



Cortisol and melatonin in the cutaneous stress response system of fish

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ARTICLE INFO

Keywords:

Cortisol
European flounder
Fish
In vitro
Melatonin
5-Methoxykynuramines
Skin
Stress

ABSTRACT

The stress hormone cortisol, together with antioxidants, melatonin (Mel) and its biologically active metabolites, 5-methoxykynuramines, including AFMK, set up a local stress response system in mammalian skin. Our *in vitro* study of the European flounder (*Platichthys flesus*) was designed to examine whether Mel and AFMK would respond to cortisol while a glucocorticoid is added to the incubation medium. The concentrations of cortisol in the incubation medium mimic plasma cortisol levels seen in fish exposed to different types of stresses such as handling, confinement, high density, food-deprivation or air-exposure. We measured Mel and AFMK in skin explants and culture media using high-performance liquid chromatography (HPLC) with fluorescence detection. We also analysed melanosome response (dispersion/aggregation) in the explants subjected to the different treatments. Cortisol stimulated the release of Mel and AFMK from skin explants in a dose-dependent manner. Melanosome dispersion and a darkening of the skin explants were observed after incubation with cortisol. This study is the first to demonstrate the interrelationship between cortisol and Mel/AFMK in fish skin. Our data strongly suggest that the cutaneous stress response system (CSRS) is present in fish. The question remains whether Mel, AFMK or cortisol are synthesized locally in fish skin and/or transported by the bloodstream. The presence of the CSRS should be taken into account during elaboration of new indicators of fish welfare both in aquaculture and in the wild.

1. Introduction

The skin of vertebrates is an organ of protection and sensation. It acts as a biological barrier defending the organism against harmful environmental factors, such as mechanical impacts, variations in pressure and temperature, radiation, chemicals, microorganisms, and external parasites. Moreover, the skin contains an extensive network of nerve cells to detect changes in the environment and relay information to the central nervous system. It has been proposed by Slominski and colleagues, as early as 1995, that the human skin has a local mechanism of response to stress which is an equivalent to the hypothalamic–pituitary–adrenal axis (HPA) (Slominski et al., 1995). At that time, they introduced the term “skin stress response system”. Recent studies of mammalian skin cell biology have revealed many unexpected functions of the skin (Watt, 2014). There is substantial evidence that many hormones and other biologically active molecules are synthesized in specialized cells of the epidermis and dermis or released by adjacent neurons in response to suitable stimuli. For instance, mammalian skin is a source of stress hormones related to HPA, such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol, as well as being a site of melatonin (Mel) synthesis (Slominski

et al., 1996; Slominski and Wortsman, 2000; Slominski et al., 2008, 2013; Acuña-Castroviejo et al., 2014). Melatonin, beyond its activity in circadian rhythm, is a highly sensitive and effective scavenger of hydroxyl and peroxy radicals (Hardeland, 2005), and can stimulate the activity of antioxidative enzymes (Dzięgiel et al., 2003). Therefore, significant amounts of Mel are found in organs such as the skin, which are exposed to unfavourable environmental conditions (Tan et al., 2007). Because Mel metabolites such as N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK) are also potent scavengers, an inactivation of free radicals in cells is performed *via* the antioxidative cascade: Mel → AFMK → AMK (Hardeland, 2008; Galano et al., 2013). The efficiency of Mel, AFMK and AMK for scavenging depends on the polarity of the analytical medium and the type of free radical with which they are reacting (Hardeland et al., 2009; Galano et al., 2013).

In mammals, the cells synthesizing the hormones together with the nerve endings constitute the cutaneous neuroendocrine system which can respond to changes in the environment, and Mel and its derivatives, CRH, ACTH, and cortisol, are interconnected parts of this system (Slominski et al., 2005, 2012). It is also well established that multiple glucocorticoid receptors (GRs) are potent modulators of cutaneous

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homeostasis (Schmuth et al., 2007). Current evidence suggests that fish skin also has a stress response system. Firstly, cortisol, the main stress hormone in vertebrates including fish (Wendelaar Bonga, 1997), and Mel, an important antioxidant, are both present in fish skin (Bertotto et al., 2010; Kulczykowska et al., 2017). There is also evidence for GR localization in epidermal cells in the skin of zebrafish (*Danio rerio*) (Cruz et al., 2013). Secondly, the interactions between cortisol or stress exposure, and Mel production or its plasma levels have been shown in rainbow trout (*Oncorhynchus mykiss*), Mozambique tilapia (*Oreochromis mossambicus*), and gilthead sea bream (*Sparus aurata*) (Kulczykowska, 2001; Larson et al., 2004; Mancera et al., 2008, 2009; Nikaido et al., 2010; López-Patiño et al., 2014). Moreover, in several fish species, administration of cortisol or exposure to stress induces stress-related changes in the skin such as melanosome dispersion (Iger et al., 1995; Höglund et al., 2000; Ruane et al., 2005).

The purpose of this *in vitro* experiment with European flounder (*Platichthys flesus*) skin explants was to find out if Mel and its biologically active metabolites, AFMK and AMK, respond to cortisol while the glucocorticoid is added into the incubation medium. We investigated the release of Mel, AFMK, and AMK from the explants after incubation with different concentrations of cortisol mimicking stress conditions in fish. We also analysed the response of melanosomes while the explants are exposed to different treatments in order to validate the physiological relevance of this *in vitro* model. We aimed to answer the question whether cortisol and Mel, with its metabolites, are functionally interconnected to set up the cutaneous stress response system (CSRS) in fish. As yet, to the best of the authors' knowledge, the CSRS has not been studied in non-mammalian vertebrates.

2. Materials and methods

2.1. Experimental fish

Adult European flounders (*Platichthys flesus*) of both sexes were caught outside the spawning season in the Gulf of Gdańsk (southern Baltic Sea). The fish ($n = 18$) were maintained at a temperature of 10 °C in water of 8 ppt salinity with a 12L:12D photoperiod in aerated aquaria at the Institute of Oceanology PAN (IO PAN Sopot, Poland), where all experiments were carried out. The fish were acclimatized for one week before experimentation and fed frozen mussels *ad libitum* once a day, at noon. The randomly selected fish were anaesthetized by immersion in 0.5% (v/v) 2-phenoxyethanol (Sigma-Aldrich, USA) solution in water, their spinal cords were sectioned and skin samples collected. Skin samples were taken from 10 fish to establish cortisol concentrations for *in vitro* study; samples from the 8 other fish were either used in *in vitro* study or stored at –70 °C until the analyses of Mel, AFMK and AMK concentrations were conducted. The *in vitro* experiment was repeated 6 times.

All experiments complied with EC Directive 2010/63/EU for animal experiments and with the guidelines of the Local Ethics Committee on Animal Experimentation.

2.2. Analysis of cortisol

Skin samples (10 mm × 10 mm) were weighed, fragmented using a scalpel blade and homogenized in 0.05 M phosphate buffer (pH 7.4) containing 0.01% Thimerosal (Sigma-Aldrich, USA) using an ULTRA-TURRAX homogenizer (IKA, USA). The extracts were centrifuged at 15000g for 20 min at 4 °C, and then supernatants were decanted and stored at –70 °C prior to the analysis of cortisol levels. Cortisol levels were determined using a solid phase enzyme-linked immunosorbent assay (ELISA) kit (DRG, Germany). A standard curve was prepared using six standard dilutions of cortisol: 20, 50, 100, 200, 400 and 800 ng/mL. The assay was conducted in microplates according to the ELISA kit manufacturer's instructions, and based on the principle of competitive binding: cortisol in standards and samples competed with

cortisol conjugated to horseradish peroxidase for the antibody binding sites in the microtiter wells. Microplates were incubated for 60 min at room temperature, and unbound components were then washed away with buffer using a HydroFlex strip-washer (Tecan, Austria). Bound cortisol enzyme conjugate was measured by the reaction of the horseradish peroxidase enzyme to the substrate tetramethylbenzidine (TMB). The reaction was carried out at room temperature for 15 min, and stopped by the addition of 100 µL of 0.5 M H₂SO₄. The absorbance at 450 nm was read using a Sunrise Absorbance Reader (Tecan, Austria) within 10 min after stopping the reaction. All samples were assayed in duplicate. The detection limit of the assay was 14.77 ng/mL. The intra-assay coefficient of variation was 0.87%. The inter-assay variation was not determined because all samples were measured in the same assay. The mean cortisol concentration in the skin was 24.99 ± 0.40 ng/g (number of fish = 10).

2.3. The dynamic culture of skin explants

Skin explants (10 mm × 10 mm) were put in sterile Petri dishes and placed in the incubator with shaking (45 cycles/min) (Heidolph, Germany) at 10 °C and aerated using atmospheric air at a pressure of 127.51 mm Hg. The explants were washed twice with Leibovitz's L-15 Medium at pH 7.4 (L-15; Sigma-Aldrich, USA) for 10 min, rinsed in L-15 Medium supplemented with Penicillin (500 UI/mL), Streptomycin (0.5 mg/mL) and Neomycin (1 mg/mL) (Sigma-Aldrich, USA) for 5 min, and in L-15 Medium for 10 min in accordance with the method applied by Sugimoto et al. (2000). All samples were then incubated in sterile 6-well plates (CELLSTAR, Greiner Bio-One, Germany) with inserts (Cell Crown, Sigma-Aldrich, USA) under the same conditions as at the beginning of the experiment (shaking: 45 cycles/min at 10 °C and aeration pressure: 127.51 mm Hg). The explants were put on the Nylon Net Filter 20 µm (NY2002500; Merck Millipore, Germany) and incubated in L-15 Medium supplemented with L-glutamine, 10 mM HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) and 1 mM NaHCO₃ (Sigma-Aldrich, USA) at pH 7.4. Each culture plate contained two control wells (medium without cortisol) and four wells with different concentrations of cortisol (50, 150, 250 or 1250 ng/mL). After a 60-min stabilization period (data not shown), all the incubations were carried out in 30-min cycles with medium replacement. During the first 30-min incubation period, the initial Mel, AFMK and AMK release was determined. The explants were then incubated with four different concentrations of cortisol (50, 150, 250, 1250 ng/mL). In the last three periods, explants were incubated in medium without cortisol. The cortisol concentrations in the incubation medium were 2, 6, and 10 times higher than the cortisol concentration measured in the skin of flounders not subjected to stress (24.99 ± 0.40 ng/g). All procedures were carried out under red light in a laminar air flow cabinet (NUAIRE Biological Safety Cabinet Class II, USA). All collected media were centrifuged at 4 °C at 3000g for 10 min and supernatants were stored at –70 °C prior to analysis.

2.4. Analysis of melanosome aggregation and dispersion in skin explants

The effect of melanosome dispersion/aggregation was studied in dorsal skin explants after 60 min of incubation in medium without cortisol, with four different doses of cortisol and a high concentration of K⁺ (60 mM). Skin explants ($n = 18$) were fixed in 4% buffered formalin and mounted with Eukitt (Sigma-Aldrich, USA). The diameter of 40 randomly selected melanophores was measured for each explant. The analysis was taken under Leica M205C Microscope with planochromatic objective connected with Leica DFC450 digital color camera, using Leica Application Suite (LAS) 4 software enhanced with Interactive Measurements module (Leica Microsystems GmbH, Germany). The melanophore index (MI) was used to estimate the degree of pigment dispersal in fish, in accordance with the method applied by Aspögren et al. (2003). Using this method, a fully dispersed

cell is rated '5' while a completely aggregated cell is rated '1'.

2.5. Analysis of Mel, AFMK and AMK

Skin samples (10 mm × 10 mm) were weighed and homogenized in 500 µL of water. The homogenate was then centrifuged at 9000g, 4 °C for 10 min. Mel, AFMK and AMK were extracted three times with 500 µL of acetonitrile:dichloromethane 1:1 (v/v). The eluate was evaporated to dryness using a Turbo Vap LV (Caliper Life Science, USA). The residue was re-dissolved in 100 µL of methanol and a 40 µL aliquot was injected into the HPLC system. High performance liquid chromatographic (HPLC) analyses were performed using a 1200 series Quaternary HPLC system (Agilent Technologies, USA) equipped with two detectors: a diode array detector (DAD) and a fluorescence detector. Chromatographic separation was achieved on Kinetex C18 column (150 mm × 4.6 mm I.D., 2.6 µm; Phenomenex, USA). A gradient elution system was applied for separation of Mel, AFMK and AMK. The mobile phase consisted of solvent A (H₂O) and solvent B (0.1% trifluoroacetic acid in acetonitrile). A linear gradient was 12–20% of eluent B during 20 min. The column temperature was 20 °C and flow rate 0.7 mL/min. The intrinsic fluorescence of Mel and AFMK was measured (Mel: excitation at 230 nm and emission at 350 nm; AFMK: excitation at 240 nm and emission at 480 nm). The identification of Mel and AFMK was performed by comparing the retention times of the sample with those of the standards: Mel (Sigma-Aldrich, Germany) and AFMK (synthesis procedure below). The concentrations of AMK were below the limit of detection (LOD; 520 pg/mL). The same procedure was used for the incubation media.

AFMK was synthesized by oxidation of Mel with 3-chloroperbenzoic acid in accordance with the method of Kennaway et al. (1988). Briefly, Mel (0.5 g, 2 mmol, Bachem, Switzerland) dissolved in dichloromethane (30 mL) was stirred with 3-chloroperbenzoic acid (2 g, 0.011 mol, Sigma-Aldrich, Germany) for 18 h at 4 °C. The solution was washed with saturated aqueous sodium bicarbonate solution, dried over anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen. The residue was purified twice by chromatography on a preparative TLC plate (Silica Gel, GF, 254, Analtech, Germany) using ethyl acetate as the eluent. The characteristic of the synthesized AFMK was as follows: UV spectra (λ_{max} = 236, 266, 355 nm) and melting point (138–140 °C).

2.6. Statistical analysis

Statistical analysis was carried out using Statistica 7.1 software. The release of Mel and AFMK from the explants after incubation with different concentrations of cortisol determined at different time points was analysed using a two-way ANOVA followed by a Newman-Keuls *post hoc* test. Data are expressed as percentage of control (Fig. 1). The dispersion of data within two sets of samples collected from different individuals and a single individual are represented by the Coefficients of Variations (CV) (Table 1). Student's unpaired *t*-test was used to detect a change in melanophore diameters in skin explants after different treatments (Table 3). Significance was accepted at $P < 0.05$.

3. Results

In our study, cortisol concentrations in the incubation medium (50, 150, and 250 ng/mL) corresponded with plasma cortisol concentrations seen in fish exposed to different types of stresses, such as handling, confinement, high density, food-deprivation or air-exposure (Arends et al., 1999; Rotllant et al., 2001; Metz et al., 2005; Mancera et al., 2008). The highest cortisol concentration used in our study (1250 ng/mL) was well above these values and should be considered supraphysiological. Cortisol stimulated the release of Mel and AFMK from skin explants in a dose-dependent manner, except at the highest concentration which was ineffective. A direct stimulatory effect of cortisol

on Mel and AFMK release was observed after 30–60 min of incubation (Fig. 1). The most pronounced effect was at 250 ng/mL of cortisol (Fig. 1). AMK was below the LOD (520 pg/mL) in the explants and incubation media. The Coefficients of Variations representing the dispersion of data within two sets of samples collected from different individuals and a single individual are shown in Table 1. Because the variabilities (low or moderate) were similar in both sets (Table 1), we felt entitled to present the changes in Mel and AFMK release for all samples together (Fig. 1). Data were presented as percentages of control values (control = incubation in cortisol-free medium). The levels of Mel and AFMK in the explants: before incubation (fresh samples) and after 180 min of incubation in medium without cortisol or with four different doses of cortisol are shown in Table 2. There were no significant differences between Mel concentrations in the explants before and after incubation in the cortisol-free medium. This confirmed the stability of Mel during prolonged incubation (180 min). However, AFMK concentrations were slightly lower after incubation in the cortisol-free medium when compared to the fresh samples (Table 2), probably due to the relatively low stability of AFMK, because AFMK can be deformed by endogenous enzyme arylamide formamidase (Tan et al., 2000; Fisher et al., 2006) and reacts easily with reactive oxygen species (Tan et al., 2007). It is also possible that AFMK were simply leaching from the explants during prolonged incubation (180 min), even in the cortisol-free medium. However, the levels of Mel and AFMK in the explants were several times lower after incubation in the medium with cortisol than without cortisol so that it was evident that cortisol, not the medium alone, stimulated Mel and AFMK release from the explants (Table 2).

The smallest melanophore diameter as a measure of melanosome aggregation was observed in the explants after incubation in medium supplemented with high K⁺ concentration (60 mM) (Table 3, Fig. 2B), in accordance with the method applied by Fujii (1959). Melanosome dispersion was presented in all skin explants after incubation in the medium with cortisol, albeit to varying degrees (Table 3, Fig. 2C–F). The highest melanophore diameter linked with melanosome dispersion was noted at 250 ng/mL of cortisol (Table 3, Fig. 2E). The melanophore index assessed the degree of pigment dispersal in melanophores (Table 3).

4. Discussion

This study was designed to examine the direct effect of cortisol on the release of Mel and its derivatives from the skin explants of the European flounder while a glucocorticoid was added into the incubation medium. Our *in vitro* model was useful for examining the relationship between cortisol and Mel/AFMK in fish skin. The cortisol concentrations which we used to simulate stress conditions were 2, 6, and 10 times higher than the cortisol concentration measured in the skin of flounders not subjected to stress (25 ng/g). In this case, the ratio of cortisol level in the incubation medium to the basic level of cortisol in the skin was the same in the plasma of fish subjected to stress as it was in fish not subjected to stress (Arends et al., 1999; Rotllant et al., 2001; Metz et al., 2005; Mancera et al., 2008). In all samples, we detected Mel and AFMK but not AMK. We found that cortisol stimulated Mel and AFMK release from flounder skin explants in a dose-dependent manner (Fig. 1). We also demonstrated melanosome dispersion and skin darkening in explants incubated in the medium with cortisol (Table 3, Fig. 2).

It is well established that cortisol is the main stress hormone in fish and circulating levels increase rapidly when an individual is exposed to stress (Wendelaar Bonga, 1997). The level of cortisol depends on several factors including the type, degree, and duration of the stress (Mommensen et al., 1999). Although there are large amounts of data on the changes in plasma cortisol seen in response to a variety of stressors in fish, the status of cutaneous cortisol is as yet uncertain. The histological effects of cortisol in the fish skin on the other hand, such as

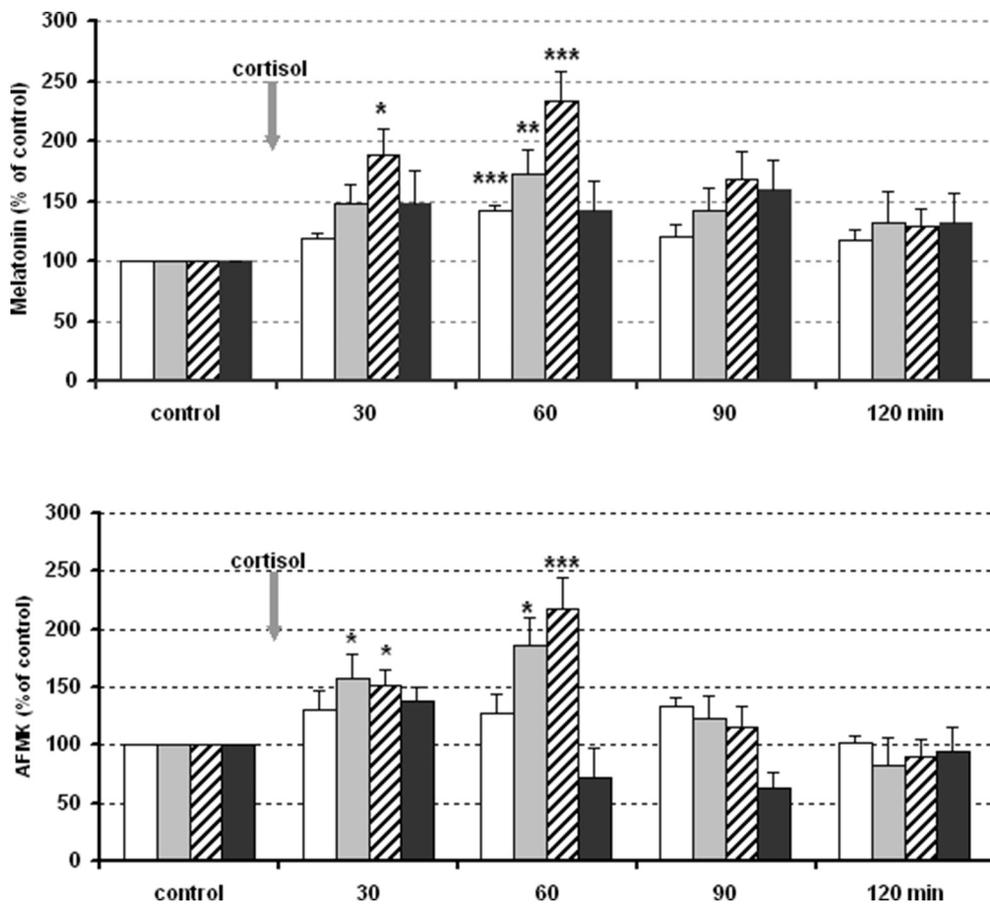


Fig. 1. The release of Mel and AFMK from skin explants after incubation with different concentrations of cortisol: □ 50 ng/mL, ▒ 150 ng/mL, ▨ 250 ng/mL and ■ 1250 ng/mL. Statistical differences are indicated as: *P < 0.05; **P < 0.01; ***P < 0.001 vs control; number of fish = 8.

Table 1
The Coefficients of Variation (CV) representing the dispersion of data within two sets of samples collected from different individuals and a single individual.

	Different individuals CV (%)		Single individual CV (%)	
	Mel	AFMK	Mel	AFMK
Fresh skin explants	11	14	7	9
Skin explants incubated in cortisol-free medium	5	23	4	14

Scale: CV < 20% – low variability; 20% < CV < 40% – moderate variability; 40% < CV < 100% – high variability; 100% < CV < 150% – very high variability; CV > 150% – extreme variability.

Table 2
The levels of Mel and AFMK in skin explants: before incubation (fresh samples) and after 180 min of incubation in medium without cortisol or with four different doses of cortisol (n – number of samples; concentrations of cortisol (ng/mL) are given in the circles; number of fish = 8).

skin explants before incubation (fresh samples)		skin explants after incubation without cortisol		skin explants after incubation with cortisol	
Mel (pg/g)	AFMK (pg/g)	Mel (pg/g)	AFMK (pg/g)	Mel (pg/g)	AFMK (pg/g)
1214 ± 46 n=5	456 ± 21 n=5	1150 ± 14 n=5	470 ± 11 n=5	199 ± 20 n=25 (50)	229 ± 50 n=25
1118 ± 49 n=5	478 ± 14 n=5	1069 ± 19 n=5	305 ± 12 n=5	227 ± 32 n=25 (150)	255 ± 46 n=25
1111 ± 42 n=5	606 ± 11 n=5	1139 ± 15 n=6	509 ± 21 n=6	182 ± 24 n=30 (250)	213 ± 55 n=30
1139 ± 20 n=5	490 ± 19 n=5	1167 ± 10 n=5	345 ± 17 n=5	254 ± 39 n=25 (1250)	245 ± 12 n=25

Table 3
Melanophore diameter (µm) and melanophore index (MI) in skin explants (scale 1–5).

Medium	Diameter mean ± SEM	Diameter minimum	Diameter maximum	MI
Cortisol-free (control)	46 ± 0.6	39	50	3
K ⁺ (60 mM)	19 ± 0.7	10	29	1
Cortisol (50 ng/mL)	44 ± 0.8	38	55	3
Cortisol (150 ng/mL)	52 ± 1.1	40	66	4
Cortisol (250 ng/mL)	79 ± 1.9	59	103	5
Cortisol (1250 ng/mL)	50 ± 1.2	32	65	4

Statistical differences are indicated as *P < 0.001 vs control (Student's t-test).

stimulation of mitosis and induction of apoptosis in the rainbow trout (*Oncorhynchus mykiss*) (Iger et al., 1995; Nolan et al., 1999, 2002; Van der Salm et al., 2002) and regulation of cells differentiation in zebrafish (*Danio rerio*) epithelia (Cruz et al., 2013), are already known. Moreover, in the rainbow trout, Arctic charr (*Salvelinus alpinus*) or post-larval *Solea senegalensis*, it has been demonstrated that administration of cortisol and stress exposure induces stress-related changes in the skin including melanosome dispersion, which results in a darkening of the skin (Iger et al., 1995; Höglund et al., 2000; Ruane et al., 2005).

We have studied the effect of melanosome dispersion/aggregation in skin explants incubated in a cortisol-free medium, a medium supplemented with cortisol (four concentrations), and a high concentration of K⁺ to test the physiological relevance of the *in vitro* model. The incubation with K⁺ caused melanosome aggregation and with cortisol

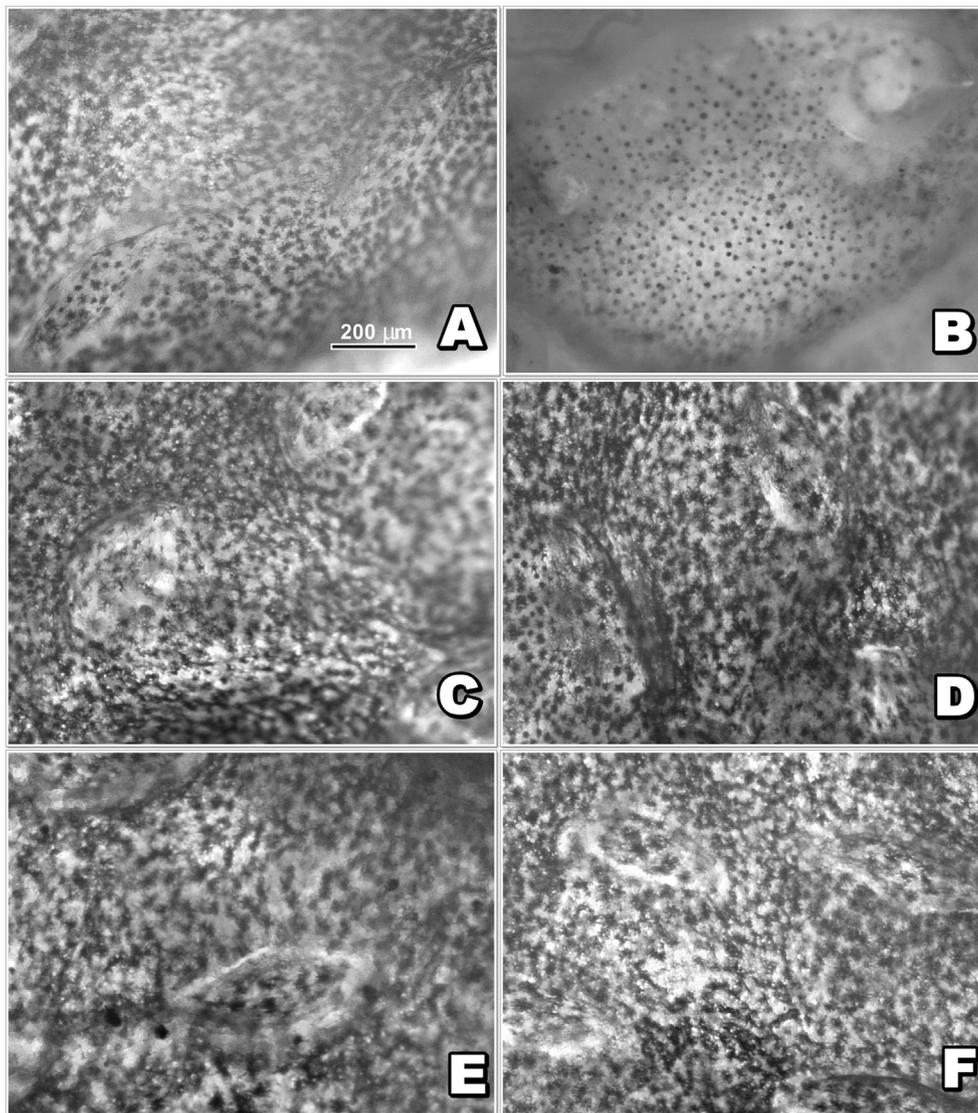


Fig. 2. The effect of melanosome dispersion/aggregation in dorsal skin explants incubated in a cortisol-free medium (A), a high concentration of K^+ (60 mM; B), and a medium supplemented with cortisol: 50 ng/mL (C), 150 ng/mL (D), 250 ng/mL (E), and 1250 ng/mL (F).

caused melanosome dispersion, as expected (Fujii, 1959; Iger et al., 1995; Höglund et al., 2000; Ruane et al., 2005). The concentration of 250 ng/mL of cortisol was most effective in both stimulation of Mel and AFMK release from the explants as well as melanosome dispersion (Figs. 1 and 2). However, the highest cortisol concentration (1250 ng/mL) was ineffective in stimulation of Mel and AFMK release (Fig. 1). It should be emphasized that this concentration far exceeded the maximum concentration of cortisol in fish subjected to stress and was, therefore, considered supraphysiological. With our current knowledge in this area, it is difficult to explain the lack of effectiveness of this treatment, however, it is well known that very high cortisol levels have a detrimental effect on many aspects of cell function (McKay and Cidlowski, 2003) and we can speculate that the supraphysiological cortisol concentration used in our study impaired some mechanisms related to stress response in flounder skin cells. On the other hand, the highest cortisol concentration remained effective in melanosome dispersion so that there are still many questions about cortisol in the skin that need to be addressed. Regarding melanosome dispersion in the explants incubated with cortisol, we suggest that this resulted from both the direct action of cortisol on melanosomes and Mel leaching from the explants, because Mel *per se* induces aggregation of the melanosomes (for review: Fujii, 2000; Goda and Fujii, 1998; Kijewska et al., 2012).

To the best of the authors' knowledge, this study is the first to investigate the influence of cortisol on Mel and AFMK in fish skin.

However, an effect of cortisol and/or stress on plasma Mel level has been shown in various fish species. For instance, plasma Mel level is depressed in rainbow trout subjected to acute disturbance stress (Kulczykowska, 2001) or when exposed to the chronic stress of high stocking density (López-Patiño et al., 2014). In the gilthead sea bream, plasma Mel concentration significantly increases in fish treated with cortisol at concentrations corresponding to the chronic stress of confinement at high density (Mancera et al., 2009). In the rainbow trout, plasma Mel concentrations are also higher in socially stressed fish (subordinated individuals) having a higher concentration of cortisol in their plasma (Larson et al., 2004). Moreover, cortisol administered in concentrations mimicking stress condition reduces Mel secretion from the pineal organ cultured *in vitro* in the Mozambique tilapia (Nikaido et al., 2010). Thus, the relationship between cortisol and plasma Mel level and/or its release from the pineal organ seems to be species-specific and dependent on the type of stress stimulation. In this study, we analysed Mel and its metabolites as parts of a cutaneous defence system. There is a lot of evidence to suggest that Mel is engaged in various lines of defence in vertebrates, acting alone and through their metabolites, AFMK and AMK, and that it is highly effective even at low concentrations in protecting organisms from oxidative stress (Tan et al., 2000; Hardeland, 2008). In mammals, Mel together with AFMK and AMK, all produced in the skin, play a role of local antioxidants (Fischer et al., 2006; Slominski et al., 2008). Quite recently, Slominski and

colleagues proposed that Mel synthesized in the skin, together with circulating Mel and metabolites such as AFMK, constitute a potent antioxidative defence system against the UV-induced solar damage to human skin (Slominski et al., 2014). Therefore, it is also justified to suppose that fish skin has its own system protecting it from oxidative stress. However, as far as the authors' are aware, no such studies on fish skin are currently available.

Although the presence of Mel and AFMK in flounder skin is evident, their origin remains uncertain. The following data strongly suggests that Mel can be produced in fish skin and higher levels of Mel in the skin than in plasma and muscles may indicate its local (cutaneous) synthesis. For instance, in the round goby (*Neogobius melanostomus*), Mel concentration in the skin is within the ranges 90–230 pg/g while in plasma and muscles it is only 50–75 pg/g and 45–90 pg/mL, respectively. In the common carp (*Cyprinus carpio*), Mel concentration in the skin is approximately 200 pg/g and 100 pg/g in the plasma (our unpublished data) and in the Atlantic cod (*Gadus morhua*), it is 120 pg/g in the skin and 90 pg/mL in the plasma (Kijewska et al., 2012). Secondly, a relative expression of aralkylamine *N*-acetyltransferase (AANAT; EC 2.3.1.87; enzyme in Mel biosynthesis) in the skin of the rainbow trout demonstrated by Fernández-Durán et al. (2007) suggests that Mel is produced in the skin. However, our study on the expression of genes encoding isozymes of AANAT in the three-spined stickleback (*Gasterosteus aculeatus*) shows that the level of *aanats* mRNA in the skin is very low and Mel is mostly derived from circulation (Kulczykowska et al., 2017). We have analysed only a constitutive expression of AANAT genes in non-stressed stickleback individuals, therefore, it cannot be excluded that AANAT genes expression in the skin would increase under stress conditions and this will be investigated in the future. Regardless of whether Mel and AFMK are derived from circulation and/or produced locally, both could participate in cutaneous antioxidative defence in fish, as has been postulated for mammals.

Taking into account that these important antioxidants, Mel and AFMK, are present in European flounder skin and the main stress hormone, cortisol, affects Mel and AFMK release in a dose-dependent manner, we postulate that cortisol and Mel/AFMK are functionally interconnected and establish the CSRS in fish. Nonetheless, the question remains whether cortisol, Mel, and AFMK are delivered by circulation or can also be produced in fish skin cells. There is no evidence to support the thesis that cortisol is synthesized in the fish skin as it is in mammals (Slominski and Wortsman, 2000; Vukelic et al., 2011); in teleost fish, cortisol is synthesized in the interrenal cells of the head kidney (Mommsen et al., 1999). In the skin of non-stressed fish, cortisol is measured in the nanogram range, for instance, it is around 25 ng/g in the European flounder and 10 ng/g in the round goby (our unpublished data) but further studies are necessary to answer the question if it is produced in the skin.

Our research opens a new perspective in the studies of defence/stress response systems in fish where cortisol together with Mel and AFMK are important components. The presence of the CSRS should, therefore, be taken into consideration during the elaboration of new indicators of fish welfare in aquaculture and in the wild.

Acknowledgments

This research was supported by National Science Centre grant UMO-2012/07/B/NZ9/02144. The authors report no conflict of interest.

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