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ROLE OF MATERNAL CORTISOL HORMONE ON GENE REGULATION OF ZEBRAFISH (*DANIO RERIO*, F. HAMILTON, 1822)

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Abstract

This study was conducted to investigate the role of cortisol, a steroid hormone on the gene expression during embryogenesis of zebrafish (*Danio rerio*). Gene expression analyses were performed using microarray hybridization. A total 80 genes were found to be up-regulated and 36 genes were down-regulated at 24 hrs post fertilization (hpf) when the fertilized eggs were treated with cortisol hormone. These findings suggest that substantial changes in the responsiveness to maternal cortisol may occur at early developmental phase.

Introduction

The physiological response of a female fish to stress could have considerable consequence in terms of gamete quality and progeny fitness. Egg cortisol contents have been reported in Mozambique tilapia (*Oreochromis mossambicus*)[1], chum salmon, (*Oncorhynchus keta)*[2] and other teleosts[3]. Specifically, the maternal transfer of cortisol could be modified by physiological processes induced by stress, suggesting that concentrations in eggs could be affected[4]. Cortisol concentrations in coho salmon (*Oncorhynchus kisutch*), eggs were significantly higher when the fish were stressed for 2 weeks prior to spawning[5]. McCormick[6] has also published that cortisol administered to ambon damselfish (*Pomacentrus ambionensis)*, resulted in elevated ovarian cortisol concentrations, similar to those of fish experiencing stress in the natural environment. Cortisol implantation into adult tilapia resulted in reduced oocytes size and lower circulating testosterone and 17β- estradiol concentrations[7].

On the other hand, no elevation in cortisol concentrations was shown in eggs of rainbow trout that were stressed up to 3 months prior to spawning. In fact, elevated levels of maternal cortisol at ovulation were not reflected in the concentrations in ovarian fluid or eggs [8]. Furthermore, embryo of early development has the biochemical machinery to metabolize steroid hormones, including cortisol[9], which suggests that regulation of maternally contributed factors occurs post-fertilization in the embryo. On the basis of these findings, the work was aimed at identifying the effect of cortisol hormone on the gene expression of zebrafish.

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Materials and Methods

Zebrafish (*Danio rerio*) was used as experimental animal in this study. Immediately after fertilization, fertilized eggs were pooled in cortisol treatment solution and control solution for 2 hrs for development and fixation at 24 hrs post fertilization (hpf). The concentration of cortisol treatment solution was 13 μM and the concentration of control solution was ml abs. ethanol/1 litre fish water. After 2 hrs of treatment, the cortisol treatment and control solution was poured out and eggs were gently washed five-fold in water to eliminate any trace of cortisol and ethanol. Finally the eggs were fixed in liquid nitrogen at 24 hpf to perform RNA extraction analyses.

To evaluate gene expression, total RNA was extracted from cortisol treated and control embryos using Trizol reagent, according to the manufacturer's instructions (Invitrogen, Milan, Italy). At the end of the procedure, the RNA pellet was dried under a chemical hood for 5 - 10 min and re-dissolved in 1 2 - 20 μl of RNase-free water. The RNA samples were stored at –80°C until future use.

Highly pure RNA is the key requirement for microarray hybridization, in which A_{260}/A_{230} must be higher than 2.2 and A_{260}/A_{280} should be higher than 2. Extracted RNA contained different types of contaminants like polysaccharides, carbohydrates, peptides and solvents (phenol, aromatic compounds). These contaminants were removed by LiCl precipitation.

After LiCl precipitation, the Agilent 2100 bio-analyzer and RNA LabChip were used to perform RNA quality assessment and quantification. The bio-analyzer software automatically generates the ratio of the 18S to 28S ribosomal subunits. This ratio plays an important role in determining the level of sample degradation in gel electrophoresis. The Agilent technologies have also introduced a new tool for RNA quality assessment through RNA Integrity Number (RIN).

Two-color Microarray-based gene expression analysis (Agilent Technologies, Santa Clara, CA) was performed to analyze the gene expression of embryos treated with cortisol at 24 hpf. The analysis was performed at CRIBI, Italy using Agilent Whole Zebrafish Genome Oligo Microarrays 4 × 44K slide.

The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files. The software determines feature intensities and ratios (including background subtraction and normalization), rejects outliers and calculates statistical confidences (p values). Subsequently, the genes were classified into functional groups using gene ontology and analyzed individually at the site "NCBI Gene"[10].

Results and Discussion

The quality of RNA was controlled by analysing RIN using Agilent 2100 bio-analyzer. RIN was assessed on the base of the presence or absence of degradation

products in the entire electrophoretic trace of the RNA sample. In this way, interpretation of an electropherogram was facilitated, comparison of samples was enabled and repeatability of experiments was ensured. The results of single analyzed sample were described as an electropherogram and a virtual image of an internal standard agarose gel. Excellent quality of RNA was assessed when the bands of 28S and 18S rRNAs were well separated and their correspondent peaks were in good evidence. RNA samples used in this experiment obtained RIN values 8.6 for control sample (Fig. 1A) and 8.4 for cortisol treated sample (Fig. 1 B). From this result it is clear that RNA quality was good and could be used for further analysis because RIN value greater than 8 is acceptable for microarray hybridization[11].

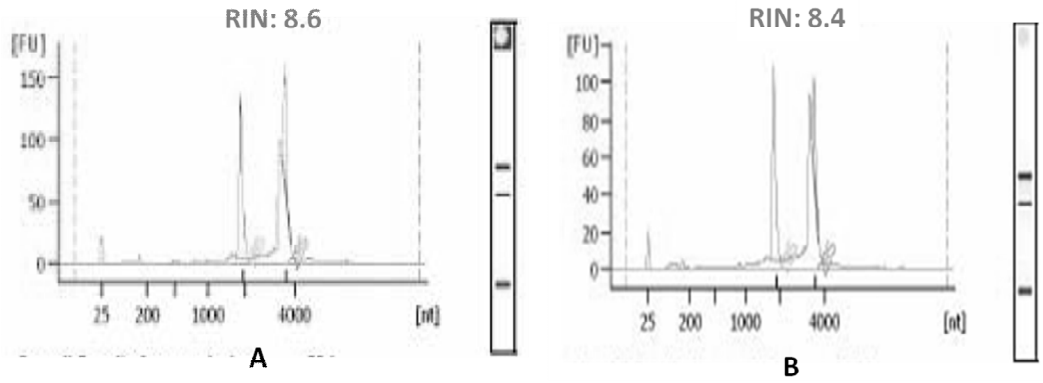


Fig. 1. Electropherogram and virtual gel of quality of RNA samples analyzed by Agilent 2100 Bioanalyzer. The two highest peaks in the electropherogram correspond to 18S and 28S rRNAs, from left. Axis y describes the fluorescence (FU), while axis x the nucleotide size (nt). 1A: Control and 1B: Cortisol treated samples.

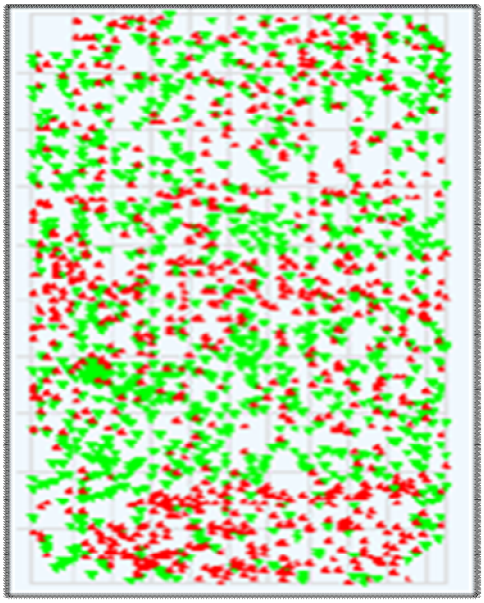
The microarray experiment was carried out according to the experimental design whereas the cRNA quantification and incorporation of Cy3 (green) and Cy5 (red) is shown in Table 1. Treated samples were labeled with Cy5 and the control with Cy3.

Table 1. Quantification of cRNA and incorporation rates of cyanine Cy3 (green) and Cy5 (red) for control and treatment samples, respectively.

Samples	Labeling	Concentration cRNA (ng/μl)	Volume (μl)	Dye (picomol/μl)	Incorporation rate (pmol/μg)
1.1 Control 24 hpf	Cy3	296	30	3.1	12.5
1.1 Cortisol-treated 24 hpf	Cy5	371	30	4.5	12

High throughput gene expression data from microarray experiments were collected by scanning the signal intensities of the corresponding spots on the array by dedicated fluorescence Agilent's DNA microarray scanner. The spatial distribution of significantly

up- and down-regulated features is shown in Fig. 2, where red spot indicates that the fluorescence intensity of the Cy5 signal is higher than that of Cy3, which means that the corresponding gene is over-expressed. Green spots indicate that the fluorescence intensity is higher in the control sample than treatment sample, which means that the corresponding gene is down-regulated.



Red: Up-regulated. Green: Down-regulated

Fig. 2. Spatial distribution of significantly up- and down-regulated features.

Image processing was performed using Agilent's Feature Extraction Software (FES) and the normalization of data was performed automatically. This software offers, among other features, the possibility to visualize the results of the data analysis in a log ratio versus log processed signal scatter plot (Fig. 3).

Finally, the up- and down-regulated genes due to cortisol treatment were filtered on the basis of their fold change. Only transcripts that were at least four-fold up- or down-regulated were selected. After this selection a total 80 genes were found to be up-regulated and only 36 genes were down-regulated. Specific gene description and functions were extracted from public databases "NCBI Gene"[10]. The annotations used were derived from Gene Ontology (GO), which provides information on molecular function, as well as from various pathway resources for information on involvement in biological signaling pathways.

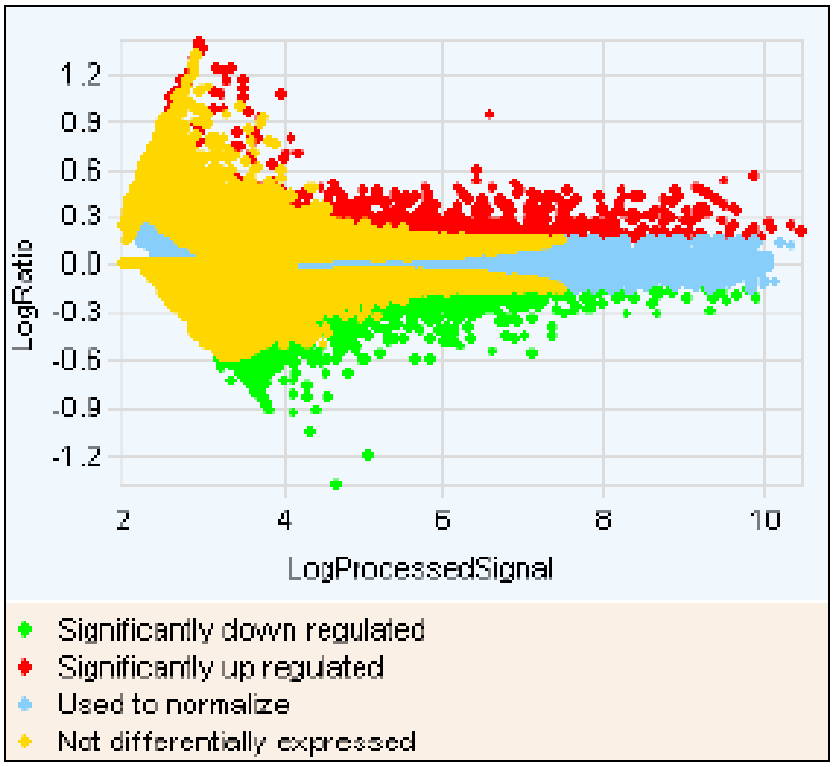


Fig. 3. Scatter plot of log ratio versus log processed signal intensities. Red spot: significantly up-regulated genes ($p < 0.05$). Green spot: significantly down-regulated genes ($p < 0.05$). Yellow spot: Not differentially expressed genes. Blue spot: Genes used to normalize.

The up- and down-regulated genes were grouped on the basis of their functions and annotations. The unknown and known function genes that found to be up- and down-regulated at 24 hpf are shown in Tables 2 - 4, respectively. The not annotated up- and down- regulated genes are as follows: up- regulated (wu:fe37g12, wu:fc16h11, LOC100002951, zgc:153953, zgc:153629, zgc:153345, zgc:113442, sc:d169, zgc:194392, wu:fd44e01, LOC100149690, zgc:110286, LOC798435, wu:fc45e01 and si:ch211-137c4.4); down- regulated (wu:fc06a09, zgc:66160, zgc:63694, zgc:63694, zgc:171673, si:ch211-13c6.2, wu:fc55e03, zgc:171445 and im:7138239). This result is an agreement with the results reported earlier [12] where 114 and 37 highly expressed transcripts were up- and down-regulated, respectively by knocked down of glucocorticoid receptor mRNA. In addition, intelectin, TLR-5M and TLR-5S mRNA transcripts were poorly and highly expressed at different embryonic stages when oocytes immersed 3 h prior to fertilization in cortisol-enriched ovarian fluid at either 100 ng/ ml or 1000 ng /ml[13].

Table 2. Unknown function up- and down-regulated genes at 24 (hpf) hour post fertilization. Descriptions of genes were extracted from public database "NCBI Gene"[10].

Up-regulated genes	Descriptions	Down-regulated genes	Descriptions
si:ch211-206k20.6	BEN domain-containing protein 3	zgc:110152	chromobox homolog 7a
LOC557191	guanylate cyclase soluble subunit beta-2-like	LOC568176	DCP1 decapping enzyme homolog B (S. cerevisiae)
LOC563738	Fetuin-B-like	ankrd10a	ankyrin repeat domain 10a
lsm8	LSM8 homolog, U6 small nuclear RNA associated	wu:fb11c07	Rho GTPase activating protein 28
zgc:103632	thioredoxin-like 4A	sumf2	sulfatase modifying factor 2
LOC557848	uncharacterized LOC557848	zp3	zona pellucida glycoprotein 3
numb	numb homolog (Drosophila)	lamb1	laminin, beta 1a
zgc:174160	p90 autoantigen	LOC100148642	phosphatidylinositol glycan anchor biosynthesis, class V
LOC100002858	zinc finger protein 850-like	rpp38	ribonuclease P/MRP 38 subunit
ttc39c	tetratricopeptide repeat domain 39C	wnt8-2	protein Wnt-8a ORF2
vasp	vasodilator-stimulated phosphoprotein		
LOC799071	aarF domain containing kinase 3		
LOC799923	FK506-binding protein 15		
si:ch211-222k6.3	SLAM family member		
LOC100148935	Usher syndrome type-1C protein-binding protein 1-like		
os9	amplified in osteosarcoma		
zgc:153456	NEDD4 binding protein 2		
jph1a	junctophilin 1a		
ENSDART00000114690	von Willebrand factor		
and3	actinodin3		
si:dkeyp-84f11.5	oxidation resistance protein 1		

Table 3. Up-regulated genes with functions at 24 (hpf) hour post fertilization. Description and functions of genes were extracted from public databases "NCBI Gene"[10]

Up-regulated genes	Descriptions	Functions
zgc:110816	UPF0364 protein C6orf211 homolog	
cog7	component of oligomeric golgi complex 7	
sncgb	synuclein, gamma b (breast cancer-specific protein 1)	
zgc:113491	PQ-loop repeat-containing protein 2-like	molecular_function
fam46c	family with sequence similarity 46, member C	
sec14l1	SEC14-like 1 (S. cerevisiae)	
ncoa6	nuclear receptor coactivator 6	transcription coactivator activity
ctsf	cathepsin F	cysteine-type peptidase activity
th	tyrosine 3-monooxygenase	iron ion binding, monooxygenase activity
plxnd1	plexin D1	Receptor activity
bcat1	branched chain aminotransferase 1, cytosolic	L-isoleucine transaminase activity
hsd3b1	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1	isomerase activity, nucleotide binding
ralgapa1	ral GTPase-activating protein subunit alpha-1	GTPase activator activity
zgc:153893	XIAP-associated factor 1	zinc ion binding
hoxa1a	homeo box A1a	DNA binding, sequence-specific DNA binding,
dcc	deleted in colorectal carcinoma	netrin receptor activity involved in chemoattraction
zgc:153452	CWC22 spliceosome-associated protein homolog (S. cerevisiae)	DNA , RNA binding
msxa	muscle segment homeobox A	DNA binding, sequence-specific DNA binding,
dsel	dermatan sulfate epimerase-like	sulfotransferase activity
zgc:154042	cytochrome P450, family 4, subfamily V, polypeptide 7	iron ion binding, metal ion binding
LOC555229	sodium-dependent neutral amino acid transporter B(0)AT3-like	

(Contd.)

(Contd.)

ehf	ets homologous factor	DNA binding, sequence-specific DNA binding,
ckba	creatine kinase, brain a	ATP binding, catalytic activity, kinase activity,
LOC558601	pipecolic acid oxidase	oxidoreductase activity
sh2b1	SH2B adaptor protein 1	phospholipid binding, signal transducer activity
gata2a	GATA-binding protein 2a	sequence-specific DNA binding
slc34a2aas	solute carrier family 34 (sodium phosphate), member 2a antisense	
fynb	tyrosine-protein kinase fynb, FYN oncogene related to SRC, FGR, YES b	ATP binding, kinase activity
zgc:77816	deoxyribonuclease gamma-like	deoxyribonuclease activity, endonuclease activity,
foxi2	forkhead box I2	DNA binding, bending,
ctbs	di-N-acetylchitobiase	catalytic activity, chitinase activity,
taf1b	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, subunit B	DNA binding,
ap1s1	adaptor-related protein complex 1, sigma 1 subunit	protein transporter activity
rsad1	radical S-adenosyl methionine domain containing 1	4 iron, 4 sulfur cluster binding, catalytic activity,
vps8	vacuolar protein sorting 8 homolog (S. cerevisiae)	metal ion binding
serac1	serine active site containing 1	hydrolase activity, acting on ester bonds
dguok	deoxyguanosine kinase	ATP binding, phosphotransferase activity, alcohol group as acceptor
drp2	dystrophin related protein 2	calcium, Zinc ion binding
dmrt1	doublesex and mab-3 related transcription factor 1	DNA binding, metal ion binding, sequence-specific DNA binding
chrna2b	cholinergic receptor, nicotinic, alpha polypeptide 2b (neuronal)	extracellular ligand-gated ion channel activity, ion channel activity
pfn2l	profilin 2 like	actin binding
stau1	staufen, RNA binding protein, homolog 1 (Drosophila)	RNA binding, double-stranded RNA binding
ube2r2	ubiquitin-conjugating enzyme E2R 2	acid-amino acid ligase activity
ust	uronyl-2-sulfotransferase	sulfotransferase activity, transferase activity

Table 4. Down-regulated genes with functions at 24 (hpf) hour post fertilization. Description and functions of genes were extracted from public databases "NCBI Gene"[10].

Down-regulated genes	Descriptions	Functions
ube2q2	ubiquitin-conjugating enzyme E2Q family member 2	acid-amino acid ligase activity
scyl3	SCY1-like 3 (S. cerevisiae), protein-associating with the carboxyl-terminal domain of ezrin	ATP binding, protein kinase activity,
zgc:173443	fish-egg lectin-like	molecular function
tead1a	TEA domain family member 1a	sequence-specific DNA binding transcription factor activity
wnt8a	wingless-type MMTV integration site family, member 8a	G-protein coupled receptor binding
zgc:154116	beta-1,4-galactosyltransferase 1	lactose synthase activity, transferase activity,
ppm1g	protein phosphatase 1G (formerly 2C), magnesium-dependent, gamma isoform	catalytic activity, hydrolase activity, metal ion binding,
zgc:162964	Golgi resident protein GCP60	fatty-acyl-CoA binding
rbbp6l	retinoblastoma binding protein 6	metal ion binding, nucleic acid binding, zinc ion binding
egln3	egl nine homolog 3	L-ascorbic acid binding, iron ion binding, oxidoreductase activity,
rem1	rad and gem related GTP binding protein 1	GTP binding, GTPase activity, nucleotide binding
zgc:153154	prostaglandin D2 synthase-like	small molecule binding, transporter activity
mespb	mesoderm posterior ba	protein dimerization activity, sequence-specific DNA binding transcription factor activity
zp2.3	zona pellucida glycoprotein 2.3	carbohydrate binding
zgc:152898	two pore segment channel 2	NAADP-sensitive calcium-release channel activity,
six7	sine oculis homeobox homolog 7	DNA binding, sequence-specific DNA binding transcription factor activity
cdx1a	caudal type homeo box transcription factor 1 a	DNA binding, sequence-specific DNA binding

In conclusion, the maternal glucocorticoid receptor transcript dosage should be regarded as the major integral sensor of the stressor load on the mother's life during oogenesis while the cortisol deposit would act as an ancillary sensor.

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