [10^{-12} (□), 10^{-10} (▩) and 10^{-8} M (■)] for 6, 24 and 48 h of incubation. Values are mean ± S.E. AVT and IT values are expressed as % of control (□). Lowercase letters above bars indicate values significantly different *v*. control within time point of incubation; **a**, $P < 0{\cdot}001$; **c**, $P < 0{\cdot}05$. Asterisks above bars indicate values that are significantly different between applied cortisol doses within time point of incubation; $^{**}P < 0{\cdot}01$ and $^{*}P < 0{\cdot}05$.

2004). Engler *et al.* (1989) have demonstrated that an audiovisual stress (barking dog) rapidly increases the plasma levels of cortisol and AVP in sheep *Ovis aries*. Other findings indicate that the expression of AVP in parvocellular neurons of the PVN and AVP secretion into the pituitary portal circulation increases under chronic stress in rats (Holmes *et al.*, 1986; de Goeij *et al.*, 1992; Aguilera, 1994; Chowdrey *et al.*, 1995). It is also shown that stress up-regulates the number of AVP receptors in the rat anterior pituitary (Aguilera *et al.*, 1994).

The results presented here revealed that the stimulatory effect of cortisol on AVT secretion diminishes after 48 h of culture. The depletion of AVT stores without subsequent supplementation of secretory granules from arginine vasotocinergic nerves is the most likely explanation. Corticoid receptor (CR) desensitization could be another cause. Desensitization of CRs is known in mammals as a result of physiological processes, stress and disease (Meaney *et al.*; 1989; Modell *et al.*, 1997; Cole *et al.*,

2009). On the other hand, the reduction of AVT secretion after 48 h of cortisol exposure could also be linked with an increase in aminopeptidase activity responsible for nonapeptide metabolism as shown in rats and chickens *Gallus domesticus* (Wang *et al.*, 1983; Burbach *et al.*, 1987, 1998).

As in the case of AVT, *in vitro* cortisol action on IT secretion in teleosts was not known before this study. Data presented here showed that cortisol decreased IT secretion from *S. aurata* pituitary cells. In mammals, cortisol action on OT was investigated by *in vitro* and *in vivo* experiments. Di *et al.* (2003) have found that glucocorticoids exert an inhibitory effect on neurosecretory activity of parvocellular oxytocinergic neurons of rats. In rats, the increase in plasma OT levels after intravenous injection of isotonic or hypertonic saline was blocked by dexamethasone (Durlo *et al.*, 2004).

In *S. aurata*, unlike other studied fishes, CRF is not a releasing factor for ACTH (Mancera & Fernandez-Lebrez, 1995). *In vitro* experiments performed on *C. auratus* revealed that UI affects HPI axis. UI is even more potent than CRF in stimulation of ACTH secretion from superfused anterior pituitary cells (Fryer, 1989; Tran *et al.*, 1990). It is presumed that UI, instead of CRF, may regulate AVT and IT release in *S. aurata*.

The doses of UI used in the experiment were determined on the basis of the literature, considering its concentration in different tissues (Suess *et al.*, 1986; Arnold-Reed & Balment, 1989, 1994; Kelsall & Balment, 1998; Backström *et al.*, 2011). In this study, dose-dependent stimulatory effect of UI on AVT secretion was observed after 6 h of incubation. In rats, it has been demonstrated that UI slightly increases the hypothalamus AVP release *in vitro*, indicating the possible stimulatory effect of this peptide on AVT production (Bagosi *et al.*, 2011). The presented data have shown that only after 24 h the highest dose of UI elevates AVT secretion from pituitary cells. The stimulatory effect of UI completely expires after 48 h of cell culture. This may be explained by later desensitization of CRF receptors (CRFR). UI is a natural ligand of CRFRs (Lederis *et al.*, 1994; Arai *et al.*, 2001). Desensitization of CRFRs is demonstrated by a number of *in vitro* studies (Hauger *et al.*, 1997; Aguilera *et al.*, 2004; Hauger *et al.*, 2006; Teli *et al.*, 2008). In this study, UI did not affect IT secretion. The influence of UI on IT or OT secretion had never been investigated before.

It is also known that UI increases cortisol secretion (Fryer *et al.*, 1983, 1984; Arnold-Reed & Balment, 1989, 1994; Lovejoy & Balment, 1999). Thus, UI may also influence AVT secretion indirectly, stimulating cortisol release.

The opposite response of AVT and IT to UI or cortisol exposure in pituitary cell culture is in accordance with other data (Saito & Urano, 2001; Kulczykowska, 2007) showing an independent regulation of nonapeptide secretion.

This study indicates that cortisol affects AVT and IT secretion from pituitary cells of *S. aurata*. In this species, UI instead of CRF may contribute to the regulation of HPI axis and regulate AVT secretion. This study confirms previous assumptions that AVT and IT are essential components of stress response in fish.

This study was supported by Institute of Oceanology of Polish Academy of Sciences statutory research task IV.2.1. to H.K.-K. The research fellowship for H.K.-K. at the National Center for Mariculture in Eilat, Israel, was supported by the scientific exchange programme between the Polish Academy of Sciences and the Israel Academy of Sciences and Humanities. The authors wish to thank A. Colorni (Israel Oceanographic & Limnological Research, the National Center for Mariculture, Eilat, Israel) for language corrections. Special thanks go to

E. Kulczykowska (Institute of Oceanology of Polish Academy of Sciences, Sopot, Poland) for critically reading the manuscript and providing helpful advice.

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