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INDIRIZZO: BIOLOGIA DELL'INTEGRAZIONE INTERCELLULARE
CICLO XXV

Effects of egg enrichment with glucocorticoid hormone, antagonist and receptor messenger in the modulation of gene expression in zebrafish (*Danio rerio*) embryos with transgenerational follow up till adulthood

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SUMMARY

This study is relevant to the recent field of investigation on the genetic programming of embryo development by maternal glucocorticoid and its receptor messenger with lasting influences on subsequent life stages. This research has been undertaken with an articulate experimental design supported by an ample repertoire of biomolecular techniques, ranging from whole-genome microarray to relative and absolute qPCRs of glucocorticoid-dependent up- and down-regulated genes. I have found that cortisol enrichment of newly fertilized eggs by immersion in a solution of the steroid (13:M) for 2 h brings about significant up-regulation of 100 genes with no down-regulation at 5 h post-fertilization (hpf), when only maternal cortisol is available to the embryo. At 12 hpf, 143 genes were up-regulated and 6 down-regulated with only 30 genes in common with those at 5 hpf, indicating that substantial changes in responsiveness to maternal cortisol may occur at early developmental phases. At 24 hpf, responsiveness to cortisol was enlarged to hundreds of genes. Subsequently, I analyzed by relative qPCR seven target genes during development, observing a significant decrement of expression from 10 to 24 hpf of both *vasp* and *plp1a* genes, when *ef1α* was used as a calibration reference. Moreover, four genes that were up-regulated at 5 hpf (*mat1a*, *notch2*, *parn* and *stmn2a*), were no longer so at 20 hpf.

The work has been extended to the analysis of cortisol-induced changes in the expression of two target genes, *igf-2a* and *casp8*. It was demonstrated that the expression of *igf-2a* was significantly enhanced by egg microinjection with the zebrafish glucocorticoid receptor mRNA (z-*gr* mRNA), and more so by z-*gr* mRNA plus cortisol at 5 hpf and, to a lesser extent, at 10 hpf. This is of interest since *igf-2a* is considered as a major growth factor during embryogenesis. A stimulatory response was obtained also with *casp8* at 5 hpf with both treatments, with a non-significant decrement at 10 hpf. Using absolute qPCR, I confirmed an expression enhancement of *casp8* transcription by z-*gr* mRNA w/wo cortisol at 5 hpf, which was depressed by both the glucocorticoid antagonist RU486 and the z-*gr* mRNA translation knockdown by morpholino. Surprisingly, at 10 hpf, the latter negative modulations were reversed. The complexity of glucocorticoid action on embryonic gene expression was highlighted also by absolute qPCR of another gene, *mcm6*, whose transcripts were significantly increased by both cortisol and RU486 when administered alone, but decreased when in combination with z-*gr* mRNA at both 5 and 10 hpf.

Further, I examined in details the effects of the above treatments on larval survival after hatching (3 days pf) and undertook a study on the influence of cortisol enrichment of fertilized eggs on fish growth till 180 days of age by comparing a lineage in which the treatment was repeated along four generations (F1-F4) with another lineage acting as an untreated control. Since both

lineages were homozygous for different colour patterns, they could be co-cultured, thus eliminating any differential environmental influence. This experiment was intended to determine whether exposure of early embryos to cortisol exerts a permanent imprinting on subsequent body growth and to elucidate the transgenerational pattern of this epigenetic modulation. Through statistical analysis, I established greater growth in cortisol-treated F1 and F2 from 120 to 180 dpf in terms of length and body weight. But, in F3 and F4, the difference in length disappeared, while that in body weight was somehow reversed. This seems to suggest that the priming effect was compensated rather than intensified along generations, as if a sort of adaptation has occurred.

RIASSUNTO

Questo studio è rilevante dal momento che recenti ricerche si sono concentrate sul ruolo svolto dai glucocorticoidi di origine materna e dai messaggeri codificanti per il loro recettore nella programmazione genetica dello sviluppo embrionale, con particolare attenzione alle fasi di vita successive. Tale ricerca è stata effettuata seguendo un articolato disegno sperimentale supportato dall'utilizzo di molteplici tecniche biomolecolari, quali, ad esempio, il microarray e la real time PCR sia relativa che assoluta. E'stato dimostrato che l'arricchimento delle uova di zebrafish appena fecondate con cortisolo, mediante immersione per 2 ore in una soluzione 13 :M dello steroide, provoca una significativa up-regolazione di 100 geni a 5 ore dopo la fecondazione (hpf), quando nell'embrione è presente solo il cortisolo di origine materna. Nessun gene è risultato invece down-regolato allo stesso stadio di sviluppo. A 12 hpf, 143 geni sono risultati up-regolati e 6 down-regolati, con solo 30 geni in comune con l'esperimento delle 5 hpf: questo indica che probabilmente nelle fasi precoci di sviluppo embrionale avvengono dei cambiamenti sostanziali nella risposta al cortisolo materno. A 24 hpf la risposta al cortisolo interessa centinaia di geni. Successivamente ho analizzato mediante real time PCR relativa sette geni bersaglio dei glucocorticoidi durante lo sviluppo, osservando una significativa diminuzione dell'espressione dalle 10 alle 24 hpf dei geni *vasp* e *plp1a*, con *ef1α* come "gene housekeeping". Inoltre ho dimostrato che quattro geni, precedentemente risultati up-regolati a 5 hpf (*mat1a*, *notch2*, *parn* e *stmn2a*), non risultano più sovraespressi a 20 hpf.

Successivamente ho esteso l'analisi anche ai cambiamenti indotti dal cortisolo sull'espressione dei geni target *igf-2a* e *casp8*. In particolare ho potuto dimostrare che l'espressione di *igf-2a* aumenta significativamente in seguito alla microiniezione delle uova con il messaggero codificante per il recettore dei glucocorticoidi di zebrafish (z-*gr* mRNA), e più ancora se alla microiniezione viene abbinato il trattamento con cortisolo. Questo è stato dimostrato a 5 hpf e, in misura minore, anche a 10 hpf. Questo dato è interessante dal momento che *igf-2a* viene considerato il principale fattore di crescita durante l'embriogenesi. Un aumento di espressione è stato ottenuto anche per il gene *casp8* con entrambi i trattamenti a 5 hpf, mentre a 10 hpf c'è una diminuzione di espressione non significativa. Mediante real time PCR assoluta ho confermato l'aumento di trascrizione del gene *casp8* a 5 hpf successivamente alla microiniezione con z-*gr* mRNA, indipendentemente dall'aggiunta di cortisolo; una diminuzione di espressione è stata invece ottenuta sia con l'utilizzo dell'antagonista dei glucocorticoidi RU486, sia dopo silenziamento del recettore tramite microiniezione con morfolino. Sorprendentemente, a 10 hpf, le modulazioni che erano risultate precedentemente negative hanno avuto un'inversione di tendenza.

La complessità dell'azione dei glucocorticoidi sull'espressione genica durante lo sviluppo embrionale è stata messa in evidenza anche dall'esperimento di real time PCR assoluta per il gene *mcm6*, i cui trascritti sono risultati notevolmente aumentati sia dopo trattamento con cortisolo che con RU486, quando questi reagenti sono stati utilizzati da soli; una diminuzione dei trascritti è stata invece osservata per entrambi i reagenti, sia a 5 hpf che a 10 hpf, quando sono stati impiegati contemporaneamente alla microiniezione con z-*gr* mRNA.

Ho poi esaminato in dettaglio gli effetti di tutti i trattamenti descritti precedentemente sulla sopravvivenza delle larve dopo la schiusa (3 giorni pf) ed ho condotto uno studio sull'influenza dell'arricchimento con cortisolo sulla crescita dello zebrafish, effettuando misurazioni ad intervalli di tempo definiti, fino ai 180 giorni pf. L'analisi è stata fatta comparando una linea in cui il trattamento con cortisolo è stato ripetuto per quattro generazioni successive (F1- F4) con un'altra utilizzata come controllo non trattato. Poichè entrambe le linee erano omozigoti per differenti pattern di colorazione si è potuto anche allevarle assieme, eliminando ogni possibile influenza ambientale. Lo scopo dell'esperimento era determinare se l'esposizione dell'embrione al cortisolo, a stadi di sviluppo precoci, esercitasse un'influenza permanente sulla crescita ed inoltre spiegare il pattern transgenerazionale di tale modulazione epigenetica. Attraverso analisi statistica ho dimostrato che c'è una maggiore crescita nei trattati con cortisolo delle generazioni F1 e F2 dai 120 ai 180 giorni pf in termini di lunghezza e peso corporeo. Nelle generazioni F3 e F4 scompaiono le differenze di lunghezza rispetto ai controlli, mentre il peso corporeo risulta addirittura diminuito. Questi risultati sembrano suggerire che l'effetto iniziale del trattamento viene compensato, e non intensificato, nel corso delle generazioni, come se avvenisse una sorta di adattamento.

INTRODUCTION

1. Zebrafish as a model animal

Zebrafish, *Danio rerio* are cyprinids native to South Asia, broadly distributed across parts of India, Bangladesh, Nepal, Pakistan and Myanmar and were first described by Hamilton (1822) in his survey of fishes of the Ganges River (Rahman, 1989; Barman, 1991; Talwar and Jhingran, 1991; Menon, 1999; Bhat, 2003). Due to monsoon weather, with dry and rainy seasons this area has profound effects on habitat parameters, including water chemistry and resource abundance. It belongs to the Phylum Chordata, Class Actinopterygii, Order Cypriformes, Family Cyprinidae, Genus Danio (Fig. 1). As early as the 1930s, the zebrafish was being used as a classical developmental and embryological model. Subsequently, it has been widely used as a vertebrate research organism primarily for developmental genetics, and increasingly for toxicological and environmental monitoring, cancer, aging, neurophysiology, and biomedicine (Kishi, 2004; Trede *et al*., 2004; Parng, 2005; Wright *et al*., 2006; Beckman, 2007; Scholz and Mayer, 2008; Sumanas and Lin, 2004).

Figure 1. Striped zebra (left) and spotted leopard (right) zebrafish, *Danio rerio*

On the basis of culture, reproduction and organogenesis, zebrafish are easy to keep and breed under laboratory conditions all the year round. The small size, high fecundity, transparent embryos and rapid development have made zebrafish highly favourable research models (Westerfield, 2007; Matthews *et al*., 2002). Adult fish spawn hundreds of eggs once every 5 days (Dahm and Geisler, 2006). Generation time is short, typically 3-4 months, making it suitable for transgenerational experiments. Moreover, fertilization is external, thus live embryos are accessible to manipulation and can be monitored through all developmental stages under a dissecting microscope. Development is rapid, with all major organs developing within 36 h at 28.5°C (Kimmel *et al*., 1995).

Importantly, the transparency of the embryo coupled with the detailed understanding of its developmental stages allows elegant embryonic manipulations to distinguish between effects due to the genetic asset *vs* influences of the surrounding environment (Spitsbergen and Kent, 2003).

The use of microinjection into the cytoplasm of one-cell-stage embryos to study gene function in the zebrafish has become widespread in recent years. This includes ectopic expression of genes by introducing DNA or RNA into embryos or injection of blocking molecules, such as RNAs encoding truncated proteins or antibodies, to perturb the function of endogenouse gene products (Holder and Qiling, 1999).

The zebrafish genome is sequenced and readily accessible on internet Ensembl (http://www. ensembl.org/ index.html), while molecular products such as high density microarray chips are available. A complete assembled sequence has already been published (http://www.ncbi. nlm.nih.gov/genome/guide/zebrafish/). A number of forward and reverse genetic methodologies are well established in zebrafish, including transgenesis, gene silencing (morpholino) and chemical-induced mutagenesis (ethylnitrosourea; ENU). Due to above advantages, zebrafish becomes one of the most important model animals today (Fig. 2).

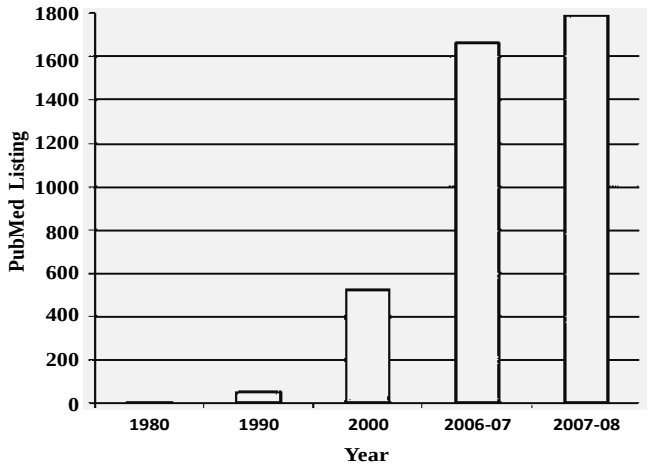


Figure 2. Zebrafish research reported in Pubmed references from the year 1980 to 2008. The term 'zebrafish' and the year like " 2007" were used for searching in Pubmed (Chakraborty *et al*., 2009)

Spotted leopard (Fig. 1), a well-known zebrafish mutant that has spotted skin pattern instead of stripes, is also a model for the study of pigment patterning (Watanabe *et al.*, 2006). Zebrafish connexion41.8 gene, Cx41.8 (Fig. 3) is responsible for pigment pattern on the body surface of leopard.

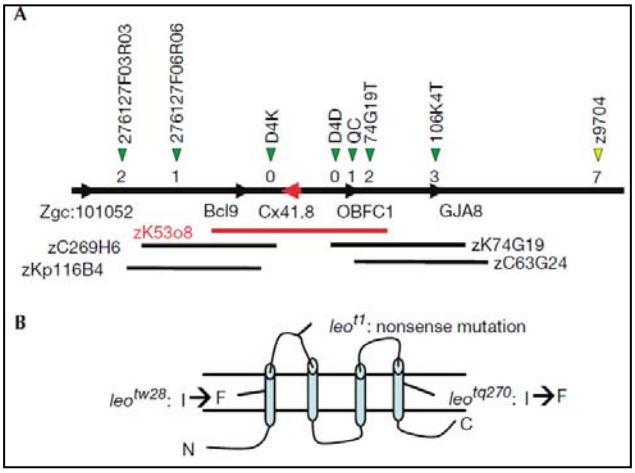


Figure 3. Zebrafish connexion41.8 as a leopard gene. (A) Map of the leopard region. The yellow arrowhead indicates the microsatellite marker z9704. The red bar indicates the bacterial artificial chromosome (BAC) clone zK53o8, which includes the critical region for leopard. The black bars show the BAC clones located around this region. Green arrowheads indicate single nucleotide polymorphism marker sites. Each site was named as shown above the arrowhead. The numbers below the green arrowheads refer to the number of recombination events (Watanabe *et al*., 2006; supplementary information online). This region includes two genes, bcl9 and Cx41.8. Cx41.8 is indicated by a red arrowhead. (B) Schematic of zebrafish connexin41.8 showing predicted structural motifs and mutations detected in leopard alleles, leo^{t1}, leo^{tw28} and leo^{tq270} (Figure and legend taken from Watanabe *et al*., 2006).

2. Zebrafish embryogenesis and organogenesis

Zebrafish develops very rapidly (embryogenesis takes only about 24 h and organogenesis is largely complete after day 5 of development), enabling the observation of defined aspects of development as well as the completion of experiments generally within a few hours to days (Dahm, 2002). Moreover, the transparency of the zebrafish's chorion and the translucency of embryos and early larval stages allow the easy visualization of internal processes, such as the formation and function of internal organs inside the living animal.

According to Kimmel *et al*. (1995), zebrafish has seven broad periods of embryogenesis, namely the **zygote, cleavage, blastula, gastrula, segmentation, pharyngula,** and **hatching periods**. These divisions highlight the changing spectrum of major developmental processes that occur during the first 3 days after fertilization. This work is focusing mainly on stages from blastula to gastrula, when most patterning and cell fate determination processes take place. Figures 4 and 5 show some stages of early embryonic development. **Zygote period (0-¾ h):** the newly fertilized

egg is in the zygote period until the first cleavage occurs (Fig. 4A), about 40 minutes after fertilization. The zygote is about 0.7 mm in diameter at the time of fertilization. The fertilized zygote contains all necessary information for its development in the zygotic genome and in maternally deposited mRNAs and proteins (Pelegri, 2003).

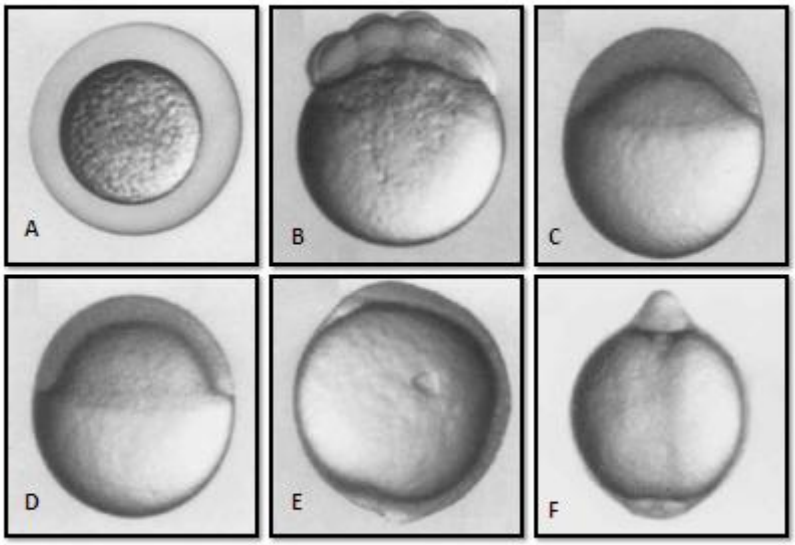


Figure 4. A: The zygote period, the zygote within its uplifted chorion, a few minutes after fertilization. B: cleavage period, 16-cell stage (1.5 h). C: blastula period, 30%-epiboly stage (4.7 h). D: gastrula period, 50%-epiboly stage (5.25 h). E: gastrula period, bud stage (10 h). F: segmentation period, 5-somite stage (1 1.7 h), ventral view. Images modified from Kimmel *et al*., 1995.

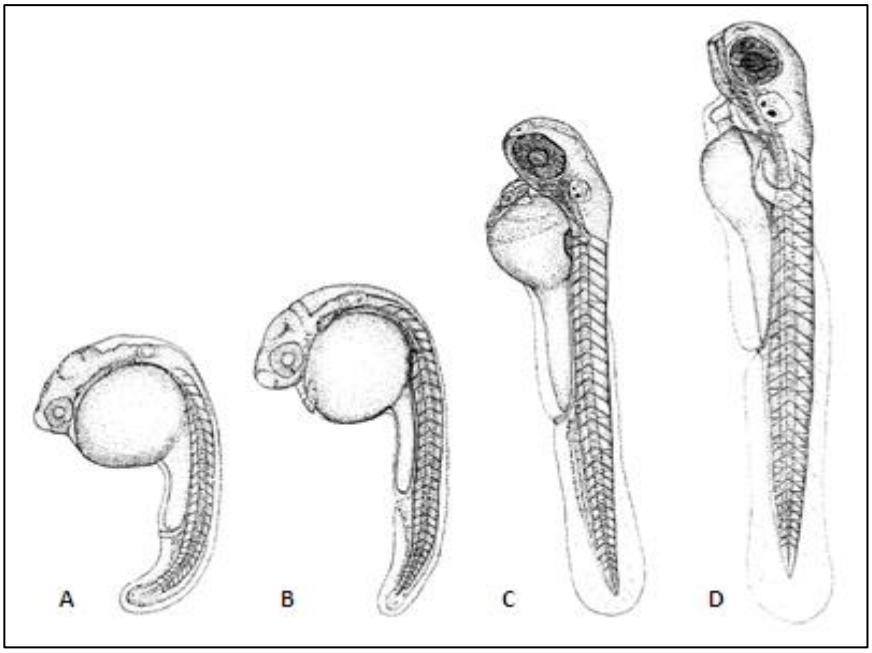


Figure 5. Developmental stages of zebrafish, A: segmentation period, 26-somatic stage (22 h). B: pharyngula period, prim-6 (25 h). C: hatching period, long pec (48 h). D: hatching period, protruding mouth (72 h). Images modified from Kimmel *et al*., 1995.

Cleavage stages (¾-2¼ h): the zygote starts to divide in a discoidal-meroblastic manner, leaving newly formed blastomeres interconnected by cytoplasmic bridges. Blastomeres are cleaved synchronously and radial-symmetrically. With the fourth cleavage (16-cell-stage), the central cells become completely divided from the others (Fig. 4B). In contrast, marginal blastomeres remain cytoplasmically connected to the yolk cell. Already during the early cleavage stages, the embryo undergoes its first subdivision into two cell lineages: (a) germ-line cells, which inherit unique maternal transcripts demarcating them as primordial germ cells (PGCs), and (b) somatic cells forming the large bulk of the embryo.

Blastula period (2¼-5¼ h): at this stage, important processes occur. The embryo enters midblastula transition (MBT), the yolk syncytial layer (YSL) forms, and epiboly begins. During the early blastula period, the cleavages are metasynchronous because mitoses do not all occur at quite the same time. The MBT starts during the tenth cell cycle (512- cell stage). As interphases lengthen, cells become motile, and RNA synthesis increases over background levels. The marginal tier of blastomeres in the early blastula lies against the yolk cell and remains cytoplasmically connected to it throughout cleavage. Beginning during cycle 10 (Kimmel and

Law, 1985), the marginal cells undergo a collapse, releasing their cytoplasm and nuclei together into the immediately adjoining cytoplasm of the yolk cell. Thus YSL arises and after forming YSL, the enveloping layer (EVL) cells that were in the second blastodisc tier now lie at the marginal position.

Epiboly starts in the late blastula (Solnica-Krezel and Driever, 1994). It consists in the thinning and spreading of both the YSL and the blastodisc over the yolk cell, as one might model by pulling a knitted cap over the head. When primary stages of this morphogenetic movement occurs, the blastodisc thins considerably, changing from a high-piled cell mound to a cup-shaped cell multilayer of nearly uniform thickness (Fig. 4C).

Epiboly also continues during the **gastrula period (5¼-10 h)**. The morphogenetic cell movements of involution, convergence, and extension occur, producing the primary germ layers and the embryonic axis. At 50%-epiboly, a thickened marginal region, termed the germ ring, appears nearly simultaneously all around the blastoderm rim. Convergence movements then, nearly as rapidly, produce a local accumulation of cells at one position along the germ ring, the so-called embryonic shield. During these time, epiboly arrests for sometimes, but after forming shield, epiboly continues; blastoderm margin continues to grow around the yolk cell to cover it completely (Fig. 4E).

When epiboly is completed at the end of gastrulation, the yolk is completely covered by the blastoderm, and the concerted cell movements have established the dorsal-ventral and anterior-posterior body axes. After gastrulation, the embryo is further patterned and elongated in the course of the segmentation period along its axes. In particular, the tail bud extends away from the yolk cell to produce the embryonic tail region. The gut tube forms in close opposition to the yolk surface and the notochord primordium separates from the adjacent somitic mesoderm, which is progressively subdivided into the segmentally arranged somites.

During the **segmentation period (10-24 h)**, morphogenetic change occurs in a amazing way, the somites develop, the rudiments of the primary organs become visible, the tail bud becomes more prominent and the embryo elongates (Figs. 4F and 5A). At the completion of the first day of development, the embryo has between 8,000 and 10,000 cells. With notable major exceptions, such as the neural crest-derived structures of the jaw and endodermally derived structures of the gut tube, the development of the major systems of the embryo is laid out. The embryos are touch-sensitive and their hearts will start beating.

During the first few hours of the **pharyngula period (24-48 h),** the embryo continues the rapid lengthening that started at 15 h, but then the rate of lengthening abruptly decreases (Fig. 5B).

The time of the change, at 31-32 h, correlates approximately with the end of the rapid morphogenetic straightening of the tail.

The embryo continues to grow at about the same rate as earlier during the **hatching period (48-72 h).** Now the morphogenesis of many of the organ is relatively finish and slows down considerably, with some remarkable exceptions including the gut and its associated organs (Figs. 5C and D). At 5 days post fertilization (dpf), larvae develop a swim-bladder and begin to swim and feed. Sexual maturity is reached within 3-4 months, and adults can live for 2.5 – 4 years.

3. Antisense techniques in developmental biology

Antisense technique was first developed over 25 years ago (Izant and Weintraub, 1984; Izant and Weintraub, 1985) to block translation, processing or stability of its endogenous mRNA complement . Exogenous RNA microinjection into oocytes of the frog *Xenopus laevis* showed successful inhibition of translation (Harland and Weintraub, 1985; Melton, 1985), as well as in inhibiting the translation of endogenous mRNA (Izant and Weintraub, 1985). However, in the zebrafish, antisense RNA has been shown to have extensive sequence-independent effects that hamper the use of this system in the research of specific gene functions during developmental stages (Oates *et al*., 2000). Usually, a gene-knockdown agent should be characterized by high sequence specificity and lack of off-target effects due to interactions with structures other than gene transcripts. Three major gene knockdown types are compared with respect to off-target effects and sequence specificities: 1) phosphorothioate-linked DNA (S-DNA); 2) short interfering RNA (siRNA); and, 3) Morpholino (Table 1).

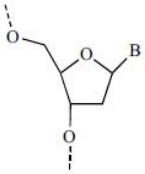
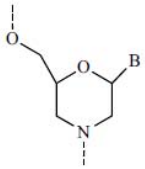
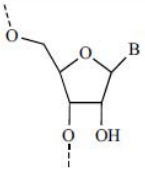
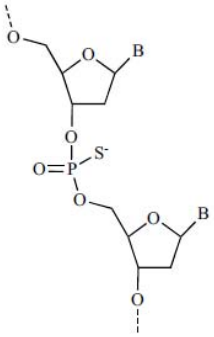
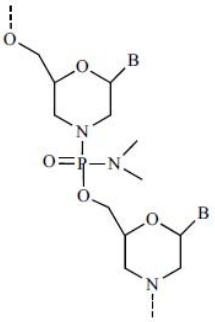
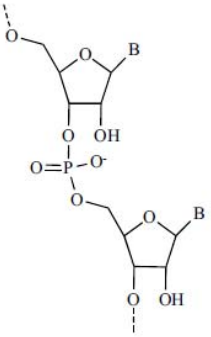
Class	RNase H-dependent	Steric block	Interfering RNA
Type	Phosphorothioate	Morpholino	Short Interfering RNA
Subunit structure B= A, C, G, T(U)	 deoxyribose ring	 morpholine ring	 ribose ring
Backbone structure	 S-DNA	 Morpholino	 siRNA
Principle sites of action	nucleus	nucleus and cytosol	cytosol

Table 1. Structure types of gene knockdown agents (Summerton, 2007).

Phosphorothioates (Table 1) are nearly iso-structural with DNA, differing only in the replacement of an oxygen atom by a sulphur atom in the phosphate linkages of the backbone (Steck *et al*., 1984). The principal advantage is that S-DNAs are degraded less quickly by nucleases (half-lives extended to multiple hours in biological systems as compared to few minutes for bare DNA oligos). On the other hand, the sulfur on the backbone phosphates significantly decreases the S-DNA's affinity for its RNA target sequence (Summerton, 2004; Summerton and Weller, 1997).

Short interfering RNA (Table 1) is a small RNA duplex about 20 to 25 base-pairs, which is processed within cells, where one of the RNA strands is disposed off while the other RNA strand is combined with cellular proteins to form the RNA-induced silencing complex (RISC) structure (Dorsett and Tuschl, 2004; Scherer and Rossi, 2004). Primarily, the location of RISC structure is in the cytosol, then efficiently it blocks partially-complementary mRNA sequences and cuts highly-complementary mRNA sequences. An alternative approach to RNA interference is the antisense Morpholino (MO) technology that has proven to be a powerful tool to knockdown specific targets in zebrafish. RNAi and MO are emerging as the most effective tools in bringing about functional silencing of genes. Both of these molecules are complementary oligonucleotides

that interfere with mRNA translation. This reduces protein levels and functionally silences the gene.

MOs are synthetic derivative of DNA and made of about 25 subunits chains which are alike to DNA and RNA oligonucleotides, but MOs have a morpholine ring rather than a ribose ring (Table 1). This structure still proves MOs to allow Watson-Crick base pairing, but it offers important benefits over conventional oligonucleotides (Corey and Abrams, 2001; Heasman, 2002). MOs do not act through an RNaseH mechanism and can be designed to inhibit translation (Summerton, 1999) (Fig. 6).

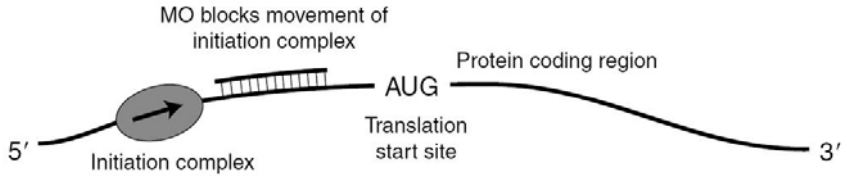


Figure 6. Translation-blocking MOs. It is targeted to sequence 5′ of the translation start site and block progression of the initiation complex (Eisen and Smith, 2008).

4. Morpholino-based screening in zebrafish

MO-based screening can produce knockdown phenotypes in wild-type embryos, significantly reducing infrastructural needs as compared to a classical mutagenesis screening due to the requirement of housing and breeding of many normal fish to produce the subset of mutant animals for analysis.

MO oligonucleotides have recently emerged as a successful and important tool for gene-specific antisence knockdown during zebrafish development owing to its rapid embryogenesis, transparency and the ease of delivery of MOs. The use of MOs in zebrafish has shown these compounds to be sequence specific and extremely potent as knockdown agents in all cells during the first 50 hours post-fertilization (hpf) (Nasevicius and Ekker, 2000).

5. Hypothalamus-pituitary-interrenal (HPI) axis and stress response in fish

Hormones are chemicals released by one or more cells that affect cells in other parts of the organisms. Only a small amount of hormone is required to alter cell metabolism. It is essentially a chemical messenger that transports a signal from one cell to another. Hormones bind to their cognate receptor proteins, resulting in the activation of a signal transduction mechanism that ultimately leads to cell type-specific responses.

The stimulation of the hypothalamus-pituitary-interrenal (HPI) axis in response to stressor exposure has been extensively reviewed in teleosts (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton *et al*., 2002; Iwama *et al*., 2006). As in other vertebrates, corticotropin-releasing factor (CRF) is the initial hormone in a signaling cascade that functions to regulate circulating glucocorticoids during stress. In fish, CRF produced in the preoptic area of the brain, stimulates the release of corticotropin (adrenocorticotropic hormone; ACTH) from the pituitary corticotrophs (Wendelaar Bonga, 1997; Fig. 7A). CRF and its related peptides, urocortin (tetrapods) and urotensin I (fish), signal via specific G-protein coupled receptors (CRF-R1 and CRF-R2) and are further regulated by a shared binding protein (CRF-BP). Together, these components are referred to as the CRF system and, in addition to their key role in the regulation of the ACTH response, they display a variety of functions ranging from food intake inhibition (Bermier, 2006) to behavioral modulation (Koob and Heinrichs, 1999; Lowry and Moore, 2006). In turn, ACTH binds to melanocortin type 2 receptors (MC2R, also called the ACTH receptor) on head kidney interrenal cells to stimulate the production and release of glucocorticoids (Aluru and Vijayan, 2008; Fig. 7B). This pathway, known as the hypothalamus-pituitary-interrenal (HPI) axis (Figs. 7A and B) is homologous to the HP-adrenal (HPA) axis of tetrapods (Flik *et al*., 2006).

In teleosts, as in humans, cortisol is the major glucocorticoid secreted by the interrenal cells (analogous to the adrenal cortex in tetrapods), whereas corticosterone, the only glucocorticoid in rodents, plays a minor role. These hormones are synthesized from either circulating cholesterol in association with low-density lipoproteins or from cholesterol synthesized *de novo* from acetate in these cells (Fig. 8) (Boron and Boulpaep, 2006).

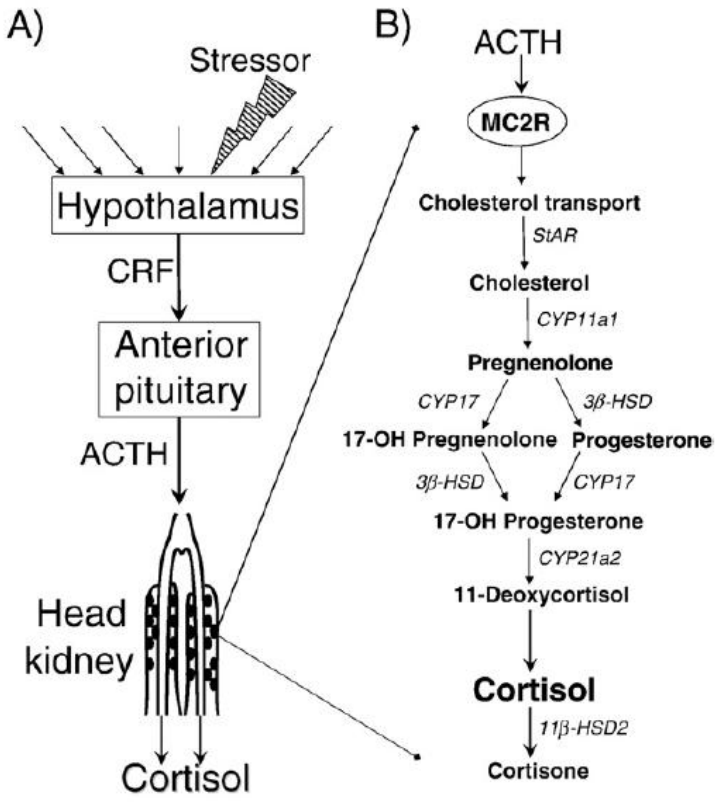


Figure 7. A) The image displays an overview of the anatomy of hypothalamus–pituitary–interrenal (HPI) axis and signaling cascade. The release of corticotropic releasing factor (CRF) in response to stressor stimulates the secretion of adrenocorticotropic hormone (ACTH). This ACTH binds to the ACTH receptor, (melanocortin 2 receptor; MC2R) and stimulates the production and secretion of cortisol into the circulation. B) The pathway of cortisol synthesis. An oxidative reaction leads to the synthesis of cortisone (Alsop and Vijayan, 2009).

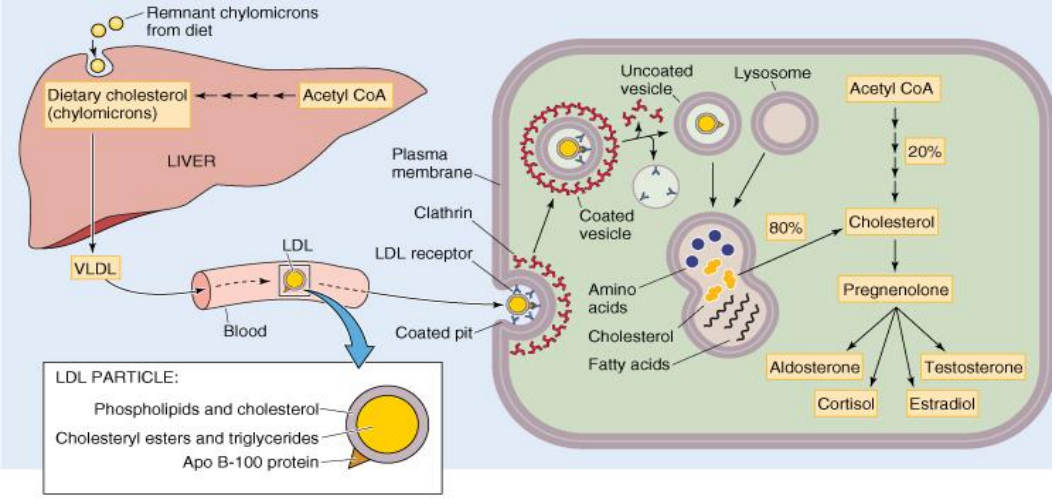


Figure 8. Cholesterol uptake and steroid hormone synthesis. Cholesterol is the initiator of steroid hormone synthesis. About 80% is from LDL particles *via* receptor-mediated endocytosis. The cell synthesizes the remaining cholesterol *de novo* from acetyl coenzyme A (Acetyl CoA). LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein (Boron and Boulpaep, 2006).

6. Cortisol actions

In fish, as in other vertebrates, the cellular responses associated with cortisol signalling are thought to be mediated by a glucocorticoid receptor (GR). Figure 9 displays the cortisol-GR-mediated molecular changes in the gluconeogenic and protein catabolic pathways in trout hepatocytes (Aluru and Vijayan, 2007). Cortisol is also known to play an important role in modulating a vast array of physiological processes, including organ development, protein and fat metabolism, carbohydrate homeostasis, immune response, neural activity, memory and behaviour and bone formation in both unstressed and stressed animals (Mommsen *et al*., 1999; Barnes, 2006; De Kloet *et al*., 2005 ; Wang, 2005; Migliaccio *et al*., 2007). Since cortisol exerts different effects on the various organs of the body, it is likely that the functional targets of GR are different in each tissue and these tissue specific mechanisms needs to be elucidated in order to understand the functional genomics of the stress response (Phuc Le *et al*., 2005; Vegiopoulos and Herzig, 2007).

Mammalian studies using microarrays and bioinformatics tools have identified GR is responsible for gene regulatory networks in the liver of mice treated with dexamethasone. Some genes are directly linked to GR signaling, while other genes are regulated indirectly by interaction of GR with other transcription factors, including estrogen receptor (ER) and CCAAT/enhancer binding

protein beta (C/EBPb) (Aluru and Vijayan, 2007). Due to their various actions, glucocorticoids is now become an attractive for therapy. As a class of compounds including synthetic analogues, they are among the most prescribed drugs in the world. Clinically, because of their immunosuppressive and anti-inflammatory activity, glucocorticoids are widely prescribed in the treatment of chronic autoimmune/inflammatory and allergic diseases, such as asthma, inflammatory bowel disease, rheumatoid arthritis, and skin disorders (Schaff *et al*., 2009).

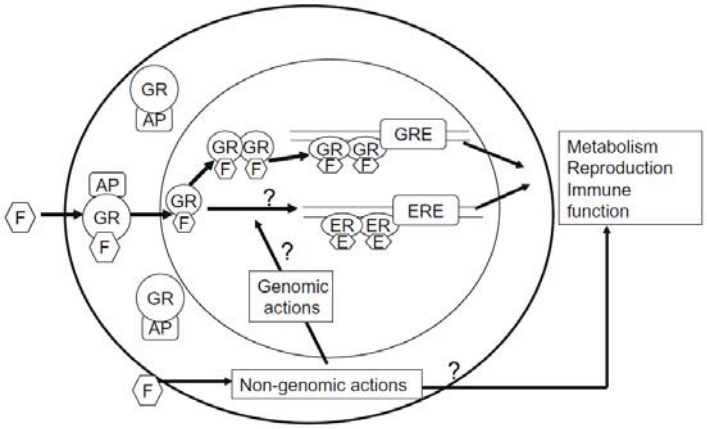


Figure 9. A role for cortisol signaling in stress transcriptomics. Glucocorticoid receptor exists as a complex with accessory proteins (AP), including heat shock protein 90, which gives stability and ligand binding conformation to the receptor. Cortisol (F) binds to GR and the GR-ligand heterocomplex translocates to the nucleus where it forms a homodimer and binds to the glucocorticoid-response element (GRE) on the promoter of glucocorticoid-responsive genes, modulating either transactivation or transrepression of genes encoding proteins involved in metabolism, reproduction and immune function. Also, ligand-bound GR may modulate transactivation and/or repression by other transcription factors, including ER. Moreover, another mechanism of action of cortisol may involve signaling by binding to membrane proteins and the associated non-genomic and genomic signalling. However, all these additional mechanisms of action are poorly understood in fish (Figure and legend taken from Aluru and Vijayan, 2009).

7. Zebrafish as a model for stress research

Now zebrafish has become a useful model in the study of the corticosteroid stress axis on the basis of both developmental and genetic traits. The Tuebingen 2000 large-scale ethylnitrosourea (ENU) screen has produced zebrafish mutants have been described that lack pituitary corticotropic cells and hence do not capable to synthesize ACTH (Herzog *et al*., 2004). So there

is opportunity to study the effects of ACTH in the stress response and in the ability of a fish to cope with a stressor.

While the teleost-related genome duplication event that occurred in the taxon 350 million years ago is very interesting from the fish evolutionary and comparative points of view, duplicate genes can create problems in some technical points of view. For example, morpholino injections in gene silencing studies, have to contend with knocking down two genes and also face the complication of functional partition between duplicate genes. But corticoid research in zebrafish can avoid this problem; zebrafish is the only fish currently known to possess a single gene for all three of the HPI components: CRF, (Chandrasekar *et al*., 2007), ACTH (POMC genes are duplicated, but only one can synthesize ACTH; De Souza *et al*., 2005) and GR (Alsop and Vijayan, 2008, Schaaf *et al*., 2008). This is also interesting in comparative study with species, such as the common carp (*Cyprinus carpio*), that possesses two genes for CRF (Huising *et al*., 2004), POMC (Arends *et al*., 1998) and GR (Stolte *et al*., 2008).

Hence, the ease of genetic manipulation, optically clear embryos and larvae for microscopic imaging study especially in combination with fluorescent labelling of specific cells and proteins, along with the single gene systems for several key components of the HPI axis, makes zebrafish a powerful animal model for studies pertaining to the development of the stress axis and corticosteroid function in vertebrates.

8. Glucocorticoids and the glucocorticoid receptor in zebrafish research

At the cellular level, the actions of glucocorticoids are mediated by intracellular GR, which functions as a hormone-activated transcription factor that regulates the expression of glucocorticoid-responsive genes. This receptor is a member of the family of steroid receptors, which in turn belongs to the superfamily of nuclear receptors (Zhang *et al*., 2004). Like all nuclear receptors, the GR acts as a ligand-activated transcription factor, and it is well conserved among vertebrate animal species (Bridgham *et al*., 2006). It consists of a large N-terminal domain, involved in transcriptional activation, a small DNA binding domain which contains two zinc-fingers and a C-terminal ligand-binding domain (Giguere *et al*., 1986). In the absence of hormone, the GR resides in the cytoplasm, where it forms a complex with heat shock proteins and immunophilins (Pratt and Toft, 2003).

Most teleostean fish species contain two glucocorticoid receptor genes, called GR1 and GR2 (Stolte *et al*., 2006). These isoforms have been recognised for rainbow trout (Bury *et al*., 2003), burton's mouthbrooder (Greenwood *et al*., 2003), green spotted puffer, fugu (Stolte *et al*., 2006),

common carp (Stolte *et al*., 2008), and sea bass (Terova *et al*., 2005; Vizzini *et al*., 2007). But in some fish species, like the Japanese flounder and brown trout (Stolte *et al*., 2006) contains one GR gene has been found thus far, still it is unclear if they contain a second GR gene, since most of these fish species are poorly studied.

The structure of fish GR1 and GR2 genes is highly similar to the organization of the human GR gene (Stolte *et al*., 2006). At the protein level, fish GRs display a high level of similarity to the human GR as well. In the ligand-binding domain, between 85% and 95% of the amino acids of fish GRs are similar to those in the human GR and in the DNA binding domain this number exceeds 98% for most fish GRs studied (Fig. 10).

Figure 10. The human and zebrafish GR α-isoforms. GR possesses the four functional domains common to nuclear hormone receptors: a large N-terminal domain, involved in transcriptional activation, a DNA binding domain (DBD) which contains two zinc-fingers and a ligand-binding domain (LBD). Percentages indicate the fraction of amino acids similar between human and zebrafish per domain. The overall level of similarity is 59.3% (Schaaf *et al*., 2009).

As said, the zebrafish genome only contains one GR gene (Stolte *et al*., 2006; Schaaf *et al*., 2008; Alsop and Vijayan, 2008). The analysis of the syntenic regions of the fish GR genes shows that the genomic region surrounding the zebrafish GR gene is well conserved and is highly similar to the region surrounding the GR2 gene of fugu, green spotted puffer, medaka and stickleback in a phylogenetic tree (Fig. 11).

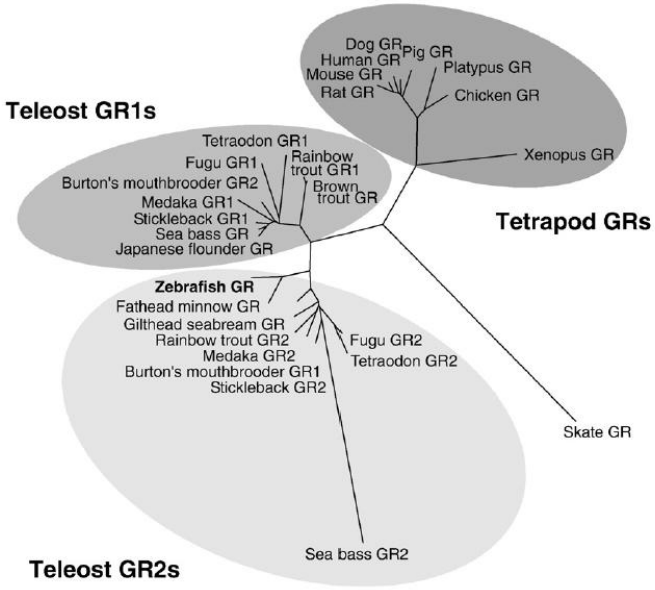


Figure 11. Phylogenetic tree of the teleost fish (GR1 and GR2) and tetrapod GRs. The zebrafish GR clusters within the GR2 clade of teleostean GRs (Schaaf *et al.*, 2009).

Thus, zebrafish could be a important research tool for GR research by using techniques for transient or stable genetic manipulation in combination with imaging-based phenotypic readouts to advance our knowledge on the molecular mechanisms underlying the effects of GR activation *in vivo*. Its potential could be exploited in studies towards the discovery of novel drugs and drug targets (Zon and Peterson, 2005; Mathew *et al.*, 2007). Because of its small size and suitability for imaging studies, the zebrafish could be an ideal tool for the screening of novel glucocorticoid drugs. In addition, using forward genetic screens for glucocorticoid responsiveness as a readout, novel drug targets may be discovered that may increase the effectiveness of glucocorticoid treatment.

9. Impact of stress on fish reproduction

Jalabert (2008) observed that the many aspects of fish physiology are potentially vulnerable to the effects of stressors as fish mature and reproduce, mainly due to the redirection of energy resources associated with the stress response (Schreck and Li, 1991). One important aspect is that a fish has to decide whether or not to reproduce under stressful conditions. When a fish reproduces, it should maintain fecundity with good quality eggs. Fish adopt different tactics to cope with stress during their reproductive life histories. The comparative results about the effects

of stress on the reproduction of tilapia (multiple spawning-throughout the year) and rainbow trout (single spawning per year, at most) are shown in Table 2.

Reproduction performance varies, when a fish is exposed to a stressor during the maturation period. For example, when tilapia is exposed to a moderate stressor during early vitellogenesis, it displays delayed spawning whereas, in mildly stressed rainbow trout, there was no effect on spawning time but great variation on egg size (Contreras-Sanchez, unpublished data; Contreras-Sanchez *et al*., 1998). Interestingly, spawning occurred 2 weeks earlier when trout were mildly stressed during the entire vitellogenic period.

On the other hand, severe stress may greatly affect folliculogenesis and ovulation. Trout under severe stress during the entire vitellogenesis showed smaller eggs and 3 weeks delayed spawning (Campbell *et al*., 1992). In the female striped trumpeter, *Latris lineate*, under frequent handling, more than half of the oocytes failed to complete vitellogenesis, but laid eggs were greater than in non-stressed fish (Morehead *et al*., 2000). Alternatively, fish may produce larger numbers of gametes to compensate for their poor quality (Gowaty *et al*., 2007).

	Tilapia, moderate stress (Contreras-Sanchez, unpublished data)	**Rainbow trout, mild stress (Contreras-Sanchez *et al*., 1998)**	**Rainbow trout severe stress**
Stress during early vitellogenesis	Lower growth rate No spawning	Slight effect on growth rate No effect on spawning No effect on absolute fecundity Smaller eggs Large variation on egg size No effect on progeny survival	
Stress during late vitellogenesis	Fast spawning	No effect on growth rate Early spawning (weeks earlier) No effect on absolute fecundity No effect on egg size Large variation on egg weight No effect on progeny survival	No effect on size (Campbell *et al*., 1994) No effect on absolute fecundity Smaller eggs Lower progeny survival
Stress during entire Vitellogenesic Period	n.a.	Slight effect on growth rate Early spawning (2 weeks earlier) No effect on absolute fecundity No effect on egg size Large variation on egg weight No effect on progeny survival	No effect on size (Campbell *et al*., 1992). Delayed spawning (~3 weeks) No effect on absolute fecundity Smaller eggs Lower progeny survival

Table 2. The effects of stress at various times on reproductive traits of two species with differing reproductive strategies (Schreck *et al*., 2001).

Moreover, chronic stress could affect offspring viability due to restricted energy stores in the eggs for development or mechanical damages caused by specific stressors (Campbell *et al*.,

1992, 1994). Hatchlings from smaller eggs produced by mildly stressed trout were smaller, but exhibited no abnormal mortality (Contreras-Sanchez *et al*., 1998). Interestingly, McCormick (1998) found that female ambon damselfish stressed by the presence of a predator produced eggs with higher amounts of cortisol. So, the quality of progeny in fish is affected by the stressful situations and behavioural interactions (Schreck *et al*., 2001).

Social factors can affect fish reproduction through interactions with the endocrine stress response. When Siamese fighting fish, *Betta splendens*, and African cichlid, *Haplochromis burtoni*, were stressed by threatening social encounters, their testicular steroidogenic capacity was inhibited (Leitz , 1987). It was reported that the size of GnRH neurons in the hypothalamo-preoptic area and testis size were inversely related to social condition (Fox *et al*., 1997).

Brown trout, *Salmo trutta*, stressed by crowding had elevated plasma ACTH and cortisol and decreased circulating testosterone and 11-keto-testosterone. This finding suggested that stress can adversely affect reproduction in fishes (Pickering *et al*., 1987). Subsequent studies confirmed that stress does indeed have a negative impact on reproductive fitness, as outlined above (Campbell *et al*., 1992, 1994; Contreras-Sanchez *et al*., 1998; Schreck *et al*., 2001). Castranova *et al.* (2005) demonstrated that striped bass, *Morone saxatilis*, despite genetically determined low cortisol responses to stressors, still manifested lower androgen levels and lower spermiation response to gonadotropin treatment when subjected to stressors. Stressful handling of mature jundia, *Rhamdia quelen*, resulted in lowered 17β-estradiol; fewer oocytes could be stripped from the stressed fish and their quality appeared reduced (Soso *et al*., 2008).

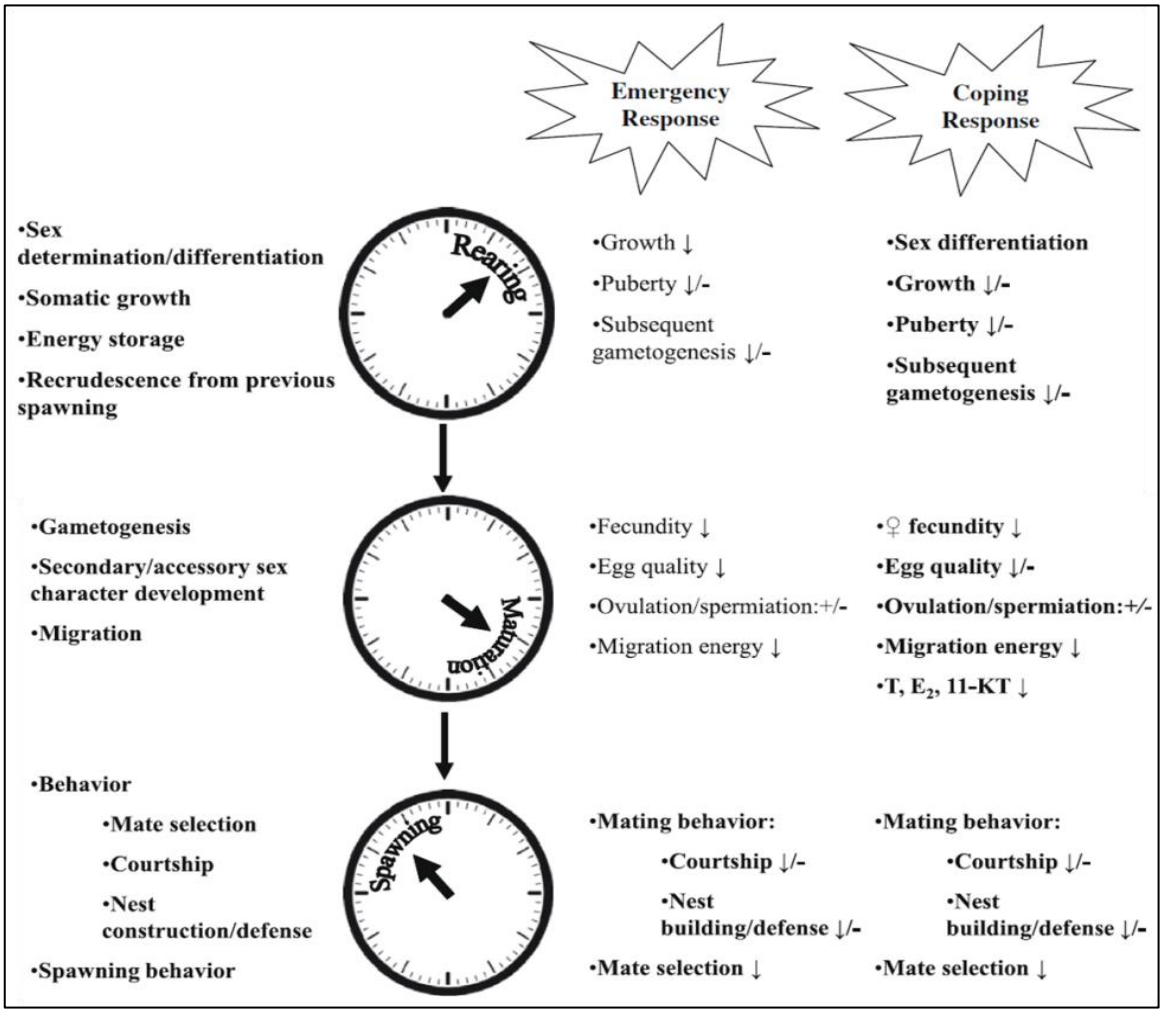


Figure 12. The main phases of the reproductive clock and the key reproductive events that happen during each phase (shown to the left of each respective clock). The organism-level response to stressors causing emergency responses and coping responses as part of allostatic load are shown to the right of the clocks. Responses in bold lettering are based on solid information; responses not in bold lettering are based on strong inference. A down arrow indicates a decrease. A minus sign (-) indicates inhibition (Figure and legend adapted from Schreck, 2010).

10. Impact of perinatal stress on the cortisol

The influence of perinatal stress on the cortisol response of young and adult subjects has been extensively explored in mammals. It has been observed that depending on the development stage at which the young are exposed to stress, the opposite regulation of the HPA axis activity operates in the adults. Indeed, when an expectant rats under prenatal stress, a prolonged post-stress corticosterone secretion is induced in the progeny once they are adults (Vallée *et al*., 1997), whereas early postnatal handling of the offspring induces a decrease in adult

corticosterone secretion in response to stress (Vallée *et al*., 1997). In the case of prenatal stress, the observed effect is suggested to be triggered, at least in part, by direct embryo exposure to corticosteroids. Corticosterone in the stressed mother causes a down-regulation of fetal glucocorticoid and mineralocorticoid receptors and impairs the feedback regulation of the HPA axis in infancy and adulthood (Weinstock, 2005).

11. Maternal hormones in fish oocytes

The yolk of mature teleost oocytes contains large amount of liposoluble hormones such as steroid, thyroid and retinoid hormones, as found in other oviparous vertebrates. These maternal hormones plays important role in regulating developmental processes of post-fertilization (Lam, 1985; Brown *et al*., 1989; Brown and Bern, 1989; Feist *et al*., 1990; Schreck *et al*., 1991; Ayson and Lam, 1993; Yeoh *et al*., 1996a,b; McCormick, 1999; Irie and Seki, 2002), are taken up from the maternal circulation or the follicular envelope. Studies on several fish species have shown that maternal steroid hormones may be involved in the early development of the offspring. For example, sex steroid hormones were found in the eggs of coho salmon (*Oncorhynchus kisutch*) (Feist *et al*., 1990) and testosterone in medaka (*Oryzias latipes*) (Iwamatsu *et al*., 2006). So, the maternal transfer of sex steroids, to the yolk constitutes a potentially significant source of maternal impact over embryogenesis and adult phenotype in oviparous fish.

The physiological response of a female fish to stress could have considerable consequence in terms of gamete quality and progeny fitness. Egg Cortisol contents were reported in Mozambique tilapia (*Oreochromis mossambicus*) (Shiraishi *et al*., 1999), chum salmon, *Oncorhynchus keta*. (De Jesus and Hirano, 1992) and other teleosts (Hwang *et al*., 1992). Specifically, the maternal transfer of cortisol could be modified by physiological processes induced by stress, suggesting that concentrations in eggs could be affected (Campbell *et al*., 1992, 1994; Yeoh, 1993). Cortisol concentrations in coho salmon, *O. kisutch*, eggs were significantly higher when the fish were stressed for 2 weeks prior to spawning (Stratholt *et al*., 1997). McCormick (1998) 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, *O. mossambicus*, resulted in reduced oocytes size and lower circulating testosterone and 17β-estradiol concentrations (Foo and Lam, 1993). 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 (Contreras-Sanchez, 1995). Furthermore, embryo of early development has the biochemical machinery to metabolize steroid hormones, including cortisol (Yeoh *et al*., 1996a,b), which suggests that regulation of maternally contributed factors occurs post-fertilization in the embryo.

But in mammals, it is not clear whether exposure of fish to cortisol variations during the early stages of development can influence the stress sensitivity later in life. Basal endogenous cortisol changes during development have been studied in several species: *Paralichthys olivaceus* (De Jesus *et al*., 1991), *Oncorhynchus keta* (De Jesus and Hirano, 1992), *Oncorhynchus mykiss* (Pottinger and Mosuwe, 1994; Barry *et al*., 1995a,b), *Dicentrarchus labrax* (Sampath- Kumar *et al*., 1995), *Cyprinus carpio* (Flick *et al*., 2002), and *Gadus morhua* (King and Berlinsky, 2006). During embryonic development, egg cortisol decreased, and endogenous cortisol production began around hatching, as shown from the increase in whole-body cortisol concentration. The development of a mature hypothalamic– pituitary–interregnal (HPI) axis able to generate cortisol in response to an external stressor occurred in the first weeks after hatching **(**Auperin and Geslin, 2008**);** cortisol production as an instant response to stress was observed 2 weeks after hatching in turbot and rainbow trout (Stephens *et al*., 1997; Barry *et al*., 1995a,b; Pottinger and Mosuwe, 1994), whereas it was observed only one week after hatching in yellowperch (Jentoft *et al*., 2002).

Alsop and Vijayan (2008) demonstrated that cortisol does not increase after a stressor exposure at 25, 49 or 73 hpf (Fig. 13). A cortisol stress response was first detected at 97 hpf, 2 days after hatching (Fig. 13). The absence of a stressor-mediated response may not be due to the lack of MC2R, as this receptor transcripts were up-regulated between 25 and 49 hpf (To *et al*., 2007; Alsop and Vijayan, 2008). Similarly, although basal cortisol levels were increasing in rainbow trout larvae at hatch (4 weeks post fertilization), a cortisol stress response was not seen until 2 weeks post-hatch (Barry *et al*., 1995a).

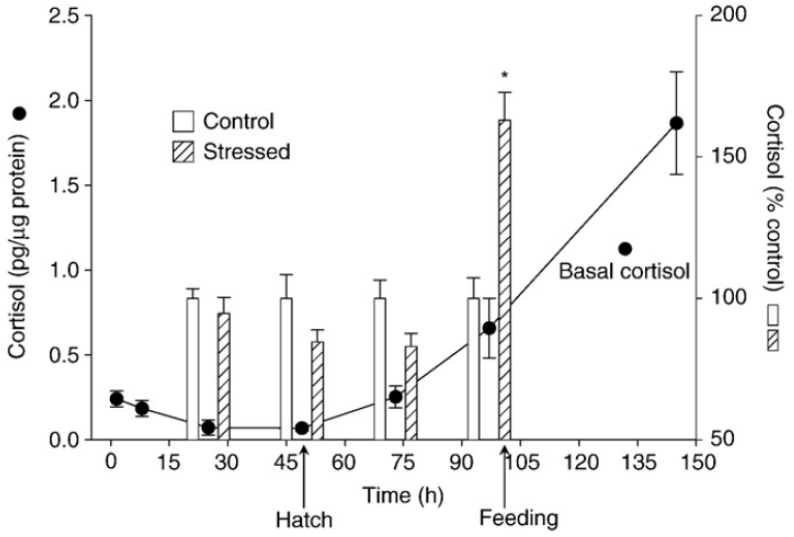


Figure 13. Basal cortisol levels from 1.5 to 146 hpf (closed circles; left axis) and changes in whole body cortisol 5 min after exposure to a stressor (control-open bars, stressed hatched bars; right axis) in zebrafish at 25, 49, 73, and 97 hpf. An asterisk (*) denotes a significant change in cortisol from control animals (t-test) (Alsop and Vijayan, 2008).

12. Objectives of the study

The objectives of this study were: A) to verify whether the cortisol treatment of zebrafish eggs affects the growth rate of the progenies as compared to controls in F1-F4 treated generations (ontogenetic programming). It was to be established whether: 1) this is due to a short-term effect of maternal cortisol, directly producing an initial growth retard that is not compensated later on in lifetime (short-term epigenetic effect); or 2) this is due to a long-term priming of growth rate by maternal cortisol that persists later on in lifetime (long-term epigenetic effect); B) to verify whether the ontogenetic programming is inheritable, being transmitted also to the untreated F4 generation. It was to be established whether the programmed genes are developmental and/or growth genes that are inhibited, or genes encoding hormones of the corticoid stress axis (hypothalamo-hypophyso-interrenal axis) that are amplified, or both gene clusters.

MATERIALS AND METHODS

13. Zebrafish maintenance

13.1. Sources

About 100 couples of adult homozygous striped (called zebra, Z) and spotted (called leopard, L) zebrafish (Fig.1) were obtained from Prof. Francesco Argenton's laboratory (Department of Biology, University of Padova, Italy). These fish were bred to produce my parent collection (parent generations, F0).

13.2. Housing and care

As zebrafish can be easily reared and bred under laboratory conditions, a facility was arranged for their proper housing, care and breeding. Transparent polycarbonate plastic tanks of different capacities were used to hold zebrafish. One litre-capacity tanks were used to hold juvenile fish and 5-L tanks for adult fish. The stocking density was about 80 juveniles/tank and about 60 adults/tank. There was a continuous water circulation system in each tank to provide clean and highly oxygenated freshwater. Daily inspection of fish was performed. Tanks were cleaned regularly by siphoning and replaced with new tanks. Diseased and dead fish were promptly removed.

13.3. Lighting

Light triggers zebrafish spawning and breeding, so appropriate lighting is an important factor for successful spawning. Darkness is crucial for animals to rest and to minimize their stress Vargesson, 2007). However, constant darkness delays normal embryo development and hatching may still not being observed at 7 dpf (Bilotta, 2000). By considering all aspects, a 14 h-light and 10 h-dark photoperiod was adopted to raise zebrafish.

13.4. Temperature

Temperature is a vital factor for development of fish. The zebrafish is recognised as a eurythermal species, which means it can tolerate a wide thermal range, surviving in nature at temperatures as low as 6°C in winter and to over 38°C in summer (Spence *et al*., 2008). Under laboratory conditions, its maximum thermal tolerance was reported to span from 6.2°C to 41.7°C (Cortemeglia and Beitinger, 2005). But 28.5°C is widely cited as the optimum temperature for

zebrafish culture and breeding. So this temperature was maintained for growing and breeding the experimental fish.

13.5. Water quality

Water quality is the most important factor for the health and wellbeing of fish. Poor water quality can lead to stress and disease, and may affect breeding (Kreiberg, 2000; Bilotta *et al*., 1999). Level of contaminants was minimized by maintaining proper water exchange, removal of excess food, and keeping tanks and the water filtration system clean. The pH level was maintained at 7 - 7.4 as measured with a pH meter for proper growth and reproductive performance. Other water quality parameters, such as dissolved oxygen, excreted ammonia, nitrates, nitrites etc were regularly monitored.

13.6. Catching and handling

The majority of zebrafish in research facilities are the descendents of many generations of captive bred animals. Although their 'nervousness' or predator avoidance behaviour, as a prey species, appear to be reduced, still handling represents a potentially dangerous stressor. Even following a brief stressful event, the physiological response may significantly affect blood chemistry for as much as 24 h (Kreiberg, 2000). Hence, catching and handling were minimised to avoid unnecessary stress to fish.

13.7. Feeding

All larvae were fed with the commercial diet Novotom, Premium Baby (JBL, Neuhofen, Germany) four times a day. Francis (2008) suggests that a quality diet specifically developed for zebrafish should be used. Some commercial feeds claim to offer a nutritionally complete food. However, it was preferred to integrate the dry feed with live prey. So, adult fish were daily fed twice with dry food flakes (AZ300, Tetra) and once with brine shrimp nauplii and metanauplii hatched from commercially purchased brine shrimp cysts incubated into saline water for 48 h.

14. Breeding of F0 brood stock for embryo studies

The first mating of F0 brood stock was performed in 1-L transparent plastic breeding boxes containing a single male and a single female of the same strain kept separated during night-time, and allowed to mate in the morning, after light switch on, by removing the separation screen.

15. Reagents for Egg Treatment

Cortisol: 362.46 Da molecular mass from Sigma (Milan, Italy).

Concentration of mother solution: 5 mg cortisol/ 2 mL abs. ethanol (6.897 mM).

Concentration of cortisol treatment solution: 2 mL mother solution/ 1 L fish water (13.794 µM)

RU486: 429.60 Da

RU486 treatment solution: 2 mg in 2 ml EtOH/ 1 L fish water

Control (EtOH) solution: 2 mL abs. ethanol/ 1 L fish water.

Time of egg exposure to treatment or control solutions: 2 h.

Dextran-charcoal suspension: 100 mg Dextran T70 + 1 g activated charcoal/ 100 mL fish water.

16. Eggs treatment with cortisol

Immediately after fertilization, Z strain eggs were pooled in cortisol treatment solution and L strain eggs in control solution for 2 h for development and fixation up to the fourth generation. Other fertilized eggs were treated as four groups with cortisol, RU486 and the mRNA of the zebrafish GR (z-*gr* mRNA) for 2 h as follows:

1. RU486
2. z-*gr* mRNA+Cortisol
3. z-*gr* mRNA +RU486
4. z-*gr* mRNA +EtOH

Eggs were pooled as a single layer on the bottom of the container. Each egg is about 1 mm ∅, covering an area of 0.78 mm^2, so theoretically 1 dm^2 (roughly the container's area) should harbour 12,800 packed eggs. However, egg packing was avoided and enough area was provided to avoid egg clumping and to expose more lateral egg surface to the steroid. The container were gently tilted during the 2 h of treatment.

17. Treatment termination

After 2 h of treatment, the cortisol treatment solution was poured out and Z eggs were gently washed 5-fold in fish water to eliminate any trace of cortisol and ethanol. They were then exactly counted and 300 intact eggs were fixed in liquid nitrogen for cortisol immunoassay. To check for any persistent cortisol contamination on the egg surfaces, a 300-egg sample was immersed in 10 mL of dextran-charcoal suspension for 5 min, washed 5-fold until clean and fixed in liquid nitrogen for cortisol immunoassay. The control solution containing L eggs was terminated as above.

18. Embryo development of the F1 progeny

The remainder of pooled embryos of the Z and L strains were kept separately in Petri dishes (100 embryos/dish) till 2 dpf. While developing, normal embryos of both strains were fixed in liquid nitrogen for RNA analysis at the following developmental stages:

- 50 +50+50 embryos at 5 hpf
- 50 +50+50 embryos at 10 hpf
- 50 +50+50 embryos at 12 hpf
- 50 +50+50 embryos at 24 hpf

19. Breeding of F0 fish for sampling of F1 juveniles and adults

Breeding of F0 adult fish of both strains, cortisol treatment of collected Z eggs and its termination were performed as indicated above. The same was done with the control of L eggs.

20. Juvenile and adult development of the F1 progeny

To equalize the culture environment, the cortisol-treated eggs and larvae of the Z strain and control eggs and larvae of the L strain were kept together in Petri dishes as 50 Z+ 50 L in number till 6 dpf, in 1 L-boxes till 20 dpf and in 5 L-tanks till adulthood. Eggs of each strain obtained from matings carried out within 3 days were considered as belonging to the same stock.

21. Testing of puberty onset

The first oviposition, marking the onset of puberty, begins after 80 dpf and is generally not fertile. From 80 dpf onwards, pairs of male and female of each strains were tested for first mating and the day of first oviposition and the number of laid eggs were checked.

22. Cortisol extractions and radioimmunoassay (RIA)

In order to evaluate the tissue cortisol levels as a stress-response indicator, embryos (n = 50) and fish (n = 3) were captured and immediately frozen in liquid nitrogen and stored at −80°C until the cortisol extraction. Whole-body cortisol was measured by a specific microtitre radioimmunoassay (RIA), as described by Simontacchi *et al.* (2009). Each sample was weighed, thawed out and pulverized in liquid nitrogen, and the resulting powders were suspended in 1 ml phosphate buffer (PBS, pH 7.2). Then the suspension was extracted with 8 ml of diethyl ether and the supernatant was evaporated to make dry. The dry pellet extracts were then dissolved in 0.5 ml of PBS and varying aliquots were used for radioimmunoassays (RIAs). Finally RIAs were performed in the Department of Experimental Veterinary Sciences, Faculty of Veterinary Medicine, University of Padova, Italy.

The anti-cortisol serum showed the following cross-reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.95%, corticosterone 3.5%, prednisone 2.7%, 17-hydroxyprogesterone 1.0%, 11-deoxycorticosterone 0.3%, dexamethasone 0.1%, progesterone < 0.01%, 17-hydroxypregnenolone < 0.01%, pregnenolone < 0.01%.

To validate steroid determination in whole-body larvae, competitive dose-response binding curves were created by serial extract dilutions of larvae (parallelism test) and the intra-and interassay tests were performed.

23. Production of second (F2), third (F3) and fourth (F4) generations

At least 50 males and 50 females of Z and L fish of F1, F2 and F3 generations were used to produce the F2, F3 and F4 generations, respectively. Egg cortisol treatment and development were carried out as described in section 16-20.

24. Length and weight measurement

While producing F1, F2, F3 and F4, length and weight of fish at 60, 90, 120, 150 and 180 dpf were measured. Before measurement 60 to 70% fish of each tank were randomly collected using scoop net and anesthetized with 0.004% tricaine (ethyl 3-aminobenzoate methanesulfonate salt). After measurement, fish were restocked in the same tank for further development.

25. RNA isolation

To evaluate gene expression, total RNA was extracted from pools of 50 embryos of Z and L strains at the desired developmental stages using TRIZOL reagent, according to the manufacturer's instructions (Invitrogen, Milan, Italy). The experimental phase of extraction and manipulation was carried out under a chemical hood using sterile glassware or sterilized at 200°C in oven for the entire night.

Reagents:

- TRIzol
- Chloroform
- Isopropanol
- 70% Ethanol

25.1. Procedure

For each sample, three replicates were taken. Embryos in individual tubes were kept in liquid nitrogen until processing. Briefly, 1 ml of Trizol reagent was added to each tube and the sample was crushed with sterilized glass beads (Sigma) in a Mini-Beadbeater^{TM} stirrer for 10 s. The homogenized sample was incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was then added and the tubes were vigorously shaken for 15 s and left on ice for 1 min. The samples were centrifuged at 12,000 *g* for 15 min at 4°C. Following centrifugation, the mixture separates into a lower red, phenol-chloroform phase, and an upper colourless aqueous phase. RNA remains exclusively in the aqueous phase. The volume of the aqueous phase is about 60% of the volume of Trizol reagent used for homogenization. The aqueous solution was transferred to a new 1.5-ml tube and 0.55 ml

of isopropyl alcohol was added to each tube for RNA precipitation. Tubes were shaken by inverting and incubated at -20°C for at least 3 h.

After incubation, the samples were centrifuged at 12,000 *g* for 20 min at 4°C. The RNA precipitate forms a gel-like pellet on the side and bottom of the tubes. The supernatant was discarded and RNA was washed twice with 70% ethanol and centrifuged at 7,500 *g* for 15 min at 4°C. At the end of the procedure, the RNA pellet was dried under a chemical hood for 5-10 min and re-dissolved in 12-20 µl of RNase-free water. The RNA samples were stored at -80°C until future use.

26. Agarose gel electrophoresis

RNA and cDNA samples, obtained from embryos and PCR **(**Polymerase Chain Reaction) reactions were analysed by agarose (Fisher Molecular Biology, USA) gel electrophoresis. This was carried out until the marker dye (bromophenol blue, added to the sample prior to loading) reaches the end of the gel. The nucleic acids in the gel are visualised by staining with the intercalating dye gel red and examined under ultraviolet (u.v.) light. The quality of extracted RNA is indicated by two bands: an upper 28S rRNA and a lower 18S rRNA (Fig. 14). The presence of additional bands or a smear is the sign of RNA degradation.

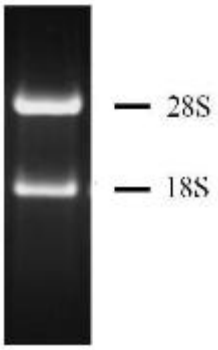


Figure 14. Traditional separation of total RNA on denaturing agarose gel electrophoresis followed by gel red staining. The 28S and 18S rRNA bands in this figure have an intensity ratio around 2 considered to be a sign of good quality RNA. (http://biomedical genomics. org/RNA_quality_control_Bioanalyzer.html)

27. Quantification of total RNA

The concentration of total RNA was measured by NanoDrop Spectrophotometer (Celbio, Milan, Italy) at CRIBI, University of Padova, Italy, which allows an assessment of the state of purity or protein contamination. The concentration of 1.5 µl solution of nucleic acid was determined by measuring the absorbance at 260 nm. An A_{260} of 1.0 is equivalent to a concentration of 50 µg/ml

for double-stranded DNA, or 40 µg/ml for single-stranded DNA or RNA. The A_{260}/A_{280} ratio should be 1.8 for pure DNA and 2.0 for pure RNA preparations (Nicholl, 1996).

28. Purification of extracted RNA with LiCl precipitation for microarray hybridization

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 contains different types of contaminants like polysaccharides, carbohydrates, peptides and solvents (phenol, aromatic compounds). These contaminants were removed by LiCl precipitation. RNA samples were heated at 40°C for 6 min and centrifuged at 12000 xg for 15 min at 4°C. The pellet contains contaminants, so the supernatant was transferred to a new clean tube. Then, 9.48 µl of 4 M LiCl was added to each tube, which was left overnight at 4°C for precipitation. The sample was then centrifuged at 12000 xg for 20 minutes at 4°C. Following centrifugation, the supernatant was discarded and 200 µl of 75% EtOH were added to the pellet for washing. The mixture was again centrifuged at 12000 xg for 20 minutes at 4°C and the supernatant discarded. The RNA pellet was dried under a chemical hood for 10 minutes and re-dissolved in RNase-free water. Finally, the RNA was re-quantified by NanoDrop Spectrophotometer.

29. Reverse transcription of RNA

After qualification and quantification of extracted RNA, reverse transcription was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) protocol (Invitrogen), according to the manufacturer's instructions. M-MLV RT uses single-stranded RNA or DNA in the presence of a primer to synthesize a complementary DNA strand.

The M-MLV reverse transcriptase PCR is a two-step process. Briefly, 2µg of the purified total RNA was mixed with Mix I reaction (Table 3), denatured by incubation at 70^{o}C for 5 min and then placed on ice for 5 min. Then, 15 µl of Mix II (table 4) were added to each sample. The samples were incubated in a PCR machine at 25°C for 10 min, 50°C for 50 min and 70°C for 15 min. Then, the cDNA reactions were either stored at -20^{o}C or used for PCR immediately.

Mix I reaction

Compoments	**Amount**
Random hexamer	2 µl
H_2O mQ	up to 10 µl
Total	10 µl

Table 3. Reverse transcriptase PCR MixI.

Mix II reaction

Compoments	**Amount (µl)**
cDNA synthesis Buffer (5X)	5
dNTP Mix (10 mM)	1.25
M-MLV reverse transcriptase enzyme	0.3
H_2O mQ	up to 15 µl
Total	**15 µl**

Table 4. Reverse transcriptase PCR MixII.

30. Amplification of DNA by Polymerase Chain Reaction (PCR)

PCR is an enzymatic reaction, used to produce many copies of a specific DNA fragment. PCR involves thermal denaturation of DNA strands, annealing of primers to the complementary sequence of DNA and synthesis of a new DNA strand complementary to the DNA template. The BioTherm™ Taq DNA Polymerase exhibits deoxynucleotidyl transferase activity, which frequently results in the addition of extra adenines at the 3'-end of PCR products.

31. Touchdown PCR

Touchdown PCR is a method of polymerase chain reaction by which primers will avoid amplifying nonspecific sequences. The annealing temperature during a polymerase chain reaction determines the specificity of primer annealing. The melting point of the primer sets the upper limit on annealing temperature. The annealing temperature of the initial cycle is 5-10°C above the melting temperature of the primers and the temperature is gradually reduced by 1°C for each following cycle down to the lowest melting temperature value of the primer. The amplification condition of touchdown PCR is as follows:

Touchdown PCR condition

Steps	***Time***	***Temperature***	***Cycles***
Initialization	2 min	95°C	1
Denaturation	30 sec	95°C	40
Annealing	30 sec	62°-50°C	
Extension	1min per kb	72°C	
Final extension	10 min	72°C	1
Termination	2 min	20°C	1

Table 5. PCR thermal cycler condition.

The number of cycles depends on the temperature range chosen for the reaction, according to the melting temperature of the primers used. The duration of the extension depends on the expected length of the amplification.

Reaction mix

Compoments	**Amount (µl)**
Buffer 10X	2.5
$MgCl_2$ (50 mM)	1
dNTPs *Mix* (10 mM)	0.5
Taq Biotherm (5 U/µl)	0.2
Oligo *forward* (10 µM)	0.5
Oligo *reverse* (10 µM)	0.5
cDNA	1
H_2O mQ	up to 25
Total	**25 µl**

Table 6: PCR reaction mix.

32. Purification of PCR product by ExoSAP-IT®

The ExonucleaseI/Shrimp Alcaline Phosphatase (ExoSAP-IT®) method treats PCR products, ranging in size from less than 100 bp to over 20 kbp, to remove unused primers and nucleotides with absolutely no sample loss. The post-PCR reaction product was mixed with 2 µl of Exosap-IT in a final volume of 7 µl. Then, the reaction was carried out as follows:

Time	***Temperature***	***Function***
15 min	37°C	Degradation of primers and nucleotides
15 min	80°C	Inactivation of ExoSAP-IT
10 min	4°C	Hold final

Table 7. PCR conditions.

Finally, 2 µl (3.2 pmol) of specific primer was added to the purified PCR DNA product sample for sequencing.

33. DNA sequencing

DNA sequencing was performed by BMR Genomics (Padova, Italy). For this purpose, a mix containing the specific primer (3.2 pmol) and the required amount of purified DNA was prepared. The mix was dried at 65°C and then delivered to BMR for sequencing.

34. Cloning

34.1. Ligation

Ligation of DNA fragment was performed into pGEM®-T Easy vector (Fig. 15) (Promega, Milan, Italy), according to the manufacturer´s instructions. The pGEM®-T Easy vector is a linearized vector with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. The promoter and multiple cloning sequence of pGEM®-T Easy vector is shown in figure 15.

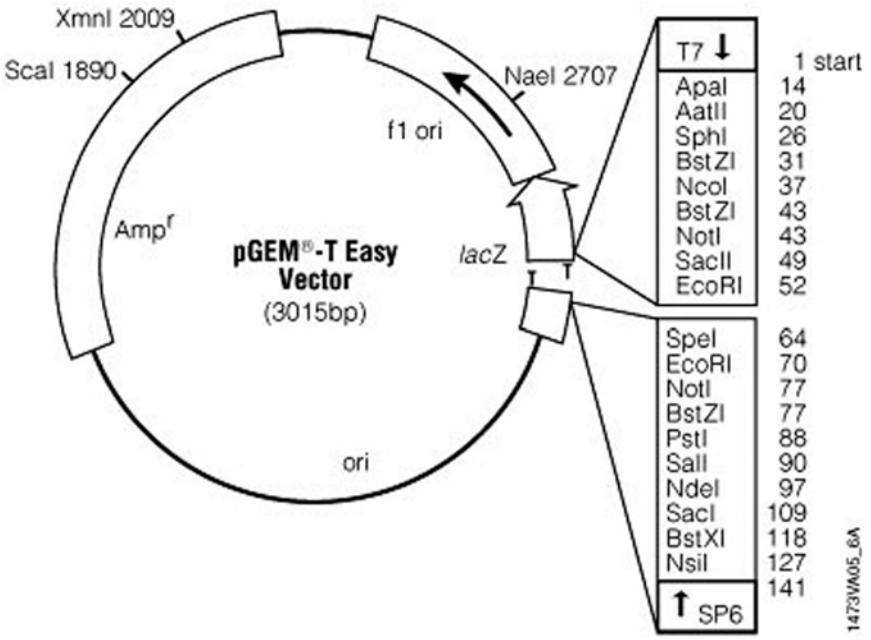


Figure 15. Map of *pGEM® Easy* plasmid vector.
(http://wwwpromega.com/paguide/images/1473VA05_6A.jpg)

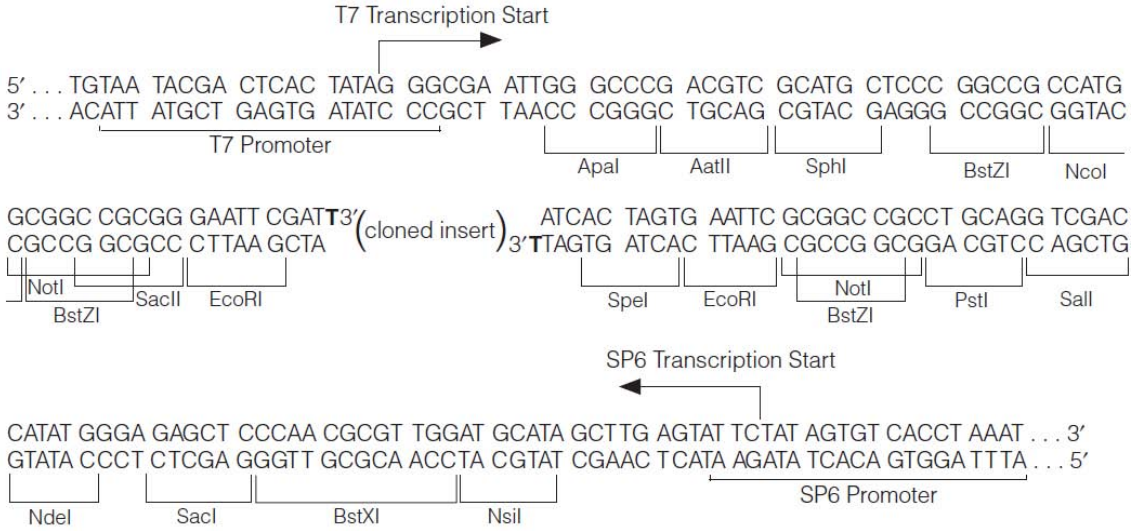


Figure 16. The promoter and multiple cloning sequence of the pGEM®-T Easy Vector.The top strand corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

The appropriate amount of DNA fragments for using in the ligation reaction was calculated by the following equation:

$$\frac{ng\ of\ vector\ \times\ kb\ size\ of\ insert}{kb\ size\ of\ vector} \times insert : vector\ molar\ ratio = ng\ of\ insert$$

Usually, the ligation reaction was performed as follows:

Compoments	**Amount (µl)**
pGEM®-T Easy vector (50 ng)	1
2X rapid ligation buffer, T4 DNA ligase	5
PCR product	X
T4 DNA ligase (3 U/ µl)	1
H_2O mQ	up to 10
Total	**10 µl**

Table 8. Ligation reaction mix.

The reaction was incubated at 4°C overnight and then used to transform chemically competent bacteria.

34.2. Bacterial transformation

Plasmid (with DNA) is inserted into competent bacteria by a process called transformation. To obtain transgenic bacteria, DNA plasmids from the ligation step were transferred into competent *E. coli* cells (JM109 from Promega) by heat shock-induced transformation. Briefly, about 200 µl of competent bacteria were mixed with 5 µl of ligation reaction and the tubes incubated on ice for 30 min. The suspension was then heated at 42°C for 40 sec and incubated on ice for 1 min. Then 1 ml of LB Broth was added and the suspension was shaken at 200 rev/min speed for 1 hour at 37°C. In the meantime, the ampicillin LB-Agar plates were prepared with 80 µl of X-Gal (20 mg/ml) and 100 µl of the inductor IPTG (0.1 M). The suspension of bacteria was then centrifuged at 12000 xg for 1 min. The upper solution was discarded and the lower transformation culture was plated onto LB-plates. The plates were incubated at 37°C overnight. According to the Technical manual of Promega, bacteria carrying a plasmid without the insert possess a functional β-galactosidase gene and produce blue colonies due to the X-Gal reaction. In contrast, bacteria containing a plasmid with the insert of interest have a disrupted β-galactosidase gene and produce white colonies.

34.3. Identification of positive colonies by PCR

Following bacterial transformation, single white colonies were picked from incubated plates with pipette tips. The single colony present on the tip was streaked onto a fresh replicate LB-plate and also added to the PCR Mix I (Table 9). The PCR tubes were incubated at 98°C for 10 min to destroy the bacterial cells. Then the PCR Mix II (Table 10), containing the Taq polymerase, was added.

PCR Reaction MixI

Compoments	**Quantity (µl)**
10X buffer reaction	2
$MgCl_2$ (50 µM)	1
dNTPs (10mM)	0.6
Primer forward (10µM)	0.6
Primer reverse (10µM)	0.6
H_2O mQ	Up to 20
Total	**20 µl**

Table 9. Reaction MixI.

PCR Reaction MixII

Compoments	**Quantity (µl)**
10X buffer reaction	1
Taq Biotherm (5U/µl)	0.2
H_2O mQ	Up to 10
Total	**10 µl**

Table 10. Reaction Mix II.

After adding Mix II, controlled PCR reaction was performed according to the Table 11. The controlled PCR product was then analysed by gel electrophoresis for identification of positive colonies. Positive colonies were then inoculated into 5 ml of LB Broth with 7 µl of ampicillin (50 mg/ml) in a shaker incubator at 37^{o}C overnight.

Steps	***Time***	***Temperature***	***Cycles***
Initialization	2 min	95°C	1
Denaturation	45 sec	95°C	40
Annealing	1.1 min	55°C	
Extension	2min	72°C	
Final extension	10 min	72°C	1
Termination	2 min	20°C	1

Table 11. Control PCR program.

34.4. Purification of plasmidic DNA (Miniprep)

Purification of plasmidic DNA that contained the gene of interest was performed using Eurogold Plasmid Miniprep (Euclone) kit according to the manufacturer's instructions. Briefly, inoculated bacteria were taken into a 1.5-ml tube and centrifuged at 6800 xg for 3 min at room temperature. The upper liquid phase was discarded and 25 µl of Buffer P1 (50 mM Tris-HCl, 10 mM EDTA,

pH 8.0, 25°C, 50 ug/ml RNase A) and 250 µl of Buffer P2 (0.2 M NaOH, 1% SDS) were added to the tube, which was kept at room temperature for 5 min. Then, 350 µl of Buffer N3 (4 M guanidine hydrochloride, 0.5M Potassium acetate, pH 4.2) was added to the tube and centrifuged at 17,900 xg for 10 min at room temperature. After centrifugation, one high pure filter was inserted into one collection tube. The entire supernatant was transferred onto the filter and centrifuged for 1 min at full speed. After centrifugation, the filter tube was removed from the collection tube, the follow-through liquid was discarded and the filter tube reinserted in the same collection tube. Then, 500 µl of Buffer PB (5 M guanidine hydrochloride, 20 mM Tris-HCl, pH 6.6, 25°C and isopropanol to 38%) was added, centrifuged for 1 min and the flow-through was discarded. Again, 750 µl of Buffer PE (mM NaCl, 2 mM Tris-HCl, pH 7.5, 25°C, and ethanol to 70%) was added and the flow-through was discarded after 1 min centrifugation. Finally, plasmidic DNA was eluted with 25 µl of H_2O and screened with NanoDrop quantification and sequencing (see section 27 and 33).

35. Quantitative Real-Time Polymerase Chain Reaction (qPCR)

35.1. Absolute quantification

In absolute quantification assays, the concentration of a target molecule is expressed as an absolute value. This is based on a standard curve, which is prepared from samples of known template concentrations. To create the standard curves for absolute qPCR, sequenced PCR products were cloned into the pGEM-T Easy vector (see section 34). The copy numbers of the plasmid DNA templates were calculated according to the plasmid molecular weight and then converted into copy numbers on the basis of Avogadro's number. Serial dilutions of 10^8–10^2 plasmids/µl were used to generate the calibration curves.

Absolute qPCR was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) and GoTaq® qPCR Master Mix kit (Promega) (Table 12) according to the manufacturer's protocol. All samples were analyzed in triplicate in 20 µl volume.

Compoments	**Quantity (µl)**
GoTaq® qPCR Master Mix, 2X	10
Primer forward (10µM)	0.4
Primer reverse (10µM)	0.4
CXR dye	0.2
cDNA	X*
H_2O mQ	Up to 20 µl
Total	**20 µl**

Table 12. Absolute qPCR reaction mix.

* 1 µl of plasmid for standard curves and 2 µl of cDNA per each sample

Threshold values for threshold cycle (Ct) determination were generated automatically by the 7500 Real-Time PCR System software. Specificity of the reaction was checked by analysis of the amplification curve of the final amplified product. The results were reported as expression, after normalization, of the transcript amount with respect to the reference gene (*ef1a*). The reaction conditions were performed as shown in the Table 13.

Steps	***Time***	***Temperature***	***Cycles***
Taq activation	2 min	95°C	1
Denaturation	15 s	95°C	45
Annealing	1 min	56°C	

Table 13. Absolute qPCR thermal cycler protocol.

35.2. Relative quantification

In relative quantification method, the target concentration is expressed as a ratio of target-to-reference gene in the same sample, rather than as an absolute value. The reference gene is an unregulated nucleic acid that is found at constant copy number in all samples. Relative qPCR mix and thermal cycler protocol for relative qPCR are shown in the following tables:

qPCR Reaction Mix

Components	**Quantity (µl)**
qPCR Master Mix	10
Primer forward (10µM)	0.4
Primer reverse (10µM)	0.4
ROX dye	0.2
cDNA	1
H_2O mQ	Up to 20 µl
Total	**20 µl**

Table 14. Realtive qPCR reaction mix.

Relative qPCR condition

Steps	***Time***	***Temperature***	***Cycles***
Taq activation	10 min	95°C	1
Denaturation	30 s	95°C	45
Annealing	1 min	57°C	

Table 15. Relative qPCR thermal cycler condition.

Each cDNA sample was amplified using specific forward and reverse primers that were derived from mRNA sequences contained in the NCBI database (www.ncbi.nlm.nih.gov). The primers were designed to cross exon/exon boundaries of coding regions in order to prevent co-amplification of genomic DNA, which may compromise assay specificity and dynamic range. *ef1a* was used as housekeeping gene to verify the quality of cDNA synthesis. The list of primers used in relative qPCR is as described in Table 16.

Primer	Orientation	Sequence (5′ to 3′)	Accession number
ef1a-F	Sense	GACAAGAGAACCATCGAG	NM_131263
ef1a-R	Antisense	CCTCAAACTCACCGACAC	
rplp0-F	Sense	CTGAACATCTCGCCCTTCTC	NM_131580
rplp0-R	Antisense	TAGCCGATCTGCAGACACAC	
vasp-F1	Sense	GTAAGATGCAGACTGACCAGC	NM_001017790
vasp-R2	Antisense	CTCTCTTGCTGCTCCAGCC	
plp1a-F1	Sense	CAGTTCGGCAATCACTCG	NM_175576
plp1a-R1	Antisense	GTACTGTCTGGCATCAATGC	
stmn2a-F1	Sense	CAGCATCACCTCTCCTCC	NM_001005923
stmn2a-R1	Antisense	GCTTTGAGCAGCACATCC	
parn-F1	Sense	CAAGCGCAGCATCAGTC	NM_201088
parn-R1	Antisense	TCCGCCTCCTCTGCCAC	
notch2-F1	Sense	GACGAATGCATCTCCAGTGC	NM_001115094
notch2-R1	Antisense	GCAGCAGCCACAGCAACC	
tsc22d3-F	Sense	AGCTCGCACTCTACCAGC	NM_200569
tsc22d3-R	Antisense	GTGCTCCTTCAAGATCTCC	
Mat1a-F1	Sense	GGGTAGCGAAGTCTCTGG	NM_199871
Mat1a-R1	Antisense	TAGCAGGCGGTGTTCTGG	
igf-2a-F	Sense	TCAAACAGCCGCCGTCCTCC	NM_131433
igf-2a-R1	Antisense	GAAAAAGTGCCTCTACTGACC	
mcm6-F2	Sense	ACCGCGTCTACAGTCTCG	NM_001082849
mcm6-R1	Antisense	AGCAGCCTGAAAGCCTCC	

Table 16. List of primers

36. Two-color Microarray hybridization

A DNA microarray is a stamp-sized piece of glass or plastic on which single-stranded fragments of DNA (also called probes) representing the genes of an organism have been attached in a microscopic array. As many as 30,000 spots can fit on one slide and each fragment can bind to complementary DNA or RNA strand. Two-color Microarray-based gene expression analysis (Agilent Technologies, Santa Clara, CA) was performed to analyze the gene expression of embryos treated with cortisol (sections 16-17) at 5, 12 and 24 hpf, as shown in Figure 17. The analysis was performed at CRIBI, using Agilent Whole Zebrafish Genome Oligo Microarrays 4x44K slide.

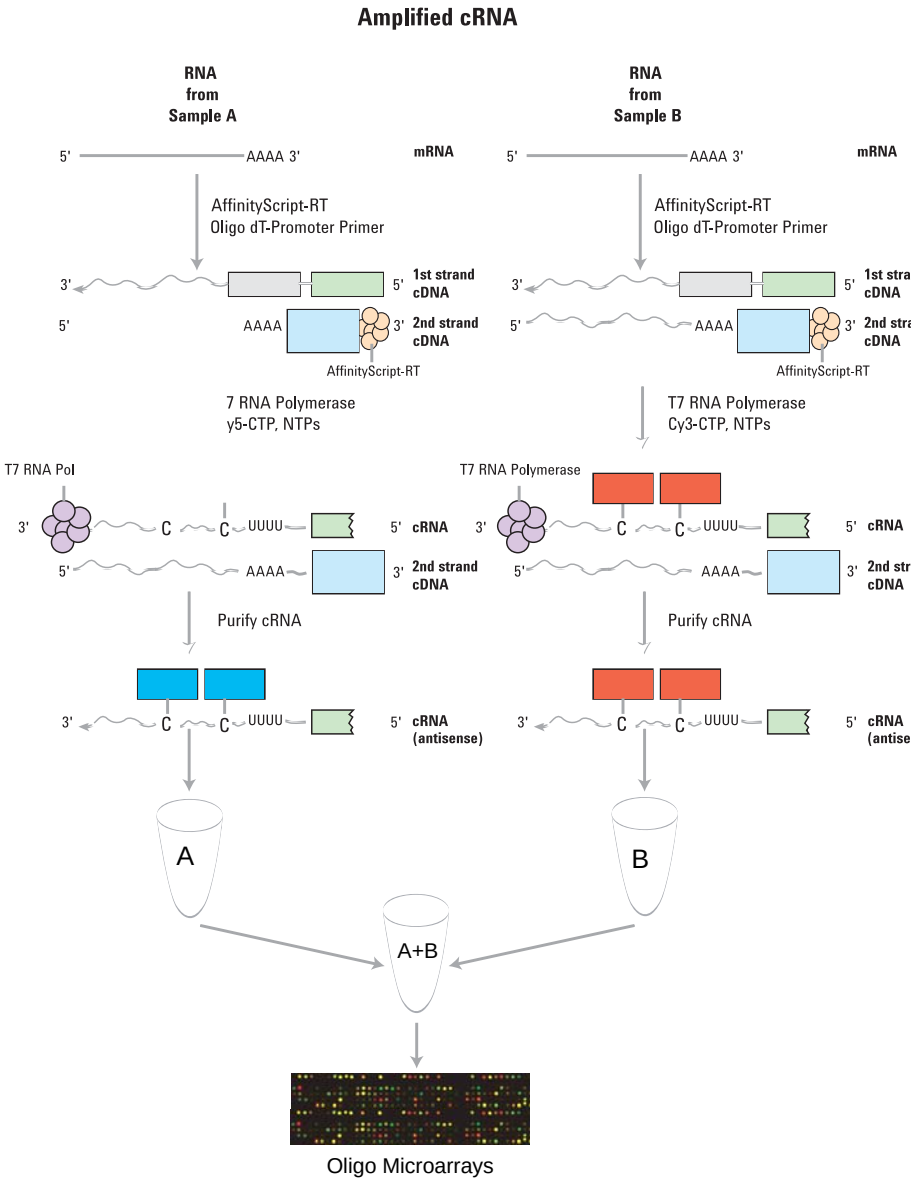


Figure 17. Scheme of amplified cRNA procedure (Protocol of Agilent Technologies, USA).

36.1. RNA quality control for microarray hybridization

The assessment of RNA integrity is a critical first step in obtaining meaningful gene expression data. Using intact RNA is a key element for successful microarray analyses. The Agilent 2100 bioanalyzer and RNA LabChip were used to perform RNA quality assessment and quantification. The bioanalyzer 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 technology has also introduced a new tool for RNA quality assessment: the RNA Integrity Number (RIN). The RIN software algorithm allows the classification of eukaryotic total RNA, based on a numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact one (Fig. 18). Only the samples having RIN value greater than 7 were used to perform microarray hybridization.

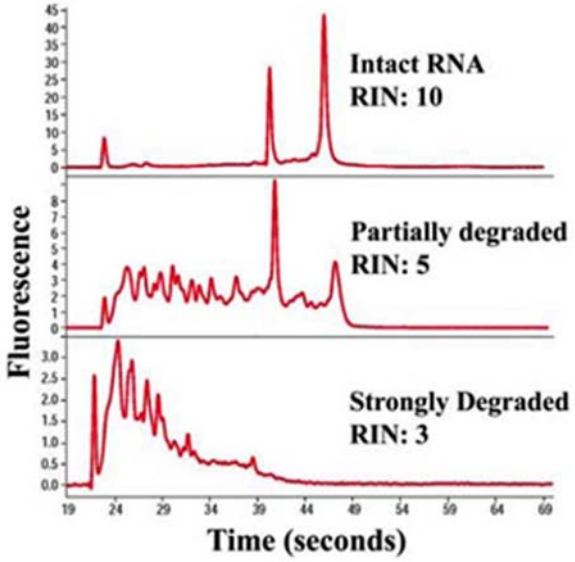


Figure 18. The RIN number was tested on samples of varying levels of intactness. The RIN software algorithm was able to accurately classify the samples.

36.2. Sample preparation, hybridization and microarray wash

Agilent's Two-Color Microarray-based Gene Expression Analysis used cyanine 3- and cyanine 5-labeled targets to measure gene expression in control and experimental samples. Figure 19 is a standard workflow for sample preparation and array hybridization design.

Briefly, after the preparation of Spike A and Spike B Mix, the synthesis of double-stranded cDNA was performed. This is followed by the synthesis of cRNA labelled with cyanine 3 (Cy3) and cyanine 5(Cy5) for control and cortisol treated samples, respectively. The cRNA was

purified and quantified with NanoDrop Spectrophotometer. Absorbance ratios 260/280 nm and 260/230 nm were measured to check a good quality (≥ 2) of samples. In addition, cyanine 3 or cyanine 5 dye concentration (pmol/µl) was also measured. The samples were then hybridized with 2x GEx Hybridization Buffer in 4x44K array at 65°C for 17 h. Then the microarray slide was washed two times with GE wash buffer 1 and GE wash buffer 2, respectively.

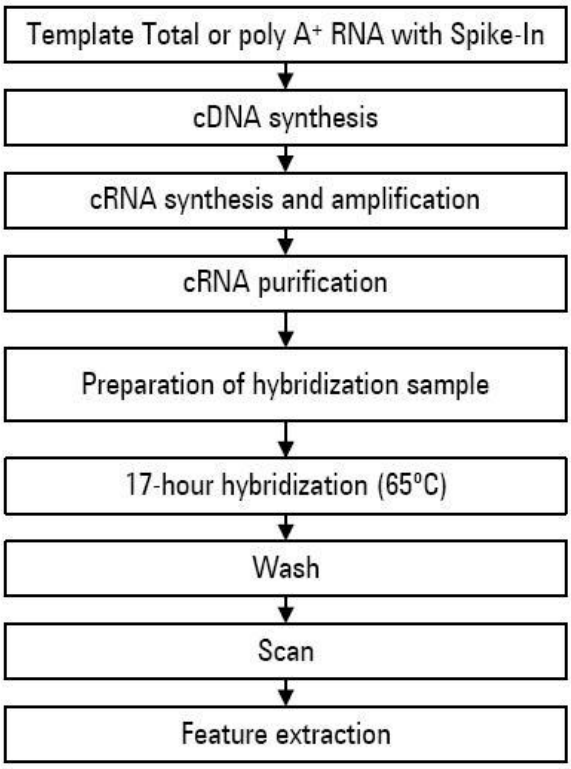


Figure 19. The workflow for sample preparation and array processing.

36.3. Scanning, feature extraction and data analysis

Fluorescence signals of the hybridized Agilent Oligo Microarrays were detected using Agilent's DNA microarray scanner. 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). For determination of differential gene expression, FES-derived output data files were further analyzed using the Rosetta Resolverâ gene expression data analysis system (Rosetta Biosoftware, Agilent). Subsequently, the genes were classified into functional

groups using gene ontology and analyzed individually at the site "Entrez Gene" (http://www.ncbi.nlm.nih.gov/gene).

37. Morpholino microinjection of fertilized eggs

Microinjections of single-cell fertilized eggs were performed with antisense oligonucleotides, called MO, and GR-mRNA. MO oligonucleotides were designed and synthesized by Gene Tools, Philomath, OR, USA (www.gene-tools.com). The sequences of MOs were as follows:

*gr*MO-ATG-1: 5'- CATTCTCCAGTCCTCCTTGATCCAT- 3'

*gr*MO-ATG-1 was designed around the start codon (ATG) of zebrafish glucocorticoid receptor coding sequence (Accession number EF567112). Morpholino oligos were delivered from a pre-quantified, sterile, salt-free, lyophilized solid in a glass vial. The stock solutions (8 mg/ml) was prepared with nuclease-free water, as advised by Genetool's protocols, and kept at RT. MO working solutions were prepared diluting the stock solutions in Danieau 1X and adding Phenol Red (Sigma) to make the solutions visible during microinjection. Different MO concentrations were tested in a range between 0.5 and 2 μg/ml.

38. Preparation of the needles

The needles for microinjection were prepared from 1.2 mm thin-walled glass capillaries (World Precision Instruments Inc., WPI, Germany) by a puller micropipette (Flaming/Brown p-97 Micropipette Puller, Setter Instruments, Crisel Instruments, Rome, Italy). The parameter of the micropipette puller was set as heat 95, velocity 60, time 50 and pull 60.

The freshly laid eggs were collected and placed in a Petri dish containing fish water. They were then aligned against a clean glass slide on the lid of a Petri dish to facilitate microinjection. The microinjection was carried out using an air pump controlled pedal (Pneumatic Picopump PV82, Word Precision Instruments Inc.). A micromanipulator (Leitz) and a stereomicroscope with 3.2 X magnification was used to monitor the procedure. The samples (MO and mRNA) were loaded with 0.5-20-μl GELoader tips (Eppendorf, Milan, Italy) into the injection needles. Then, the tip of injection needles was broken with dissection forceps. The samples (MO and mRNA) were injected into the yolk sac of 1 or 2-cell stage embryos. After injection, embryos were incubated in fish water and kept at 28.5ºC in an incubator. MO- and/or mRNA-injected embryos were raised to the desired stages for observations or fixed for RNA analysis.

39. Preparation of z-*gr* RNA

The full-length z-*gr* cDNA was amplified using primers containing overhangs with restriction sites for further specific subcloning into the vector pCS2+ (Fig. 20).

- z-*gr*-F: ***CGGGATCCCG***GCAAAATGGATCAAGGAGGA (restriction site of *Bam*HI is shown as bold italic letters)

- z-*gr*-R: ***GCTCTAGAGC***CTGCTGTTGGGAGGAGATTC (restriction site of *Xba*I is shown as bold italic letters)

Full-length z-*gr* cDNA was cloned into pGEM-T Easy and subcloned into pCS2+ expression vector using *Bam*HI and *Xba*I (Table 15) restriction enzymes. After complete sequencing to check for nucleotide changes that could alter the amino acidic sequence or interrupt the translation-reading frame, one positive clone was linearized with *Xba*I.

Restriction enzyme	**Recognition sequence**
***Xba*I**	T▼CTAGA
***Bam*HI**	G▼GATCC

Table 17. Recognition sequences for specific restriction enzyme.

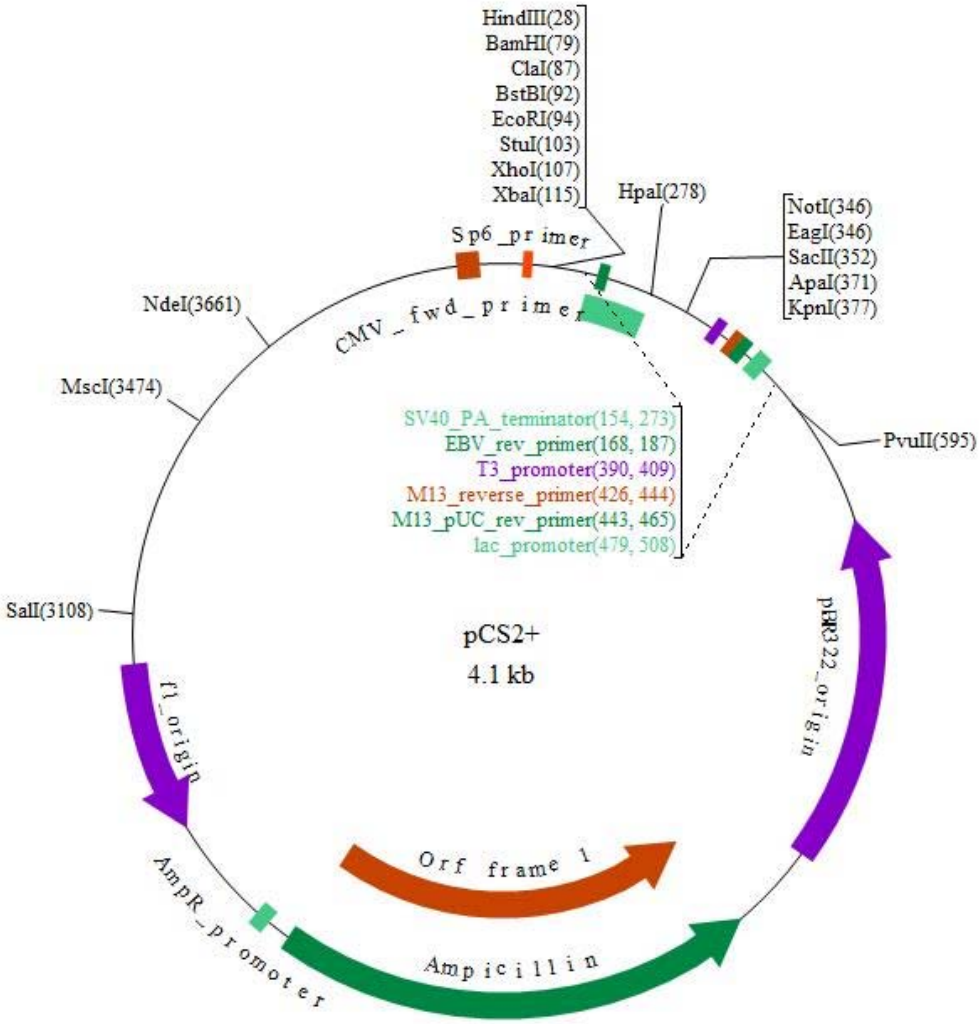


Figure 20. Map of pCS2+ vector (www.biovisualtech.com/bvplasmid/pCS2+.htm).

Efficiency of linearization was checked on agarose gel electrophoresis and the linear DNA was purified using phenol/chloroform extraction. Briefly, 50 µl of sterile water, 50 µl of chloroform and 50 µl of phenol are added to 50 µl of digestion product; the reaction mix is vortexed vigorously to mix the phases and centrifuged at 12,000 *g* for 1 min. After centrifugation, the supernatant (aqueous phase) was transferred to a new tube and mixed with 50 µl of chloroform and 50 µl of phenol. The mix was vortexed and centrifuged at 12,000 *g* for 1 min. Again, the supernatant was transferred into a new tube, mixed with 100 µl chloroform and centrifuged at 12,000 *g* for 1 min. After transferring the supernatant into a new tube, 10 µl of 4 M NaCl and 250 µl of 100% ethanol are added and gently mixed. The sample was chilled at -80°C for at least 1 h. The sample is then centrifuged at 12,000 *g* at 4°C for 20 min. The pellet obtained was washed with 70% ethanol, dried and resuspended in 10-15 µl of mQ H_2O.

Full length z-*gr* mRNA was *in vitro* transcribed and 5'-capped with the SP6 polimerase using the kit mMessage mMachine according to manufacturer's protocol (Ambion, Milan, Italy). The reaction (Table 18) was incubated at 37°C for 2 h. The template DNA was digested at the end of the reaction with DNAse I, RNAse-free. The RNA was then precipitated in LiCl and subsequently dissolved in mQ H_2O. The RNA concentration was determined using a NanoDrop Spectrophotometer (Thermo Scientific, Euroclone, Milan, Italy). The *in vitro* transcribed mRNA was stored at -80°C.

Components	**Quantity(µl)**
Linear template DNA (1 µg)	5.2
mQ H_2O	1.5
2X NTP/CAP	10
10X Reaction Buffer	2
Enzyme Mix	2
Total	**20 µl**

Table 18. Reaction Mix.

This z-*gr* mRNA was diluted with 200 ng/µl concentration and microinjected into fertilized one-cell stage embryos according to the following treatments:

Group 1. z-*gr* mRNA+ Cortisol

Group 2. z-*gr* mRNA+EtOH

Group 3. z-*gr* mRNA+RU486

Treatment termination: 2 h

After 2 h treatment, the embryos were fixed at 5 and 10 hpf for RNA analysis.

40. Statistical analysis

All values are presented as mean±standard error of the mean (sem) and mean±standard deviation (SD). Cortisol immunoassay data were analysed by using one-way analysis of variance (ANOVA) followed by LSD pair-wise multiple comparison test. Where F values indicated statistical significance ($P < 0.05$),

SAM 3.0 (Significance Analysis of Microarrays) statistical program was used to analyse expression of up- and down-regulated genes using 1% False Discovery Rate (FDR), which points out statistically significant up- and down-regulated genes from the set of a microarray experiment.

Relative qPCR results were analysed using REST 2009 (Relative Expression Software Tool) program to estimate up and down regulation for gene expression of some cortisol responsive genes. One-way ANOVA, followed by Tukey HSD multiple comparison test was performed to analyse absolute qPCR results to point out significantly up- and down-regulated genes.

Effects of different treatments on the percentage of mortality and abnormal phenotypes of embryos data were analysed using one-way ANOVA, followed by LSD multiple comparison test. Length and weight of fish at different time points and generations were analysed using paired sample Student's t-tests. For all statistical tests, a probability level of $P < 0.05$ was considered to be significant.

41. ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AP	accessory proteins
BAC	bacterial artificial chromosome
bp	base pairs
cDNA	complementary DNA
CRF	corticotropin-releasing factor
Ct	threshold cycle
Cy3	cyanine-3 (green)
Cy5	cyanine-5 (red)
Da	dalton
DBD	DNA-binding domain
DNA	deoxyribonucleic acid
dpf	days post fertilization
ENU	Ethylnitrosourea
ER	estrogen receptor
EVL	enveloping layer
ExoSAP	exonucleasei/shrimp alcaline phosphatase
F	cortisol
F0	parent stock
F1	first generation
F2	second generation
F3	third generation
F4	fourth generation
FDR	false discovery rates
FES	feature extraction software
Fig.	figure
FU	fluorescence
g	gram

g	gravity force
GO	gene ontology
GR	glucocorticoid receptor
GRE	glucocorticoid-response element
h	hour
hpf	hour post-fertilization
HPI	hypothalamus-pituitary-interrenal
HSD	Honestly Significant Difference
l	liter
kbp	kilo base pairs
kDa	kiloDalton
L	leopard
LBD	ligand-binding domain
LDL	low-density lipoprotein
LSD	least significant difference
MBT	midblastula transition
MC2R	melanocortin 2 receptor
min	minute
mL	milliliter
mM	millimolar
M-MLV RT	moloney murine leukemia virus reverse transcriptase
mmol	millimole
MO	morpholino oligonucleotides
mRNA	messenger ribonucleic acid
nt	nucleotide size
PBS	phosphate buffer
PGCs	primordial germ cells
pH	potential of hydrogen
Pmol	picomole
qPCR	quantitative real-time polymerase chain reaction

REST	relative expression software tool
RIA	radioimmunoassay
RIN	RNA integrity number
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT	room temperature
RU486	mifepristone
s	second
SD	standard deviation
S-DNA	phosphorothioate-linked DNA
SEM	standard error of mean
siRNA	short interfering RNA
StAR	steroidogenic acute regulatory protein
U	unit
VLDL	very-low-density lipoprotein
WT	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
YSL	yolk syncytial layer
Z	zebra
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar

RESULTS

42. Cortisol immunoassay

The cortisol immunoassay showed an acceptable parallelism test for both embryos of zebrafish. For embryos just after treatment, the parallelism test showed: $y=16.59x-0.23$; r^2= 0.996, and reproducibility CV % intra-assay = 5.29. Cortisol concentrations of cortisol-treated embryos and control for F1, F2, F3 and F4 generations are shown in Table 19.

	F1	**F2**	**F3**	**F4**
Cortisol- treated embryos (pg/egg)	4.3 ± 1.1^{a}	2.9 ± 0.49^{a}	4.0 ± 2.5^{a}	3.6 ± 0.80^{a}
Control embryos (pg/egg)	0.2 ± 0.01^{b}	0.27 ± 0.05^{b}	0.53 ± 0.3^{a}	0.5 ± 0.2^{b}

Table 19. The cortisol concentration of cortisol-treated embryos and control embryos for F1, F2, F3 and F4 generations. Cortisol concentrations (mean±SD) with different letters in each column indicate statistically differences from one another ($p < 0.05$, paired sample t-test).

The cortisol-treated embryos displayed significantly higher ($p < 0.05$) cortisol concentration than control in F1, F2 and F4 generations. But there was no statistical significant difference in F3 between cortisol-treated and control embryos.

43. Microarray analysis of the effect of cortisol hormone on gene expression

Microarray analysis of cortisol-treated embryos and control was performed to investigate glucocorticoid-dependent gene expression in zebrafish. Samples were collected at 5, 12 and 24 hpf, corresponding to the 40%-epiboly Blastula, 6-somite Segmentation and prim-5 Pharyngula stages, respectively. Prior to analysis, fertilized eggs F1 were immersed in treatment and control solutions for 2 h. Thereafter, eggs were poured out from treatment and control solutions and washed gently 5-fold in fish water to eliminate any trace of cortisol and ethanol. Then, embryos were sampled at 5, 12 and 24 hpf for microarray analysis.

43.1. RNA quality control

After RNA extraction (according to section 25.1), the quality of RNA was controlled by means of Agilent 2100 bioanalyzer that use the RNA integrity number (RIN) software tool. RIN is 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 is facilitated, comparison of samples is enabled and repeatability of experiments is 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 is assessed when the bands of 28S and 18S rRNAs are well separated and their correspondent peaks are in good evidence. All RNA samples used in these experiments obtained RIN values between 8.4 and 10.

43.2. cRNA quantification and incorporation of cyanine

The microarray experiment was carried out according to the experimental design illustrated in Table 20 for the 12- and 24-hpf samples, whereas the cRNA quantification and incorporation of cyanine Cy3 (green) and Cy5 (red) is shown in Table 21. Treated samples were labeled with Cy5 and the control with Cy3. Replicates (n=3) were performed for 5 and 12-hpf samples by dye swap to get a better selection of significant variation of gene expression.

Experiments	Cy3	Cy5
Array 1_1	**1.1 Control 12 hpf**	**1.1 Cortisol-treated 12 hpf**
Array 1_2	**1.2 Control 12 hpf**	**1.1 Cortisol-treated 12 hpf**
Array 1_3	**1.3 Control 12 hpf**	**1.1 Cortisol-treated 12 hpf**
Array 1_4	**1.1 Control 24 hpf**	**1.1 Cortisol-treated 24 hpf**

Table 20. The experimental design of microarray analysis. Cy3= Control (green colour) and Cy5=Treated (red colour).

Samples	Labeling	Concentration cRNA (ng/µl)	Volume (µl)	Dye (picomol/µl)	Incorporation rate (pmol/µg)
1.1 Control 12 hpf	**Cy3**	411	30	7.3	17.8
1.2 Control 12 hpf	**Cy3**	362	30	3.2	8.8
1.3 Control 12 hpf	**Cy3**	293	30	4.7	16.4
1.1 Control 24 hpf	**Cy3**	296	30	3.1	12.5
1.1 Cortisol-treated 12 hpf	**Cy5**	248	30	5.1	12.5
1.1 Cortisol-treated 12 hpf	**Cy5**	407	30	4.7	13.4
1.1 Cortisol-treated 12 hpf	**Cy5**	350	30	2.5	8.3
1.1 Cortisol-treated 24 hpf	**Cy5**	371	30	4.5	12

Table 21. Quantification of cRNA and incorporation rates of cyanine Cy3 (green) and Cy5 (red) for control and treatment samples, respectively.

43.3. Scanning and microarray data analysis

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. An example of the spatial distribution of significantly up- and down-regulated features is shown in Figure 21, 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.

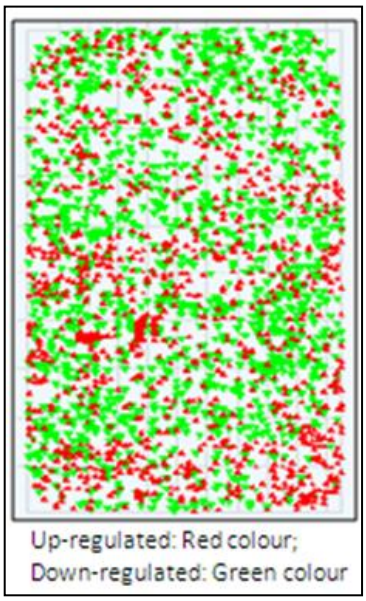


Figure 21. An example of the spatial distribution of significantly up- and down-regulated features.

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. An example of log ratio versus log processed signal scatter plot is shown in Figure 22.

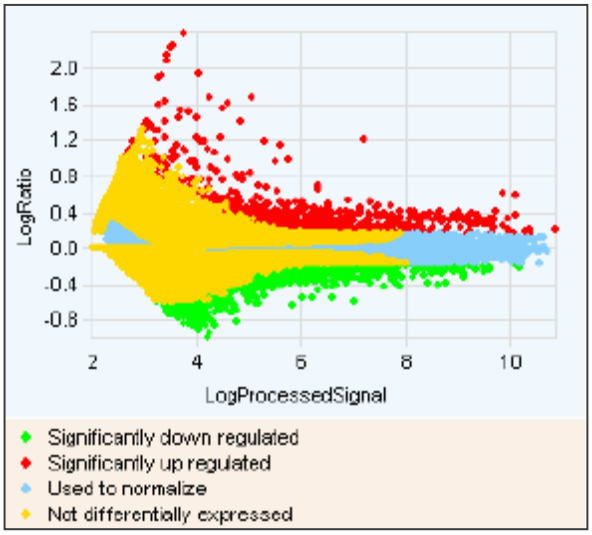


Figure 22. Scatter plot of log ratio versus log processed signal intensities (an example of one array experiment). Red spot: significantly up-regulated genes ($P < 0.05$). Green spot: significantly down-regulated genes ($P < 0.05$). Yellow spot: Not differentially expressed genes. Blue spot: Genes used to normalize.

43.4. Interpretation of microarray results

The differentially expressed up- and down-regulated genes were analysed using SAM (Significance Analysis of Microarrays) statistical program using one percent FDR, which points out statistically significant up- and down-regulated genes from the set of a microarray experiment. The results showed that 100 genes were significantly up-regulated at 5 hpf, whereas none was down-regulated. At 12 hpf, 149 genes were dys-regulated, with 143 up-regulated and 6 down-regulated. There were 33 common dys-regulated genes from 5 and 12 hpf (Table 22). A description of up- and down-regulated genes at 5 and 12 hpf with their functional groups are shown in Tables 23 and 24, respectively.

Gene ontology	5 hpf		12 hpf		Common	
	Up	Down	Up	Down	Up	Down
Embryo development	5	-	1	-	1	-
Metabolic processes	7	-	17	2	3	-
Intra-and inter-cellular signaling pathway	1	-	-	-	-	-
Transcription	6	-	9	-	2	-
RNA processing	1	-	3	-	1	-
Translation	2	-	2	-	2	-
Biological processes	5	-	8	-	1	-
Cell structure and cytoskeletal organization	3	-	-	-	-	-
Intra-and inter-cellular transport	10	-	7	-	4	-
Negative regulation of cell proliferation	3	-	3	-	2	-
DNA repair	1	-	-	-	-	-
Not annotated	12	-	23	1	3	-
Unknown	44	-	70	3	11	-
Total (P <0.05)	**100**	**0**	**143**	**6**	**30**	**0**

Table 22. The number of up- and down-regulated genes at 5 and 12 hpf and also common genes from both stages. The genes are classified on the basis of their functional groups or gene ontology.

Functional grouping analysis was performed from public databases (http://www.ncbi.nlm.nih.gov/sites/entrez) for specific gene functions in order to find common features among the genes sharing similar expression characteristics. 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. Genes were classified into 12 functional groups, as shown in Tables 22-24 and not annotated up and down-regulated genes are shown in Tables 25-26.

Function	Genes	Description	5 hpf	
			Up	Down
Translation	mrpl20	mitochondrial ribosomal protein L20	↑	-
	sepsecs	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	↑	-
Biological process	ccnd3	cyclin D3	↑	-
	ndufa1	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1	↑	-
	pawrl	PRKC, apoptosis, WT1, regulator like	↑	-
	marveld2b	MARVEL domain containing 2b	↑	-
	acot9.2	acyl-CoA thioesterase 9.2	↑	-
Embryo develpment	dscam	Down syndrome cell adhesion molecule	↑	-
	jag2	jagged 2	↑	-
	stmn2a	stathmin-like 2a	↑	-
	ptrfb	polymerase I and transcript release factor b	↑	-
	notch2	notch homolog 2	↑	-
Intra-and inter-cellular transport	vtg1	vitellogenin 1	↑	-
	slc16a12b	solute carrier family 16 (monocarboxylic acid transporters), member 12b	↑	-
	vtg6	vitellogenin 6	↑	-
	syt5a	synaptotagmin Va	↑	-
	sfxn5b	sideroflexin 5b	↑	-
	slc4a1	solute carrier family 4, anion exchanger, member 1a	↑	-
	snx16	sorting nexin 16	↑	-
	mfsd7	major facilitator superfamily domain containing 7	↑	-
	dscr3	down syndrom critical region gene 3	↑	-
	slc30a4	solute carrier family 30 (zinc transporter), member 4	↑	-
DNA repair	ino80db	INO80 complex subunit Db	↑	-
Negative regulation of cell proliferation	adra2db	adrenergic, alpha-2D-, receptor b	↑	-
	hhip	hedgehog interacting protein	↑	-

	apaf1	apoptotic protease activating factor 1	↑	-
RNA processing	parn	poly(A)-specific ribonuclease (deadenylation nuclease)	↑	-
Intra and inter cellular signalling pathway	exorh	extra-ocular rhodopsin	↑	-
Transcription	hoxd9a	homeo box D9a	↑	-
	nkx2.2a	NK2 transcription factor related 2a	↑	-
	pde8a	phosphodiesterase 8A	↑	-
	znf367	zinc finger protein 367	↑	-
	il7r	interleukin 7 receptor	↑	-
	nfe2l3	nuclear factor (erythroid-derived 2)-like 3	↑	-
Cell structure and cytoskeleton organization	tmsb	thymosin, beta	↑	-
	actc1b	actin, alpha, cardiac muscle 1b	↑	-
	epb4.1l4	erythrocyte protein band 4.1-like 4	↑	-
Metabolic processes	rnd2	Rho family GTPase 2	↑	-
	mat1a	methionine adenosyltransferase I, alpha	↑	-
	fgl2	fibrinogen-like 2	↑	-
	hnrnpab	heterogeneous nuclear ribonucleoprotein A/B	↑	-
	ripply3	ripply3	↑	-
	oxsr1b	oxidative-stress responsive 1b	↑	-
	tbk1	TANK-binding kinase 1	↑	-

Table 23. Up- (↑) and down-regulated genes at 5 hpf with their specific functions and descriptions.

Functions	Genes	Descriptions	12 hpf	
			Up	Down
Translation	mrpl20	mitochondrial ribosomal protein L20	↑	-
	sepsecs	Sep (O-phosphoserine) tRNA:Sec (selenocysteine) tRNA synthase	↑	-
Biological process	vasp	vasodilator-stimulated phosphoprotein	↑	-
	sec14l1	SEC14-like 1 (*S. cerevisiae*)	↑	-
	plp1a	proteolipid protein 1a	↑	-
	atpif1	ATPase inhibitory factor 1	↑	-
	tbl3	transducin (beta)-like 3	↑	-
	commd1	copper metabolism (Murr1) domain containing 1	↑	-
	mybpc2b	myosin binding protein C, fast type b	↑	-
	acot9.2	acyl-CoA thioesterase 9.2	↑	-
Embryo develpment	dscam	down syndrome cell adhesion molecule	↑	-
Intra-and inter-cellular transport	rbp4	retinol binding protein 4, plasma	↑	-
	slc30a4	solute carrier family 30 (zinc transporter), member 4	↑	-
	kpna3	karyopherin (importin) alpha 3	↑	-
	atp2b2	ATPase, Ca++ transporting, plasma membrane 2	↑	-
	dscr3	down syndrome critical region gene 3	↑	-
	mfsd7	major facilitator superfamily domain containing 7	↑	-
	Snx16	sorting nexin 16	↑	-
Negative regulation of cell proliferation	lppr5b	lipid phosphate phosphatase-related protein type 5b	↑	-
	hhip	hedgehog interacting protein	↑	-
	apaf1	apoptotic protease activating factor 1	↑	-
RNA processing	myoc	myocilin	↑	-
	dgcr8	DiGeorge syndrome critical region gene 8	↑	-
	parn	poly(A)-specific ribonuclease (deadenylation nuclease)	↑	-

Transcription	foxf1	forkhead box F1	↑	-
	mef2ca	myocyte enhancer factor 2ca	↑	-
	ldb1a	LIM-domain binding factor 1a	↑	-
	znf367	zinc finger protein 367	↑	-
	nfe2l3	nuclear factor (erythroid-derived 2)-like 3	↑	-
	ets2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	↑	-
	hmga1b	high mobility group AT-hook 1b	↑	-
	il7r	interleukin 7 receptor	↑	-
	pde8a	phosphodiesterase 8A	↑	-
Metabolic processes	bckdha	branched chain keto acid dehydrogenase E1, alpha polypeptide	-	↓
	tcp1	t-complex polypeptide 1	-	↓
	march8	membrane-associated ring finger (C3HC4) 8	↑	-
	lrrc4c	leucine rich repeat containing 4C	↑	-
	ate1	arginyltransferase 1	↑	-
	aplp	amyloid beta (A4) precursor-like protein	↑	-
	sh2b1	SH2B adaptor protein 1	↑	-
	arl3	ADP-ribosylation factor-like 3	↑	-
	ripply3	ripply3	↑	-
	slit3	slit (Drosophila) homolog 3	↑	-
	ankfy1	ankyrin repeat and FYVE domain containing 1	↑	-
	crfb8	cytokine receptor family member b8	↑	-
	rgs5b	regulator of G-protein signaling 5b	↑	-
	oxsr1b	oxidative-stress responsive 1b	↑	-
	adck1	aarF domain containing kinase 1	↑	-
	spag1b	sperm associated antigen 1b	↑	-
	tbk1	TANK-binding kinase 1	↑	-
	zdhhc5a	zinc finger, DHHC-type containing 5a	↑	-
	trim35-24	tripartite motif containing 35-24	↑	-

Table 24. Up (↑) and down-regulated (↓) genes at 12 hpf with their specific function and descriptions.

Name of genes	5 hpf	
	Up	**Down**
zgc:174689	↑	-
zgc:171686	↑	-
zgc:103519	↑	-
zgc:153629	↑	-
zgc:85858	↑	-
zgc:65857	↑	-
zgc:154176	↑	-
zgc:64002	↑	-
zgc:158254	↑	-
si:dkey-21k24.2	↑	-
zgc:153031	↑	-
zgc:113983	↑	-

Table 25. Not annotated up (↑) and down-regulated (↓) genes at 5 hpf.

Name of genes	12 hpf	
	Up	**Down**
zgc:114123	↑	-
zgc:66337	↑	-
si:dkey-165a24.4	↑	-
zgc:114175	↑	-
si:dkey-11e23.5	↑	-
im:6903943	↑	-
si:dke-21k24.2	↑	-
zgc:77816	↑	-
zgc:112992	-	↓
zgc:171485	↑	-
zgc:153845	↑	-
si:dkey-71l1.1	↑	-
zgc:153893	↑	-
si:dkeyp-22b2.2	↑	-
zgc:64022	↑	-
zgc:73144	↑	-
zgc:122979	↑	-

zgc:73359	↑	-
zgc:153031	↑	-
si:dkey-21k24.2	↑	-
zgc:165519	↑	-
zgc:110655	↑	-
zgc:136758	↑	-
zgc:113983	↑	-

Table 26. Not annotated up (↑) and down-regulated (↓) genes at 12 hpf.

There was no replica for 24-hpf embryos sampled for microarray hybridization, so it was not possible to perform statistical analysis with the SAM program. The up- and down-regulated genes due to cortisol treatment were filtered on the basis of their fold changes. In total, 719 genes were up-regulated having fold change greater than 2 and 955 genes were down-regulated having fold change less than -2. The percentage of up and down-regulated genes at 24 hpf is shown in Figure 23, using a pie chart.

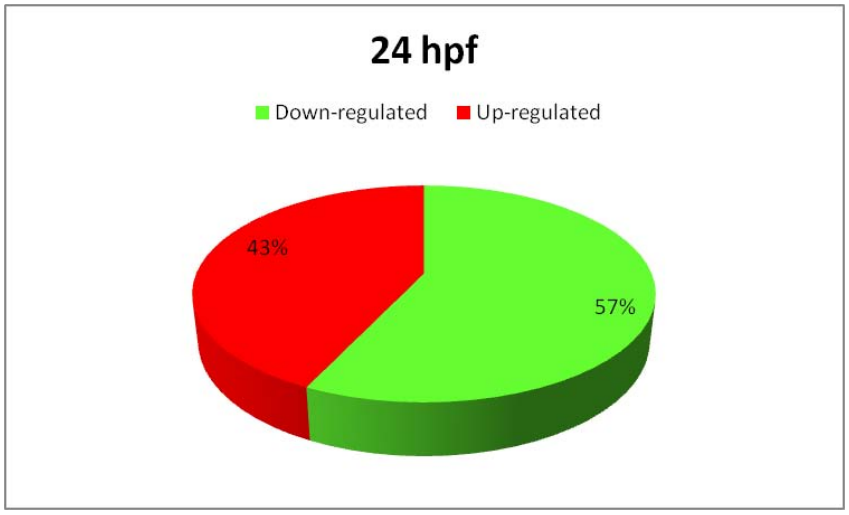


Figure 23. The pie chart displays percentages of up- and down-regulated genes at 24 hpf due to cortisol treatment. Genes in red are up-regulated and genes in green are down-regulated.

44. Analysis of gene expression by qPCR

qPCR was performed to analyse differential expression of genes. For this purpose, some cortisol-responsive genes that were identified by microarray technology were selected for performing relative and absolute qPCR.

44.1. Relative qPCR

Relative qPCR was performed to determine the change in the expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. The calibrator sample can be an untreated control or a sample at time zero in a time-course study (Livak and Schmittgen, 2001). A total of seven cortisol-responsive genes were selected to perform Relative qPCR to check the effects of cortisol in treated fish compared to control: *vasp, plp1a, stmn2a, parn, notch2, tsc22d3* and *mat1a.* Three replicate samples from cortisol-treated and control embryos were collected at 10, 12, 20 and 24 hpf.

The *vasp* and *plp1a* genes did not show significant ($p > 0.05$) high or low expression at 12 and 24 hpf of F1 when *rplp0* was used as reference gene (Fig. 24).

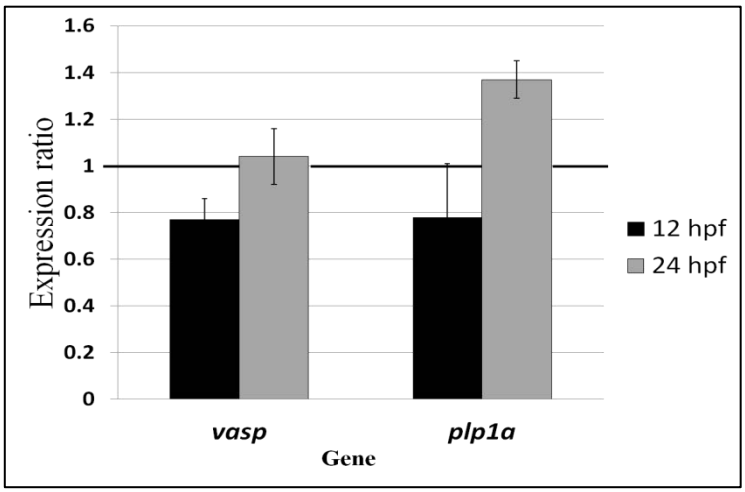


Figure 24. Expression of *vasp* and *plp1a* gene at 12 and 24 hpf of F1 compared to control (set at 1) when *rplp0* gene was used as reference gene. Values are represent as mean±SEM (n= 9).

At 10 hpf of F2 generation, *vasp* and *plp1a* did not show significant up regulation, when *ef1α* was used as reference gene (Fig. 25A). Interestingly, *vasp* gene at 24 hpf of F1 and 12 hpf of F2 was significantly down regulated ($p < 0.001$) when *ef1α* was used as reference gene, as shown in

Figures 25C and 25B. But *plp1a* gene was significantly ($p < 0.05$) down regulated only at 12 hpf of F2 generation (Fig. 25B).

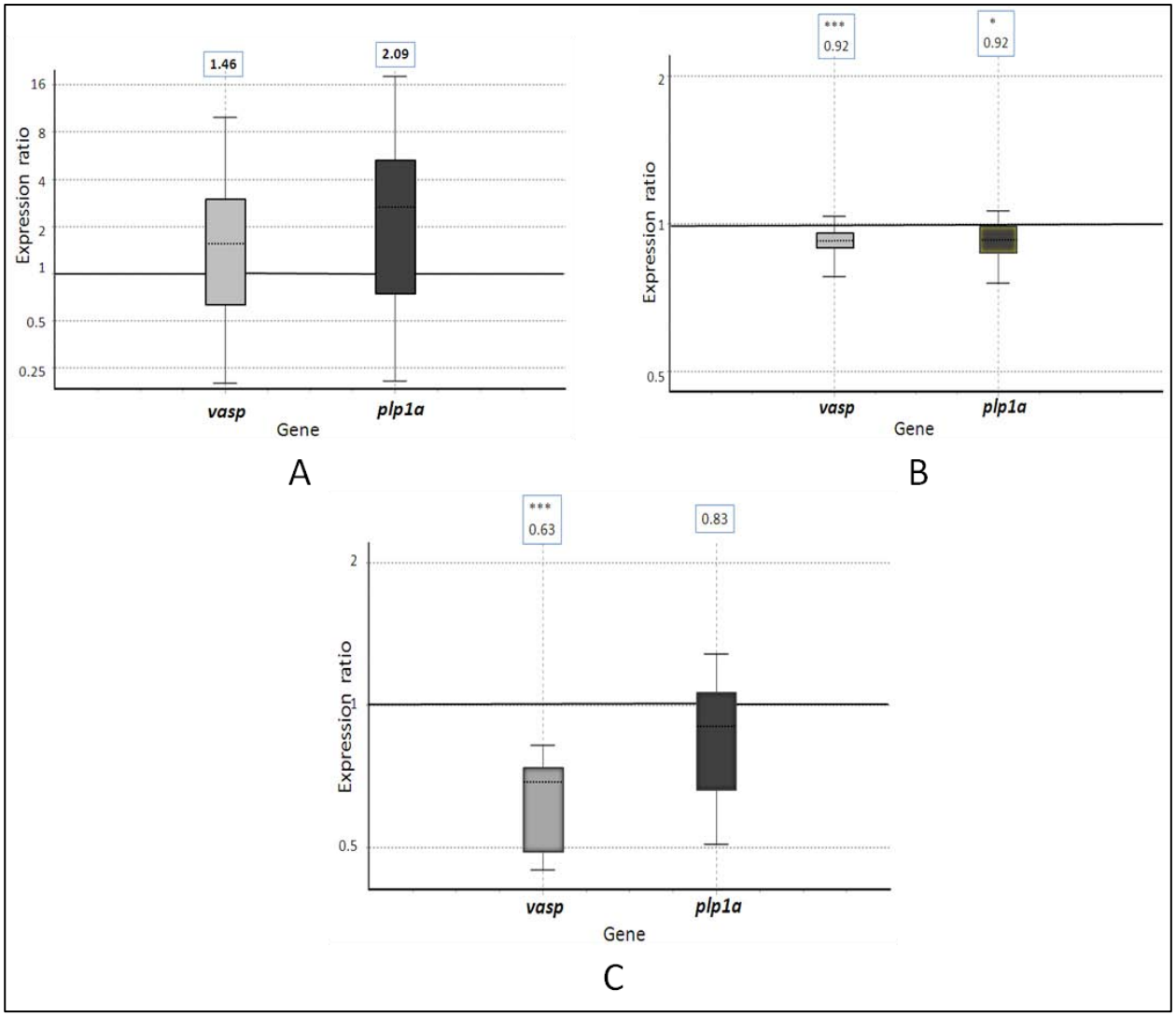


Figure 25. Whisker-box plot represents expression of *vasp* and *plp1a* gene compared to control (set at 1) when *ef1α* gene was used as reference gene. Expression ratio values are shown on the top of top whisker. A: 10 hpf F2. B: 12 hpf F2 and C: 24 hpf F1. Symbol (*) indicates that the difference in the expression levels is statistically significant ($p < 0.05$)= *; ($p < 0.01$)= **; ($p < 0.001$) = ***.

This analysis discloses that statistical significance and temporal trends of the measurements are dependent upon the choice of the reference gene. With *rplp0*, statistical significance was never achieved, whereas with *ef1α*, it was attained with both *vasp* and *plp1a* at 12 hpf and with *vasp* alone at 24 hpf. Moreover, the plots with *ef1α* suggest a decrement of expression from 10 hpf to

24 hpf for both *vasp* and *plp1a*, which is still compatible with the up-regulation of both genes observed with microarray analysis at 12 hpf.

Figure 26 shows the expression of *fkbp5, tsc22d3, mat1a, notch2, parn* and *stmn2a* genes at 20 hpf of F1 compared to control (set at 1) when *ef1α* gene was used as reference gene. In this case there were no significant up- or down-regulation of the investigated genes due to cortisol treatment.

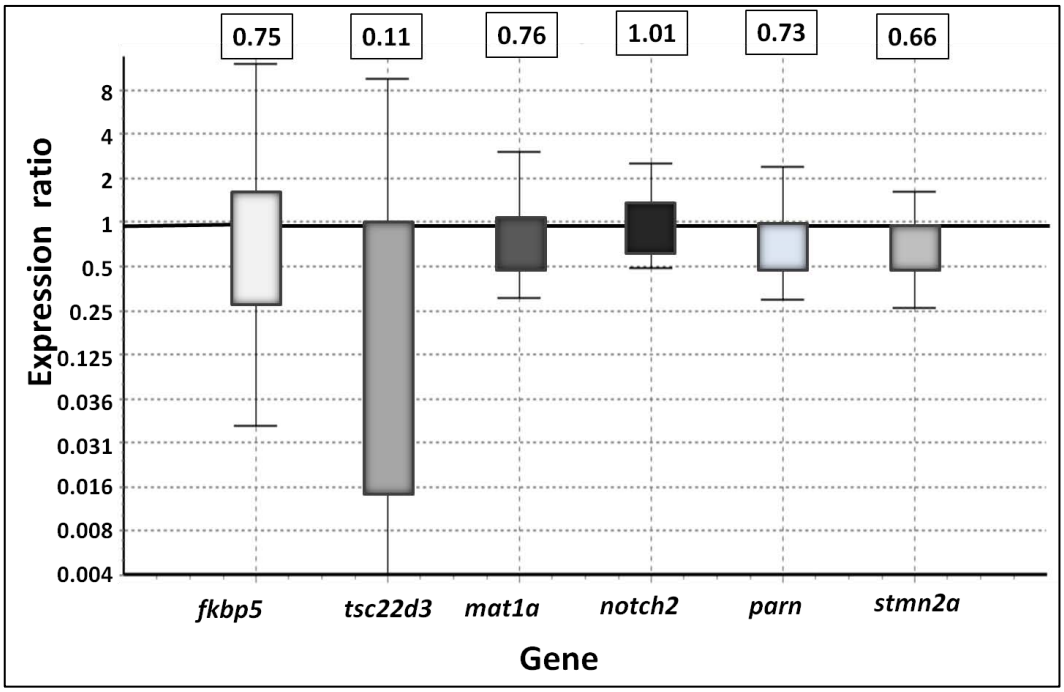


Figure 26. Expression of *fkbp5, tsc22d3, mat1a, notch2, parn* and *stmn2a* genes at 20 hpf of F1 compared to control (set at 1) when *ef1α* gene was used as reference gene. Expression ratio values are shown on the top whisker-box plots.

Relative qPCR technique was also performed to analyse *igf-2a* and *casp8* gene expression of embryos treated with z-*gr* mRNA+EtOH and z-*gr* mRNA+Cortisol at 5 and 10 hpf of F1 compared with a wild-type control (WT), whose expression level was set at 1. With z-*gr* mRNA+EtOH, the *igf-2a* gene was significantly ($p < 0.05$) up-regulated at 5 and 10 hpf, whereas the *casp8* gene was significantly ($p < 0.001$) up-regulated at 5 hpf only (Fig. 27).

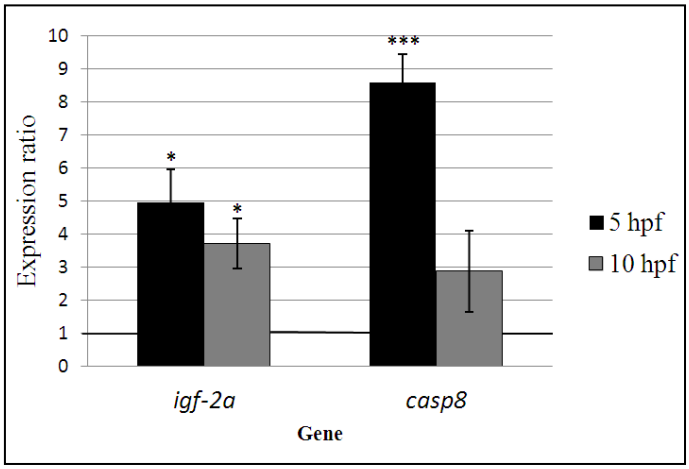


Figure 27. Expression ratio of *igf-2a* and *casp8* gene in z-*gr* mRNA+EtOH treated embryos at 5 and 10 hpf of F1 compared to WT (set at 1) when *ef1α* gene was used as reference gene. Each bar represents the mean±SEM (n = 9). Symbol (*) indicates that the difference in the expression levels is statistically significant ($p < 0.05$)= *; ($p < 0.01$)= **; ($p < 0.001$) = ***.

With z-*gr* mRNA+Cortisol, the *igf-2a* gene was significantly ($p < 0.01$ and $p < 0.05$) up-regulated at 5 and 10 hpf, respectively, whereas the *casp8* gene was significantly ($p < 0.01$) up-regulated at 5 hpf only (Fig. 28).

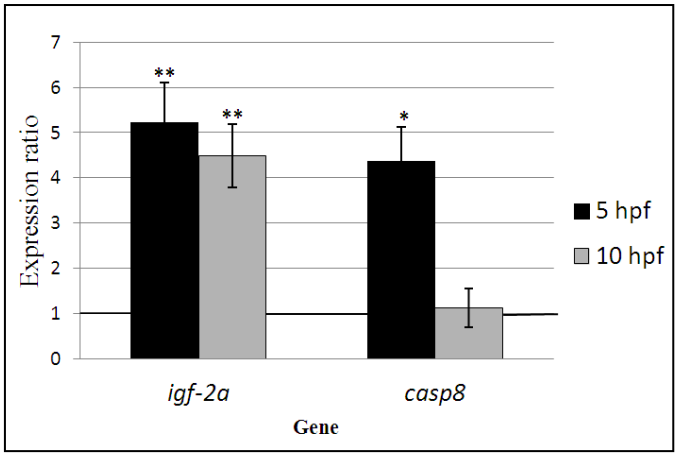


Figure 28. Expression ratio of *igf-2a* and *casp8* gene in z-*gr* mRNA+Cortisol treated embryos at 5 and 10 hpf of F1 compared to WT (set at 1) when *ef1α* gene was used as reference gene. Each bar represents the mean±SEM (n = 9). Symbol (*) indicates that the difference in the expression levels is statistically significant ($p < 0.05$)= *; ($p < 0.01$)= **; ($p < 0.001$) = ***.

44.2. Analysis of mRNA expression by absolute qPCR

The absolute qPCR was performed to investigate the levels of mRNA expression of two genes of interest: *casp8* and *mcm6*. Fertilized eggs were subjected to seven treatments by immersion in solutions containing: WT, Cortisol, RU486, z-*gr* mRNA+Cortisol, z-*gr* mRNA+RU486, z-*gr* mRNA and *gr*MO-ATG-1. Treated embryos were sampled at 5 and 10 hpf. The results of absolute qPCR were reported as mRNA copies, after normalization of the transcript amount with respect to the reference gene (*ef1a*).

Figure 29 shows the normalized gene expression of *casp8* transcripts at 5 hpf, as determined by absolute qPCR. Levels were significantly higher ($p < 0.001$) in z-*gr* mRNA+Cortisol than in the other six treatments (WT, Cortisol, RU486, z-*gr* mRNA+RU486, z-*gr* mRNA and *gr*MO-ATG-1). The transcripts of the *gr*MO-ATG-1 group were significantly lower ($p < 0.01$) than the z-*gr* mRNA+RU486 treatment group.

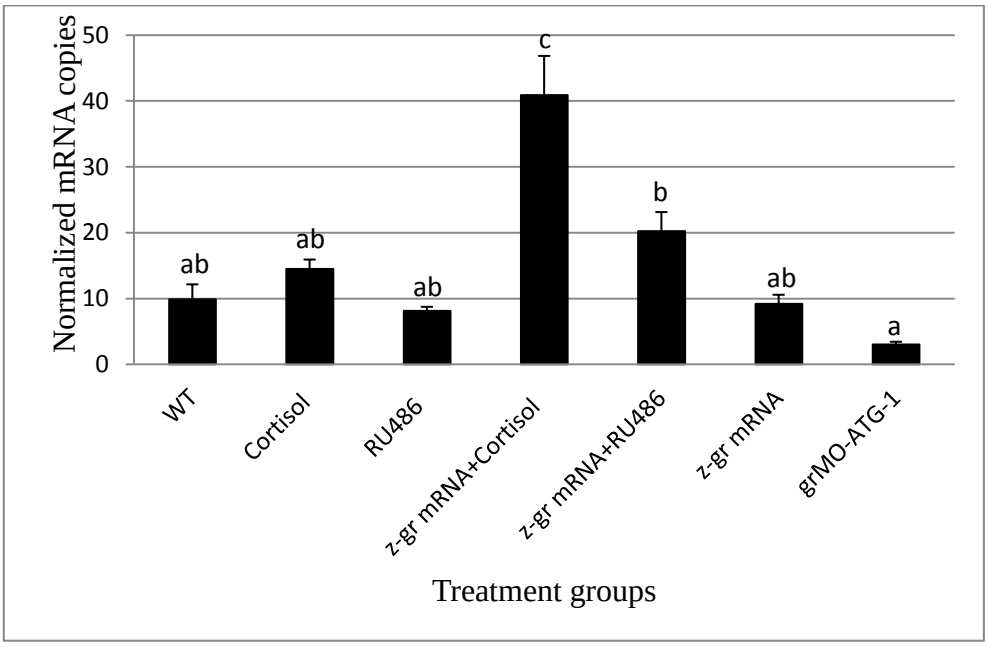


Figure 29. Normalized gene expression of ***casp8*** transcripts of different treatment groups at **5 hpf**. Values represent the means±SEM (n = 9) of expressions, as determined by absolute qPCR. Different letters indicate significant differences among treatment groups ($p < 0.05$, one-way ANOVA, followed by Tukey HSD multiple comparison test).

Surprisingly, normalized *casp8* transcript levels at 10 hpf were significantly higher ($p < 0.05$) in *gr*MO-ATG-1 than in WT, Cortisol, RU486, z-*gr* mRNA+Cortisol and z-*gr* mRNA treatment groups, whereas there was no difference between *gr*MO-ATG-1 and z-*gr* mRNA+RU486 (Fig. 30). There were no significant differences ($p > 0.05$) among Cortisol, RU486 and z-*gr* mRNA. The transcripts of RU486 group were significantly lower ($p < 0.01$) than z-*gr* mRNA+Cortisol, z-*gr* mRNA+RU486 and *gr*MO-ATG-1 groups.

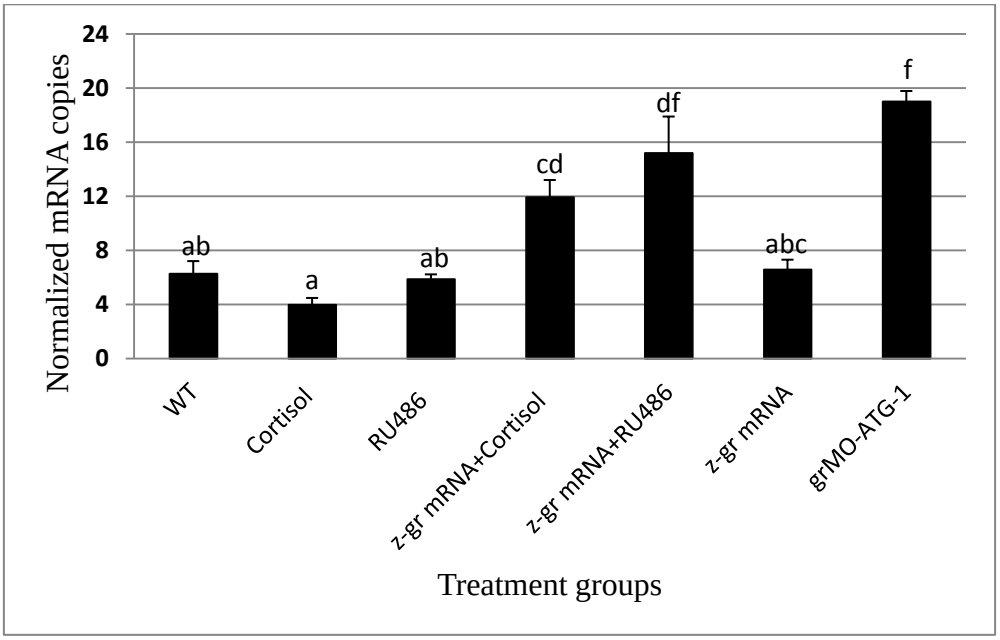


Figure 30. Normalized gene expression of ***casp8*** transcripts of different treatment groups at **10 hpf**. Values represent the means±SEM (n = 9) of expressions, as determined by absolute qPCR. Different letters indicate significant differences among treatment groups ($p < 0.05$, one-way ANOVA, followed by Tukey HSD multiple comparison test).

The normalized gene expression of *mcm6* transcripts at 5 hpf with respect to the different treatments, as determined by absolute qPCR, is shown in Figure 31. Levels in Cortisol and RU486 were significantly higher ($p < 0.01$) than in z-*gr* mRNA+Cortisol, z-*gr* mRNA+RU486, z-*gr* mRNA and *gr*MO-ATG-1 treatment groups, whereas z-*gr* mRNA+Cortisol, z-*gr* mRNA and *gr*MO-ATG-1 groups did not show any significant difference ($p > 0.05$). There were also no significant transcript differences between WT and z-*gr* mRNA+RU486 groups.

Results

Figure 32 shows the normalized gene expression of *mcm6* transcripts at 10 hpf as determined by absolute qPCR. Levels at 10 hpf were significantly higher ($p < 0.01$) in WT and RU486 than in the other five treatment groups. There were also no significant transcript differences ($p > 0.05$) among z-*gr* mRNA+Cortisol, z-*gr* mRNA+RU486 and z-*gr* mRNA treatment groups.

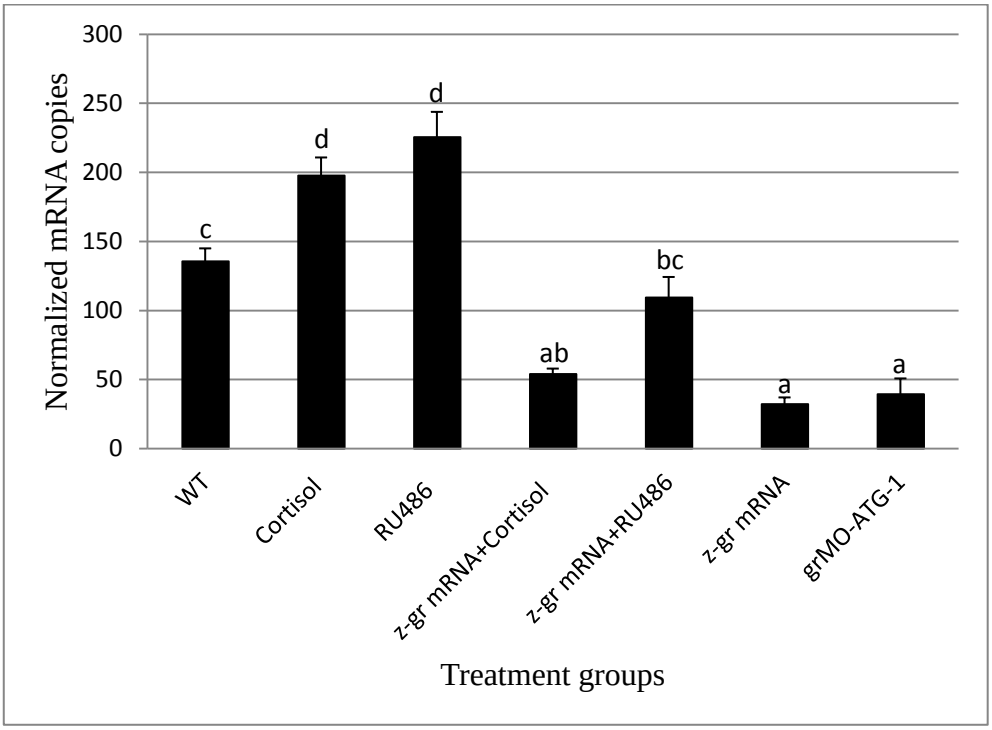


Figure 31. Normalized mRNA copies of ***mcm6*** transcripts of different treatment groups at **5 hpf**. Values represent the means±SEM (n = 9) of expressions, as determined by absolute qPCR. Different letters indicate significant differences among treatment groups ($p < 0.05$, one-way ANOVA, followed by Tukey HSD multiple comparison test).

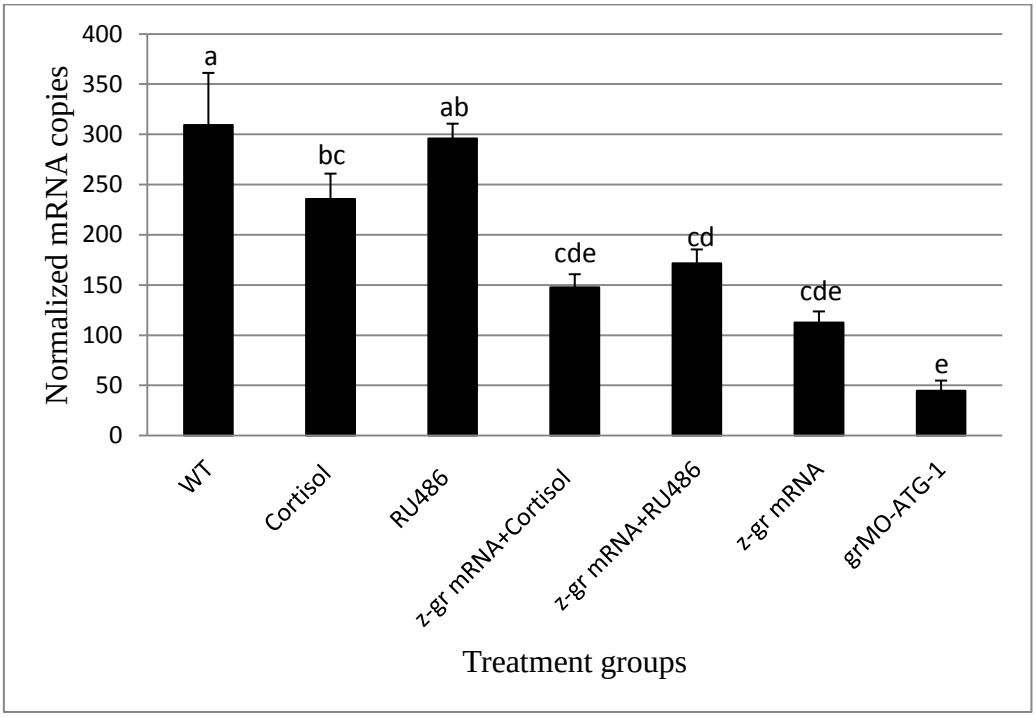


Figure 32. Normalized mRNA copies of ***mcm6*** transcripts of different treatment groups at **10 hpf**. Values represent the means±SEM (n = 9) of expressions, as determined by absolute qPCR. Different letters indicate significant differences among treatment groups ($p < 0.05$, one-way ANOVA, followed by Tukey HSD multiple comparison test).

45. Morphological effects

The effects of different treatments of fertilized eggs (WT, Cortisol, RU486, z-*gr* mRNA +Cortisol, z-*gr* mRNA+RU486, z-*gr* mRNA and *gr*MO-ATG-1) on zebrafish larvae were assessed by the observation of morphological effects. The mortality and abnormal phenotypes of treated larvae were assessed at 3 dpf. Significantly higher mortality was observed in *gr*MO-ATG-1 -injected larvae than in the other treatment groups (Table 27). There were no significant difference among WT, Cortisol, RU486 and treated larvae. Significantly higher percentage of abnormal phenotypes was observed in *gr*MO-ATG-1 and z-*gr* mRNA+Cortisol treated larvae. Some abnormal phenotypes found among z-*gr* mRNA+Cortisol, z-*gr* mRNA+RU486 z-*gr* mRNA and *gr*MO-ATG-1 treated larvae at 3 dpf are shown in Figure 33. Larvae were affected by growth delay, reduced or oedematous yolk extension, curved body shape, persistent voluminous yolk sac, uninflated swim bladder and rudimentary caudal fin with aberrant circular swimming after mechanical stimulus (Fig. 33) .

Treatment groups	Total no. of larvae	Mortality (%) (Mean±SD)	Abnormality (%) (Mean±SD)
WT	439	7 ± 2^{ab}	2 ± 1^{a}
Cortisol	273	5 ± 1^{a}	3 ± 1^{a}
RU486	265	8 ± 5^{ab}	5 ± 2^{a}
z-*gr* mRNA+ Cortisol	247	20 ± 6^{bc}	25 ± 8^{c}
z-gr mRNA+ RU486	185	13 ± 4^{c}	12 ± 4^{b}
z-*gr* mRNA	251	12 ± 3^{abc}	9 ± 5^{ab}
***gr*MO-ATG-1**	352	34 ± 9^{d}	45 ± 13^{d}

Table 27. Effects of different treatments on the percentage of mortality and abnormal phenotypes among 3 dpf larvae. Data were pooled from at least 3 experiments. Values represent the means±SD. Different letters indicate significant differences among treatment groups in each column ($p < 0.05$, one-way ANOVA, followed by LSD multiple comparison test).

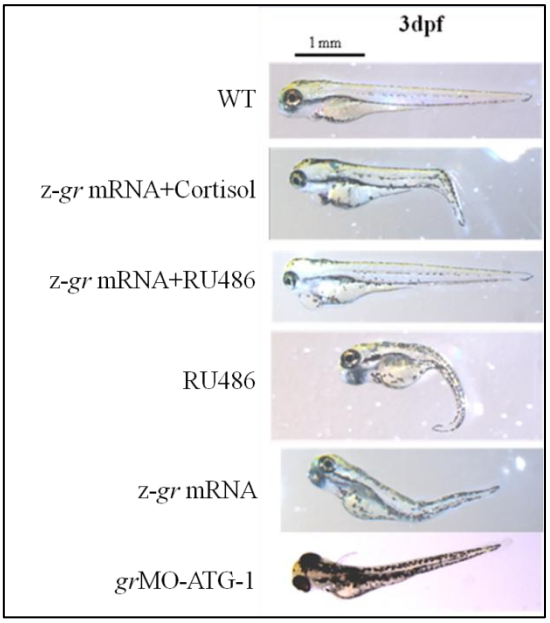


Figure 33. Abnormal phenotypes of larvae at 3 dpf after treatment with z-*gr* mRNA+Cortisol, z-*gr* mRNA+RU486, RU486, z-*gr* mRNA and *gr*MO-ATG-1 as compared to WT. Larvae are presented as lateral view.

46. Length measurement

To verify the morphogenetic effect of steroid hormone, the measurement of length for cortisol-treated fish and control fish was performed for F1, F2, F3 and F4 generation at 60, 90, 120, 150 and 180 dpf. The lengths of cortisol-treated fish of F1 generation at 150 and 180 dpf were significantly greater ($p < 0.05$) than in control fish, whereas there were no significant difference of length at 60, 90 and 120 dpf (Fig. 34).

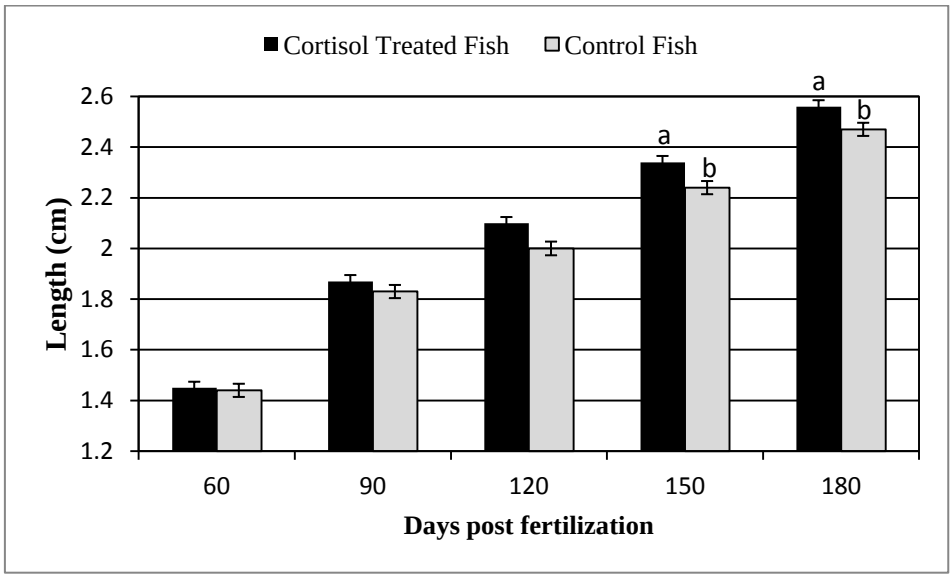


Figure 34. The length of the first generation (**F1**) fish at different time points. Values are mean ± SEM (n = 100). Symbols with different letters (a, b) are significantly different ($p < 0.05$, paired sample t-test) from cortisol-treated fish to control.

The length of second generation (F2) at 60, 90, 120, 150 and 180 dpf of cortisol-treated fish and control fish is shown in Figure 35. In the second generation, at 120, 150 and 180 dpf the length of cortisol-treated fish were significantly higher ($p < 0.05$) than control fish. In contrast, there was no significant difference of length at 60 and 90 dpf between treatment and control fish. In third generation (F3), only the length of control fish was higher ($p < 0.05$) than cortisol-treated fish at 60 dpf, which was at variance with the results of F1 and F2 generations, but there was no significant difference ($p > 0.05$) of length at 90, 120, 150 and 180 dpf (Fig. 36). In the fourth generation (F4), the lengths of cortisol-treated fish and control fish were more or less similar, as there was no difference at each time point except at 90 dpf, when the length of cortisol-treated fish was significantly higher than control (Fig. 37).

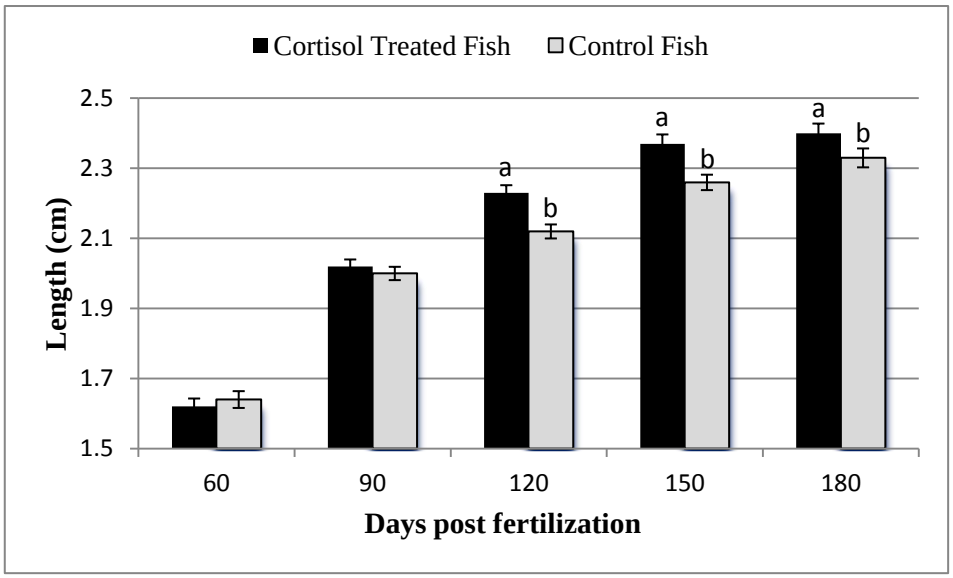


Figure 35. Length of the second generation (**F2**) fish at different time stages . Values are mean±SEM (n= 100). Symbols with different letters (a, b) are significantly different ($p < 0.05$, paired sample t-test) from cortisol-treated fish to control.

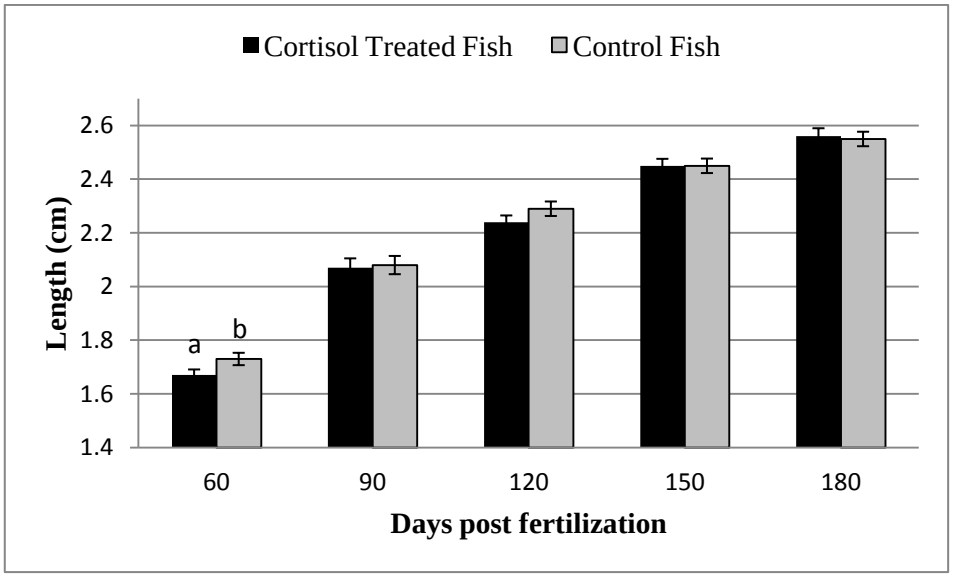


Figure 36. The length of the third generation (**F3**) fish at 60, 90, 120, 150 and 180 dpf. Values are mean±SEM (n = 80). Symbols with different letters (a, b) are significantly different ($p < 0.05$, paired sample t-test) from cortisol-treated fish to control.

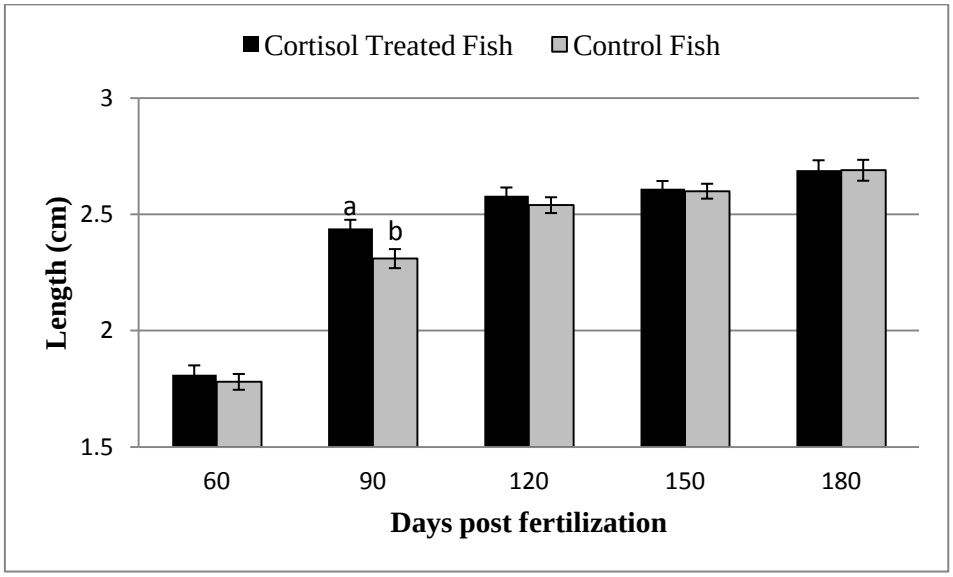


Figure 37. The length cortisol treated and control fish of the fourth generation (**F4**) at different time points. Values are mean±SEM (n= 45). Symbols with different letters (a, b) are significantly different ($p < 0.05$, paired sample t-test) from cortisol-treated fish to control.

47. Weight measurement

The weight of cortisol-treated and control fish were measured up to the F4 generation at different time points (60, 90, 120, 150 and 180 dpf) as for the length. Figure 38 shows that the weight of cortisol-treated and control fish of F1 were significantly higher ($p < 0.05$) than control at 150 and 180 dpf, whereas there were no significant weight difference at 60, 90 and 120 dpf. In F2, the weight of control fish were significantly lower ($p < 0.05$) at 120, 150 and 180 dpf than cortisol-treated fish, while, at 60 and 90 dpf, there was no statistical weight difference (Fig. 39).

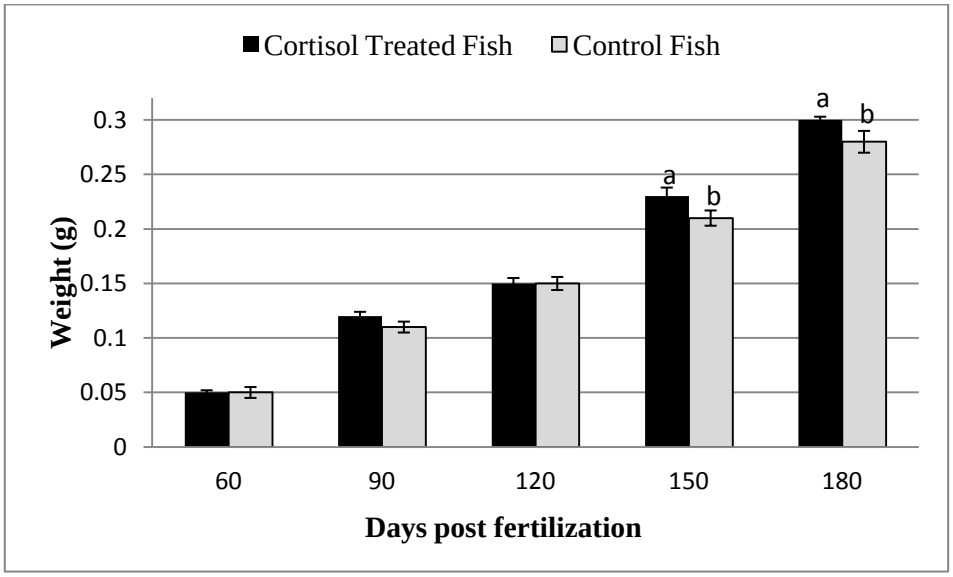


Figure 38. The weight of cortisol-treated and control fish of **F1** generation. Each bar represents the mean±SEM (n = 100). Different letters (a, b) denote statistical difference between treatment and control fish at each time point ($p < 0.05$, paired sample t-test).

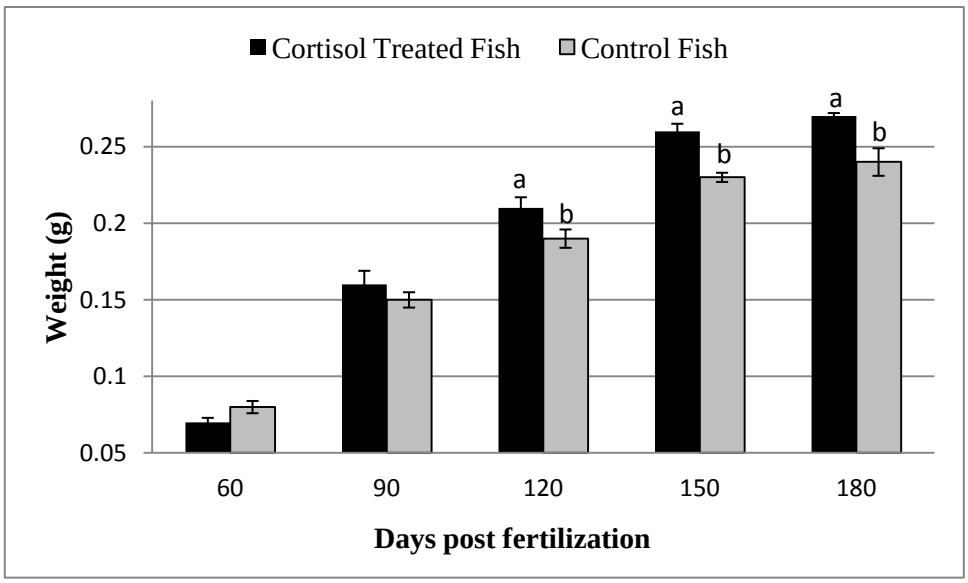


Figure 39. The weight of cortisol-treated and control fish of **F2** generation at different days post fertilization. Each bar represents the mean±SEM (n = 100). Bars with different letters (a, b) denote statistical difference between treatment and control fish at each time point ($p < 0.05$, paired sample t-test).

Surprisingly, F3 and F4 obtained higher weight in control fish than cortisol-treated fish except the weights at 60 and 90 dpf of F4 generation. But in the third generation, only the weight of control fish at 120 dpf was significantly higher ($p < 0.05$) than in cortisol-treated fish (Fig. 40).

Results

There was no significant weight difference ($p > 0.05$) at the other time points of F3 generation. In fourth generation, the weights of cortisol-treated fish were significantly lower ($p < 0.05$) at 120 and 150 dpf, whereas the weight of control fish was significantly lower at 60 dpf (Fig. 41).

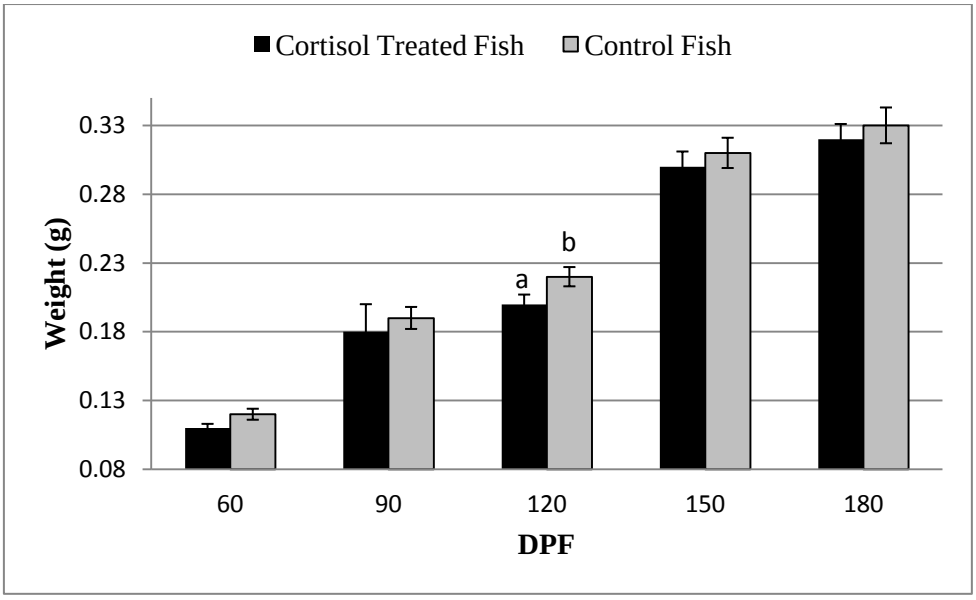


Figure 40. The weight of cortisol-treated and control fish of **F3** generation. Each bar represents the mean±SEM (n = 100). Different letters (a, b) denote statistical difference between treatment and control fish at each time point ($p < 0.05$, paired sample t-test).

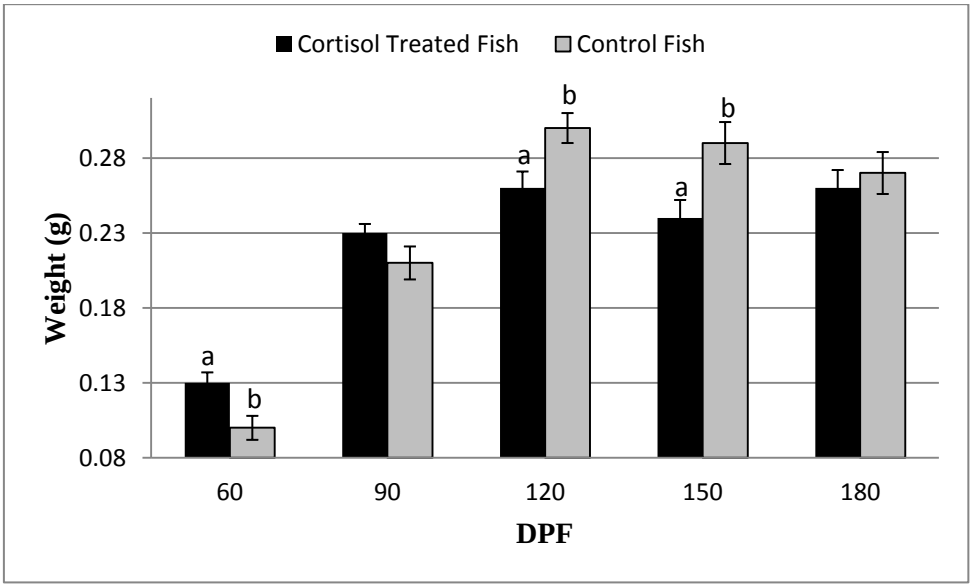


Figure 41. The weight of cortisol-treated and control fish of **F4** generation at different days post fertilization. Each bar represents the mean±SEM (n = 100). Bars with different letters (a, b) denote statistical difference between treatment and control fish at each time point ($p < 0.05$, paired sample t-test).

Results

In generation F4, zebra and leopard embryos were treated in four different ways: Z4+Cortisol, Z4+EtOH, L4+Cortisol and L4+ EtOH. Figure 42 and 43 shows the length and weight of F4 fish at different time points, respectively. There were no significant length difference among Z4+Cortisol, Z4+EtOH, L4+Cortisol and L4+ EtOH groups at 60, 120, 150 and 180 dpf, except at 90 dpf, when the length of Z4+Cortisol treatment group was significantly higher ($p < 0.05$) than L4+EtOH treatment group, while there was no significant difference between Z4+EtOH and L4+Cortisol group at same time point (Fig. 42).

The weight of the four different treatment groups did not show any significant difference at 180 dpf. At 60 and 90 dpf, the weight of Z4+EtOH group was significantly higher ($p < 0.01$) than L4+ EtOH group, but there were no significant difference between Z4+Cortisol and L4+Cortisol group fish (Fig. 43). The weight of Z4+Cortisol group was significantly lower than the weight of L4+ EtOH group fish at 120 and 150 dpf, whereas Z4+EtOH and L4+Cortisol groups did not show any significant difference (Fig. 43).

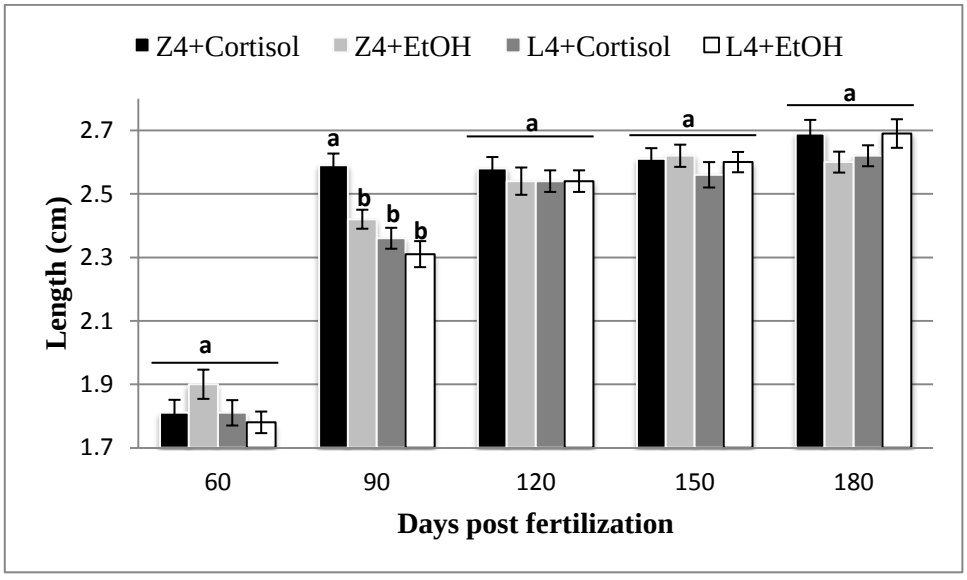


Figure 42. The length of F4 zebra and leopard fish at different days post fertilization with cortisol and EtOH treatment. Values are presented as mean±SEM, bars with different letters are statistically different at the same time points as determined by ANOVA ($p < 0.05$) followed by Tukey's post hoc multiple comparison.

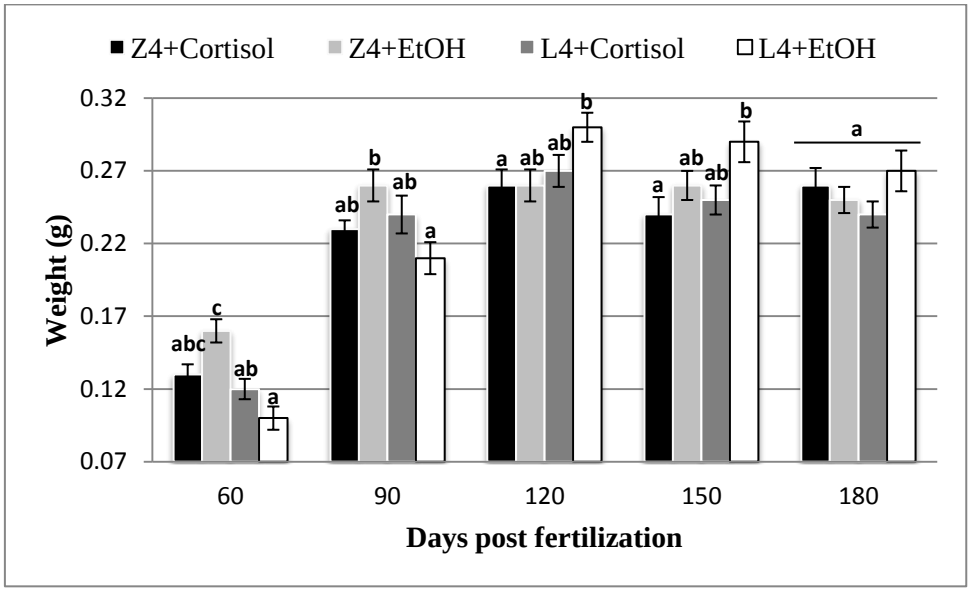


Figure 43. The weight of F4 zebra and leopard fish at different days post fertilization with cortisol and EtOH treatment. Values are presented as mean±SEM, bars with different letters are statistically different at the same time points as determined by ANOVA ($p < 0.05$) followed by Tukey's post hoc multiple comparison.

48. Analysis of puberty onset

The day of first oviposition and the number of laid eggs were checked to observe the effect of cortisol on puberty onset in zebrafish. The first oviposition of cortisol-treated fish started from 92, 91, 116 and 83 dpf for F1, F2, F3 and F4 generations, respectively (Table 28). For control fish, the first oviposition started from 90, 96, 115 and 83 dpf for F1, F2, F3 and F4 generations, respectively (Table 28). Surprisingly, third and fourth generation exhibited very later and earlier ovipositions, respectively.

	F1(dpf)	**F2(dpf)**	**F3(dpf)**	**F4(dpf)**
Cortisol treated fish	92	91	116	83
Control fish	90	96	115	83

Table 28. The first oviposition (dpf) of cortisol-treated and control fish for F1, F2, F3 and F4 generations.

The number eggs laid at first oviposition of cortisol-treated and control fish for F1, F2, F3 and F4 generations is shown in Figure 44. The number of eggs of cortisol-treated fish declined

steadily from F1 to F3 generation, with a higher declination observed at F4 generation. The number of eggs of cortisol treated fish at F4 generation was significantly lower ($p < 0.05$) than control. On the other hand, the number of eggs of cortisol-treated and control fish did not show any significant difference ($p > 0.05$) in F1, F2 and F3 generations .

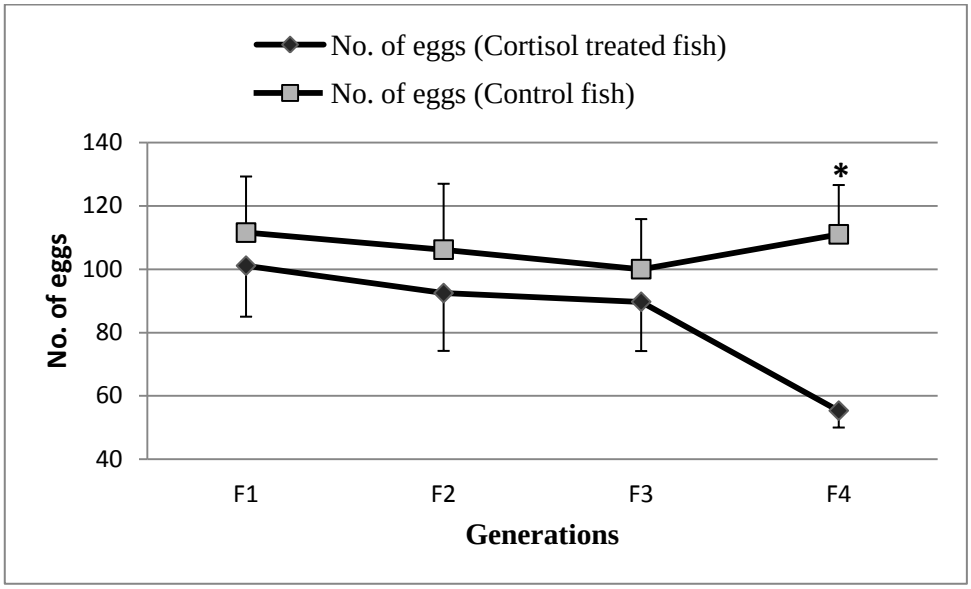


Figure 44. The number of eggs laid at first oviposition for cortisol-treated and control fish of F1, F2, F3 and F4 generations. Each values represent mean±SEM, **(*)** symbol denotes statistical difference between no. of eggs of cortisol-treated and control fish with ($p < 0.05$).

DISCUSSION

This thesis work provides experimental evidence that the enrichment of cleaving eggs with exogenous cortisol can alter the contents of a wide range of transcripts during the early stages of embryo development in zebrafish. Immunoassay showed that immersion of eggs for 2 h in a cortisol solution is a valid procedure to increase the deposition of the steroid in the embryo, thus mimicking an enhanced corticoid transfer into the blastomeres from an ooplasmic store derived from the maternal circulation in a stressogenic environment. This similarity rests on the cortisol availability to the embryo, while the involved processes are different.

Enrichment is initially facilitated by the inward flux of external medium needed for the expansion of egg volume just after fertilization (Auperin and Geslin, 2008). Yet, a relatively high concentration of external free cortisol is required to compensate for its low cellular permeability due to intermediate polarity that is complicated by chorion hardening before cleavage. The ooplasmic supply is instead the result of prolonged contact of more permeable oocytes in the ovary with low levels of circulating unbound steroid that is then retained in the yolk mass. During treatment, embryos are exposed to two steroid sources, internal and external, with superimposed transport kinetics. Afterwards, the situation becomes more similar to that of the control with progressive utilization of internalized cortisol. Throughout embryogenesis, there is no contribution by newly synthesized cortisol, because this capacity is acquired only after hatch (Alsop and Vijayan, 2008).

A comparable bathing approach was adopted in rainbow trout (Auperin and Geslin, 2008; Li *et al*., 2010), where it was found to follow first-order kinetics (Belvedere *et al*., 1999), as an alternative to stressing the mother during oogenesis (Eriksen *et al*., 2006) or direct cold stressing of embryos (Auperin and Geslin, 2008). These alternatives are impractical in zebrafish due to its asynchronous oogenesis with multiple spawning and thermal sensitivity, but cortisol microinjection into one-cell embryos was successfully applied to amplify its developmental effects in this species (Nesan and Vijayan, 2012). However, in the present work, the permeation technique was preferred in order to treat large numbers of eggs, since the time-window available for microinjection before the onset of first cleavage is short (about 40 min).

Of particular interest is the finding by microarray analysis that cortisol enrichment caused a significant up-regulation of the contents of one hundred transcripts, with no down-regulation, already in embryos at 5 hpf. Bioactivity of cortisol, either maternal or exogenous, is expected in zebrafish embryos because the mRNA encoding its main cognate receptor, GR, is the most abundant among maternal transcripts encoding nuclear and membrane steroid receptors in

ovulated oocytes (Pikulkaew *et al*., 2010). Its translation into protein to act as GR-cortisol complex on gene targets is to be assumed in the embryo, because the activation of the GR signaling pathway is essential for mesoderm formation in zebrafish (Nesan *et al*., 2012). Yet, invoking the genomic mechanism of action of cortisol to explain the observed transcript up-regulation is problematic. First of all, at 5 hpf, corresponding to the end of the blastula stage, the embryo still contains hundreds of the maternal transcripts synthesized during the diplotene stage of meiosis I from the lampbrush chromosomes of the primary oocyte (Pelegri, 2003). These include the "medium degrading group" of maternal mRNAs which start to be eliminated after the mid-blastula transition (MBT) at 3 hpf and are mostly removed during early gastrulation (Mathavan *et al*., 2005; Ferg *et al*., 2007). Secondly, up to MBT, there is barely any opportunity for zygotic transcription, because blastomeres divide synchronously every 14-15 min without interphase (Kane and Kimmel,1993). Thereafter, with the lengthening of the cell cycle, a G phase is progressively introduced and expanded allowing activation of zygotic transcription, which still remains at low levels during the blastula stage (Tadros and Lipshitz, 2009), though some zygotic transcription is required for gastrulation just after MBT (Zamir *et al*., 1997).

These facts suggest that the up-regulated transcripts at 5 hpf are likely of maternal origin and that their increased contents reflect a slowing down of their programmed degradation. The lack of down-regulation may be explained by assuming that the degradation process is already so fast that a further acceleration is unfeasible. How cortisol may delay the pace of maternal mRNA degradation is difficult to be clarified, because many agents are involved. The destruction depends initially upon maternally transcribed/translated products, but is later on reinforced by zygotic proteins and microRNAs (Tadros and Lipshitz, 2009).

This kind of cortisol interference does not apply to the dys-regulated (that is up- or down-regulated) transcripts after gastrulation, because at 12 hpf, with the onset of the segmentation period and somitogenesis, the transition from maternal to zygotic mRNAs is mostly completed. At this stage, cortisol appears to influence the rates of zygotic transcription with a prevalent up-regulation and a minor down-regulation. The important point is that, although 77% of the altered transcripts at 12 hpf are different from those at 5 hpf, there are 30 transcripts in common, which would imply that cortisol can concordantly affect corresponding maternal and zygotic transcripts by presumably unrelated mechanisms. Although zygotic transcripts can affect maternal ones, the other way around remains to be explored. In particular, it is unclear whether zygotic transcription is directly altered by cortisol mechanism of action or indirectly by links with cortisol dys-regulated maternal transcripts or both. Preliminary microarray evidence indicates that, at 24 hpf, the number of affected zygotic transcripts has grown more than ten-fold, with a prevalence of

down-regulated transcripts, suggesting both an amplification and a mode transition in the cortisol control of transcription.

Indeed, the role of the GR-cortisol complex in modulating transcript abundance in the embryo is more complex than what inferred from these results. In particular, a comparable microarray analysis carried out by Pikulkaew *et al*. (2011) on embryos microinjected with morpholinos to block translation of maternal and zygotic *gr* transcripts (*gr*MO-ATG, as in the present study), thus impairing cortisol action, showed not just a reversed, but an entirely different pattern of transcript dys-regulation, with 114 up- and 37 down-regulated mRNAs at 5 hpf, 68 up- and 52 down-regulated mRNAs at 10 hpf, and 43 up- and 6 down-regulated mRNAs in common. Besides greater proportions of down-regulated and in common transcripts, the most represented functional groups were those of embryo development, metabolic processes, signaling and negative regulation of cell proliferation while, in this thesis, metabolic processes, biological processes and intra-and inter-cellular transport were more represented. It seems that excess cortisol affects mainly dynamic processes, whereas the lack of GR is associated with regulatory drawbacks.

A crucial aspect, is that significantly higher incidence of abnormal phenotypes and mortality were observed in *gr*MO-ATG-1 larvae at 3 dpf, thus confirming a previous report by Pikulkaew *et al*. (2011), in which these defects were rescued with trout *gr2* mRNA. Since treatment with morpholino to selectively block the splicing process of zygotic *gr* mRNA yielded normal larvae, their work underlined the relevance of maternal *gr* mRNA in the control of embryogenesis. Here, the gain of function experiment on morphogenesis disclosed that also the microinjection with z-*gr* mRNA slightly compromised larval development and survival, which were significantly further worsened by the combination of z-*gr* mRNA plus exogenous cortisol. This suggests that not only maternal *gr* mRNA is essential to the embryo, but also that the amount stored in the oocyte must be precisely adjusted in the presence of maternal cortisol.

The gain of function with excess corticoid was not harmful for normal development, as documented along four generations. Such result is at variance with what described by Nesan and Vijayan (2012), who found that elevation of cortisol level by microinjection caused heart deformities in 50% of larvae, such as pericardial edema and malformed chambers, defects described also in morphant embryos and larvae after GR knockdown (Pikulkaew *et al*., 2011). The former Authors concluded that normal catabolism of maternally deposited cortisol in the embryo with progressive decrease to a minimum around hatch at 48 hpf is necessary to relieve a corticoid repression of genes involved in cardiac organogenesis. This claim is intriguing because, in embryos microinjected with 32 pg, they measured only a small increment of cortisol contents

which remained constant till hatch (3.5 – 3.8 pg/embryo from 12 to 36 hpf), whereas in the control there was a modest decline (from 2.7 to 2.0-2.2 pg during the same period). The reason why a decline was not observed after such limited enrichment (30% more) remains unclear, but the point is that, in the present study, bathing of eggs in cortisol solution increased around 10-fold the cortisol concentration in 2-hpf-embryos without greater abnormalities or mortalities in 3-dpf larvae as compared to control. In fact, the range of cortisol content increased from 0.2-0.9 pg/egg in the control to 1,7-10.9 pg/egg after enrichment along the four generations.

Microarray analysis established that presumably maternal transcripts up-regulated by cortisol at 5 hpf were not up-regulated as zygotic transcripts at 12 hpf. This result was validated by relative qPCR technique showing that the mRNAs of four genes displaying this transient pattern, namely *mat1a, notch2, parn* and *stmn2a*, were still unchanged at 20 hpf, meaning that they were not cortisol targets in the embryo. The fact that the messengers of two well-known cortisol targets, namely the genes *fkbp5* and *tsc22d3*, were also unaffected is not surprising, because they were also found to be unaltered at 10 hpf in morphant embryos deprived of GR (Pikulkaew *et al*., 2011). Apparently, the responsiveness of these targets to cortisol emerges at later developmental stages.

The relative qPCR was also applied to the *vasp* transcript, which appeared up-regulated at 12 hpf according to microarray analysis. The gene encodes the vasodilator-stimulated phosphoprotein, a member of the Ena-VASP protein family, which plays a widespread role in cell adhesion and motility (Zimmer *et al*., 1997). A non-significant up-regulation was found at 10 hpf, a slight down-regulation at 12 hpf which was followed by a significant marked down-regulation at 24 hpf (when down-regulation prevails over up-regulation, as noted above). Hence, there was a shift from up- to down-regulation during the segmentation period, when a variety of morphogenetic movements occurs. The same trend was observed for the *plp1a* transcript, also up regulated at 10 hpf and down-regulated at 24 hpf, but data were less statistically reliable. This gene encodes the proteolipid protein 1a involved in the process of myelination as an integral membrane protein of axon-enveloping glial cells (Brösamle and Halpern, 2002), whose mRNA is first detectable during the segmentation period.

Two more genes, *igf-2a* and *casp8*, were analysed by relative qPCR, despite the fact that their transcripts were not among those up-regulated by cortisol in the microassays at 5 and 12 hpf. They were included because reported to be strongly up-regulated in *gr*MO-ATG-injected early embryos compared to WT (Pikulkaew et al., 2011). Hence, their expression was assessed after treatment with z-*gr* mRNA alone or in combination with cortisol relatively to untreated control. The *igf-2a* encodes the insulin-like growth factor-2a, which is the most potent among four IGF

peptides (1a and b, 2a and b) in zebrafish. It is a critical regulator of GH-independent somatic growth during embryogenesis by stimulating cell proliferation. Notably, excess of IGF-2a signalling caused defects in the midline formation and expansion of the notochord, leading to fully or partially duplicated notochords (Zou *et al*., 2009). Despite the ineffectiveness of cortisol alone, the gain of function with z-*gr* mRNA elicited a significant up-regulation at both 5 and 10 hpf, which was reinforced after addition of cortisol. As said above, this outcome might share responsibility for compromising larval development and survival. But, in this case, the observed up-regulation seems to be imputed to zygotic transcription, because an extremely low level of maternal *igf-2a* transcript was detected at 4 hpf (Pikulkaew *et al*., 2011). This means that not only maternal *gr* transcript deposition must be carefully adjusted, as inferred above, but also that an anticipation and enhancement of *igf-2a* zygotic transcription may be harmful. In fact, it is really remarkable that both GR knockdown by morpholino as well as GR gain of function can bring about developmental defects in zebrafish, once again emphasizing the fine tuning of all molecular actors involved.

The same kind of reasoning applies to the *casp8* gene, whose caspase 8 protein exerts pro-apoptotic activity by proteolytically activating downstream caspases. Its transcripts are maternally expressed and zygotic ones continue to be present throughout embryogenesis and into larval stages (Sakata *et al*., 2007; Pikulkaew *et al*., 2011). *Casp8* over-expression is detrimental to the embryo, because it boosts the normal restrained rate of apoptosis. Up-regulation was significantly measured by relative qPCR with z-*gr* mRNA/w/wo cortisol at 5 hpf and non-significantly at 10 hpf.

Given the ample range of variation, *casp8* expression was further investigated by absolute qPCR analysis at 5 and 10 hpf after treatments with cortisol, the corticoid antagonist RU486, z-*gr* mRNA, administered alone or in combination, and *gr*MO-ATG-1. When alone, there was no difference with respect to untreated embryos, whereas z-*gr* mRNA plus cortisol caused a significant up-regulation at both times, with possible deleterious effects, as argued above. It is instructive the fact that no up-regulation of *casp8* occurred in both *gr*MO-ATG-1 injected embryos and z-*gr* mRNA-treated ones at 5 hpf, indicating that gearing up or down the content of translatable maternal *gr* mRNA *per se* does not affect maternal *casp8* transcript degradation, which is normally almost complete by 8 hpf, as shown by Pikulkaew *et al*. (2011). Conversely, at 10 hpf, in *gr*MO-ATG-1 injected embryos, there was a significant up-regulation of zygotic *casp8* transcripts, even greater than that due to z-*gr* mRNA plus cortisol at the same time, though lower than when combined at 5 hpf in terms of normalized mRNA copies. This confirms an equivalent *casp8* up-regulation in *gr*MO-ATG-1 injected embryos at 8 and 10 hpf, with no effect

at 4 hpf, as reported by Pikulkaew *et al*. (2011). This signifies that GR keeps in check apoptosis during embryogenesis by restricting zygotic caspase 8 expression, a control that is lost not only with GR knockdown, but also with an excess of the GR-cortisol complex. Apparently, there is limited tolerance about abnormal variations of controlling molecules, resulting in an autodestructive course when exceeded.

A comment is required for the response to z-*gr* mRNA+RU486, which was ineffective on maternal *casp8* transcripts at 5 hpf, while inducing a strong up-regulation of zygotic ones at 10 hpf, thus mimicking z-*gr* mRNA+cortisol action. Actually, the "antagonistic" properties of RU486 on zebrafish GR are still to be clarified but, in mammalian models, its interactions with the corticoid and progestogen receptors are known to occur at multiple levels with complex integration (Leonhardt and Edwards, 2011). This may explain why, at 5 hpf, it failed to elicit a GR-induced delay of maternal *casp8* transcript degradation (no agonistic action) while, at 10 hpf, it seems to prompt GR activation of zygotic *casp8* transcription (agonistic action).

The experimental design with absolute qPCR was adopted also for a second gene, *mcm6.* This encodes the protein component 6 of the hexameric mini-chromosome maintenance complex which is essential for the initiation of eukaryotic genome replication, owing to its DNA helicase activity as an unwinding enzyme in the formation of replication forks (You and Masai, 2008). In WT embryos, transcripts of this gene are present at low level at 4 hpf to increase dramatically thereafter (Pikulkaew *et al*., 2011). Since there is a tumultuous thrust of DNA replication before MBT, it is conceivable that maternal MCM6 protein rather than its translatable transcript be implicated in speeding up the process. Anyhow, this gene was selected because, in *gr*MO-ATG-1 injected embryos, its transcripts exhibited an opposite pattern with respect to those of *casp8*, with down-regulation from 4 to 12 hpf, hinting at a stimulatory role of GR on its transcription in the developing embryo (Pikulkaew *et al*., 2011). As a matter of fact, *casp8* and *mcm6* genes can be regarded as operating antagonistically during embryogenesis, fulfilling pro-apoptotic and pro-replicative roles, respectively.

The present results confirmed the down-regulation of *mcm6* mRNA after *gr*MO-ATG-1 treatment. Moreover, at 5 hpf, there was a significant up-regulation at high levels of normalized mRNA copies after either cortisol or RU486 enrichment, though no difference was evident at 10 hpf with respect to control. But the unexpected outcome was a significant down-regulation after gain of function with z-*gr* mRNA alone or associated with cortisol. With z-*gr* mRNA+RU486, the combination was ineffective at 5 hpf, while inducing down-regulation at 10 hpf. This pattern reminds of that of *casp8* transcripts, with obviously inverted signs, at 10 hpf. Once again, gearing down or up the embryo z-*gr* mRNA content abolishes its stimulatory role on *mcm6*

expression, which remains operative in the presence of excess corticoid alone. This implies that probing *gr* gene function by either translation knockdown or transcript enhancement by gain of function may lead to equivocal results in the absence of parallel treatments with its cognate ligand.

The above discussion indicates that, differently from alterations of the maternal *gr* transcript which can precipitate adverse consequences on embryo development and larval survival, the enrichment of maternal cortisol in the egg was associated with modifications in the rates of maternal mRNA degradation as well as changes in zygotic transcriptome that did not hamper viability and might actually be beneficial. As summarized in the Introduction, it has been demonstrated that the impact of environmental or social stressors on female fish may derange oogenesis provoking increased follicular atresia and smaller egg size at ovulation with unfavourable progeny survival (cf. Table 2). However, it is reasonable to assume that reduced ooplasmic energy stores and possibly deficient or altered transcript deposition be responsible for crippling progeny rather than the greater amount of cortisol taken up by the oocytes from the maternal circulation.

Evidence was, instead, provided that cortisol enrichment of rainbow trout eggs just after fertilization induced a dose-dependent reduction of sensitivity to stressors in 5-month-old fingerlings, as measured by lower plasma cortisol levels 1 h after stress (Auperin and Geslin, 2008). This long-lasting effect points at an imprinting action of the developmental programming by maternal cortisol on the offspring epigenome, resulting in modified threshold levels of response in the HPI axis. Sparing energy for growth through hyporesponsiveness to stressors would be a positive trait for aquaculture. Accordingly, Li *et al*. (2010) found that a 40% increase in oocyte cortisol content in rainbow trout caused a significant increase in the growth rates of embryos and adults. This result was explained by enhanced expression of the *igf-1* and *igf-2* genes during embryogenesis with subsequent maintenance of superior growth capacity.

Therefore, after demonstrating the effectiveness of cortisol in modifying embryo transcript contents, it was deemed of interest to check whether a similar auxogenic or growth-promoting imprinting existed also in the zebrafish. Taking advantage of its relatively short generation cycle and standardized conditions of culture in terms of water temperature, photoperiod and feeding regime, the experiment covered four successive generations to ascertain possible cumulative effects. The use of two strains differing merely by colour pattern allowed the co-culture of the treated lineage of fish with the untreated control, in order to eliminate interference by surrounding environment, stocking density and food availability.

Despite all these precautions and reliance on large numbers of fish, the experiment met only with partial success, owing mainly to lack of constant growth rates among generations in both lineages. The finding of significant greater body lengths and weights in the treated lineage of the first two generations was encouraging, because statistical divergence occurred at 4-5 months of age till adulthood at 6 months, revealing an imprinting effect by cortisol. But, in F3, the difference was no longer present, despite a greater growth of the treated lineage as compared to those observed in F1 and F2. Even more ambiguous was the situation in the F4, where the seemingly superior growth of the control lineage is doubtful, because bigger fish were measured in both lineages at 3 months with respect to 4 and 5 months of age. Probably, there was some sort of inadvertent sampling bias due to non-uniform growth in the F4 stocks. Even the challenge of both the treated and untreated lineages with cortisol in F4 was inconclusive, because fish failed to grow significantly after 120 days. On the other hand, although cortisol did not affect the time of puberty in the four treated generations, it did lower fertility by reducing the number of eggs laid at first oviposition, which was statistically significant in F4. Less energy diverted for reproduction might be available for enhanced growth. So, the present experiment supports, though not in a definitive way, a priming auxogenic action of maternal cortisol in zebrafish progeny.

From this thesis work, a model can be proposed as a working hypothesis for future research. First of all, maternal cortisol action in the zebrafish would mainly favour cell proliferation and development in the embryo, while taking on a catabolic, energy-mobilizing hormonal function only in post-natal life, likely by controlling different sets of genes. This functional shift is motivated by the fact that the embryo is focused on completing its auto-organizational program with finite energy available in a predetermined time, without the faculty to waste energy for reactivity to stressors, an ability which is acquired only after 97 hpf (Alsop and Vijayan, 2008). Presumably, a smooth course during embryogenesis might have a positive imprinting effect on growth till adulthood. This might occur in the embryo through higher saturation of GR derived from maternal and zygotic *gr* transcripts. Since some reports claim adverse effects by egg loading with exogenous cortisol on cardiac organogenesis (Nesan and Vijayan, 2012) and craniofacial morphogenesis (Hillegass *et al*., 2008), there might be just a tolerable range of receptor saturation or activation by too much cortisol of additional, high-threshold response mechanisms with detrimental interference. While the high concentration of maternal *gr* transcripts, with replacement by zygotic ones from 8 hpf onwards (Pikulkaew *et al*., 2010), points to a fundamental role of the GR-cortisol complex during embryogenesis, it is evident that the level of the maternal *gr* transcript content must be finely tuned in the oocyte, because not

only GR knockdown, but also GR enhancement by gain of function can endanger progeny development, as shown in this study. Conversely, the recovery of normal development after rescuing with a precisely calibrated amount of translatable trout *gr* transcript concomitantly with z-GR translation knockdown (Pikulkaew *et al*., 2011), emphasizes once more the sophistication in the adjustment of maternal *gr* gene expression in the oocyte.

In conclusion, the maternal *gr* transcript dosage should be regarded as the major integral sensor of the stressor load on the mother life during oogenesis, as proposed by Pikulkaew *et al*. (2011), while the cortisol deposit would act as an ancillary sensor. Apart from abortive *gr* transcript levels, a reduced (or anyway altered), but still viable, maternal *gr* transcript content may result in stunted progeny with over-reactivity to stressors. This imprinting, which would be advantageous in an environment experienced by the mother as full of threats, might prevail over the imprinting due to cortisol that, by ensuring better energy utilization for body growth at the expense of stress reactivity and reproduction, would be advantageous if the cortisol hypersecretion in the mother reflects a stress response to scarce food availability. From this perspective, there is ample scope for investigation not only to disentangle the underlying molecular mechanisms in the maternal programming of offspring epigenetic adaptation to the environment, but also to determine whether recurrent imprinting signalling along generations might lead to hereditary fixation of favourable epigenetic changes.

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