

Article

Effects of Maternal Stress on the Development of the Somatotrophic Axis During the Larval and Juvenile Stages in Zebrafish (*Danio rerio*)

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Abstract: Stress is recognized as an adaptive response to potentially harmful environmental stimuli. The primary physiological adaptation to stress is an increase in circulating cortisol levels, which, in excess, can be transferred and incorporated into the oocytes of maturing females, affecting the embryonic developmental program. Additionally, maternal energy availability is an essential environmental factor that modulates this program. Based on this background, we investigated the effects of maternal cortisol on the development of the somatotrophic axis in zebrafish offspring and juveniles. Zebrafish mothers were divided into two groups based on diet: Group 1 received a cortisol-enriched diet, to mimic maternal stress, while Group 2 (control) received a standard diet, for five days. On the third day after treatment, the control and treated females were bred with untreated males. Offspring were assessed at 0, 24, 48, 72, 96, 120, and 144 h post-fertilization (hpf). Morphological analyses were performed during embryonic development, including survival rate, body length, the presence of pericardial edema, and heartbeat. We examined the gene expression of key somatotrophic axis components, including *mtor*, *foxo3a*, *mafbx*, *murfl*, *mstna*, *gh*, *igf1*, *igf2a*, *igf2b*, *11hsdb2*, and *fkbp5*. The study demonstrated that cortisol-treated females significantly influenced offspring development, resulting in higher mortality rates and increased morphological abnormalities, particularly pericardial edema. Gene expression analysis revealed alterations in transcripts related to the somatotrophic axis, especially genes involved in protein synthesis, with signs of accelerated growth in the first hour post-fertilization. At 30 days post-fertilization, juveniles from cortisol-treated females displayed a marked increase in muscle bundle size and cross-sectional diameter compared to the control group. Our findings provide valuable insights into the intricate interaction between maternal factors and the development of the somatotrophic axis in offspring.

Keywords: cortisol; maternal stress; stress response; zebrafish; somatotrophic axis

Key Contribution: This study suggests that maternal treatment and environmental conditions can modulate the Hypothalamic–Pituitary–Interrenal (HPI) axis function, a key endocrine pathway, thereby impacting the growth, survival, and viability of offspring.



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1. Introduction

According to Reynolds et al. (2019) [1], developmental programming originates from maternal environment stimuli or early environmental conditions, which can lead to significant consequences in embryonic development and result in physiological changes

that persist throughout life. Epigenetic mechanisms play a pivotal role in mediating the effects of adverse environmental factors during early life, inducing changes in gene expression and organ function that influence long-term metabolic health [2,3]. The study of early programmed changes in response to environmental stress dates back several decades. A pivotal study conducted in 1957 on rats demonstrated that parental stress could alter the Hypothalamic–Pituitary–Adrenal (HPA) axis response in offspring, suggesting that early life experiences can modify the adult nervous system’s reactivity to stress [4,5]. This area of research is now being widely explored across different species, including humans [4–6]. Exposure to a stressful environment during fetal development has been associated with behavioral dysfunction and disruptions in the HPA axis [7–9], along with metabolic [10], neurological [11–13], immunological [14,15], cardiovascular [16], and aging-related dysfunctions [17]. Studies on reptiles and birds have demonstrated that maternal stress can result in abnormal deposition of corticosterone, the primary hormone involved in the stress response in vertebrates, in embryos. This abnormal corticosterone deposition has been shown to cause developmental changes in offspring following hatching [18–20]. In fish, cortisol manipulations in embryos or parents have been shown to result in significant alterations in the development of the somatotrophic axis [21,22].

The environmental conditions that fish are cultured in can subject them to a diverse array of stressors, both physical and chemical. Therefore, understanding the physiological alterations induced by stress is crucial in aquaculture [23]. Stress is an adaptive response that activates the central nervous system and endocrine pathways to re-establish homeostasis [14]. The primary response involves the release of catecholamines and corticosteroids through the Hypothalamic–Pituitary–Interrenal (HPI) axis. This is followed by secondary responses, which include the mobilization of energy substrates, increased oxygen transport capacity, and immune system modulation. Tertiary effects then manifest as changes in behavior, reproduction, development, and growth. Thus, stress responses extend beyond simple glucose mobilization, reflecting a complex metabolic adaptation that affects multiple physiological systems within the organism [24,25].

The HPI axis plays a central role in the primary stress response and releases glucocorticoids in fish. Functionally, the HPI axis is analogous to the HPA axis in mammals, sharing key similarities. In response to stress, the hypothalamus releases corticotropin-releasing hormone (CRH), which stimulates the synthesis of adrenocorticotrophic hormone (ACTH). ACTH then targets the interrenal tissue, which is homologous to the adrenal gland in mammals, leading to the release of cortisol. Cortisol is the major glucocorticoid in the circulatory system in both humans and teleost fish, mediating the physiological stress response [21].

In zebrafish, cortisol during the embryonic phase is maternally derived, as *de novo* synthesis of this steroid occurs only post-hatching [26]. Early cortisol plays a critical role in developmental programming, including the activating of the HPI axis [27]. During maternal stress, cortisol is transferred into developing oocytes and subsequently deposited into fertilized embryos. In the embryos, cortisol binds to glucocorticoid receptors (GRs). These GRs, when bound to cortisol, act as transcription factors that regulate the expression of various target genes in the embryo. Maternal cortisol is gradually deposited into the developing oocyte and is present in decreasing concentrations throughout embryogenesis. The enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD) converts cortisol into its inactive form, cortisone, thereby mitigating the potential negative effects of excessive cortisol on oocytes and embryos during maternal stress [21,27]. The interplay between maternal stress and cortisol in oocytes is both significant and complex. Therefore, research into the effects of maternal cortisol during early embryonic development is essential for

understanding how these effects influence early developmental processes and contribute to phenotypic changes in offspring.

Furthermore, muscle tissue development, or myogenesis, which begins in the early stages of fish development, can be affected by stress and cortisol. Embryonic muscle formation originates from cellular compartments known as somites, which are derived from mesodermal tissues. The dorsal region of the somites gives rise to myogenic precursor cells (MPCs) that express transcription factors Pax3 and Pax7, which are specific to muscle fiber types. This region also generates a population of reserve progenitor cells, referred to as satellite cells, which are crucial for muscle growth during adulthood [28]. Muscle tissue growth requires a balance between the self-renewal and differentiation of precursor stem cells. This balance is regulated by different factors that influence the cell cycle of precursor stem cells, including Meox1, a critical factor for their activity [29]. In zebrafish, the muscular system sustains a continuous population of self-renewing precursor cells, supporting secondary myogenesis throughout life. Secondary myogenesis occurs through hyperplasia and hypertrophy [30]. The activation of precursor stem cells, proliferation of myoblasts, differentiation, formation of myotubules, and maturation of myofibrils are regulated by myogenic regulatory factors (MRFs) such as MyoD, Myf5, myogenin, and MRF4, each with specific roles [31].

In teleost fish and other vertebrates, skeletal muscle represents a significant portion of body mass, and its growth is regulated by a balance between anabolic and catabolic processes influenced by stress and cortisol. The absence of glucocorticoid signaling has been shown to disrupt the expression of myogenic regulatory factors and muscle-specific genes, as evidenced by studies on zebrafish lacking glucocorticoid receptors (GRs) [16,32]. Additionally, stress and cortisol can affect the somatotrophic axis. The somatotrophic axis comprises the hypothalamus, the pituitary gland (which releases the growth hormone, GH-), and the liver, the primary source of insulin-like growth factors (IGFs). A study using salmonids demonstrated that acute stress and intraperitoneal cortisol administration lead to a significant decrease in GH levels in plasma [24]. Therefore, it is plausible that stress and cortisol also influence IGF1 production, as IGF1 synthesis in the liver is stimulated by GH. IGF1 is a key hormone in promoting the proliferation, differentiation, and hypertrophy of muscle precursor cells.

The importance of the somatotrophic axis in the growth of fish has been elucidated. On the other hand, perturbations in the growth axis involving environmental factors, including energy availability, stress, photoperiod, temperature, and others, can influence the development of this axis. This study aimed to explore how the maternal environment affects the development of the somatotrophic axis in offspring, using zebrafish as a model organism.

2. Materials and Methods

2.1. Animal Husbandry

Sexually mature males and females (outbred, 4–5 months old) were maintained in the aquarium facility of the Department of Structural and Functional Biology, Institute of Biosciences, Botucatu, São Paulo State University (UNESP) in 6 L tanks on a recirculating system with a 14:10 light/dark cycle and constant temperature conditions (28 °C). The following water parameters were monitored in all tanks every other day (pH: 6.8–7.2, ammonia ≤ 0.05 ppm, and nitrite ≤ 0.5 ppm). Males were fed twice a day with commercial food (Pentair Aquatic Habitats, Apopka, FL, USA), while females were fed twice a day with a commercial diet (Pentair Aquatic habitats, Apopka, FL, USA) supplemented with hydrocortisone to assess the effects of maternal stress-associated cortisol on offspring development, as described below. Handling and experimentation were performed according

to the Brazilian legislation regulated by the National Council for the Control of Animal Experimental (CONCEA) and Ethical Principles in Animal Research (Protocol n. 8520250320-CEUA).

2.2. Maternal Stress-Associated Cortisol

Ten sexually mature female zebrafish were assigned to each of the two 6 L tanks. One group ($n = 5$) was fed a control zebrafish diet, while the other group ($n = 5$) was fed a diet laced with cortisol, according to methods previously described [21]. For that, the zebrafish commercial diet was prepared by soaking food pellets in 100% ethanol, either alone (control group) or with 0.5 mg per g feed (hydrocortisone group) (Sigma-Aldrich, St. Louis, MO, USA) and allowing the ethanol to evaporate [21,33]. This concentration was chosen based on previous studies on maternal cortisol [21]. Zebrafish mothers received approximately 25 μg cortisol/g body mass. Fish were fed twice per day for 5 days. On day 3 post-treatment, five females from each group were transferred to breeding tanks (Figure 1).

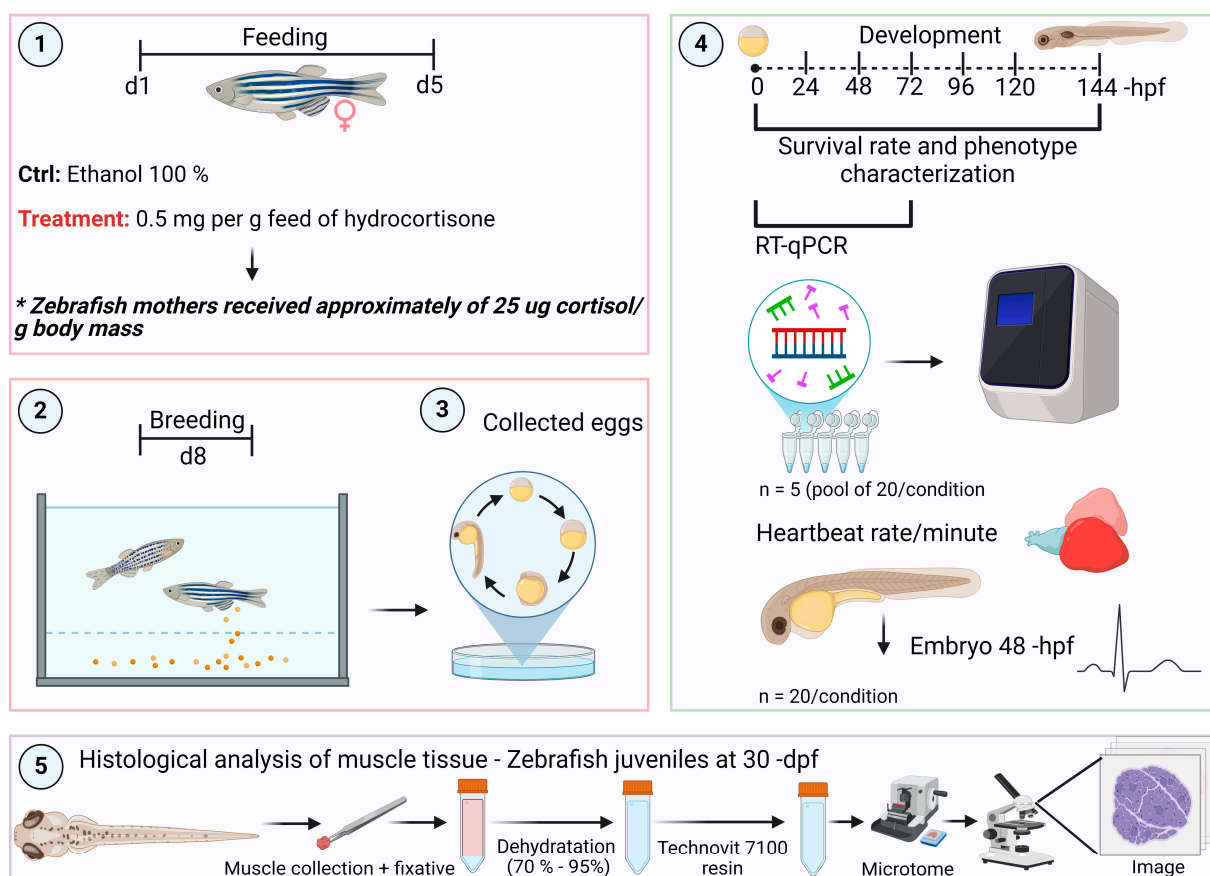


Figure 1. Experimental design. (1) Two groups of female zebrafish ($n = 30$) were assigned into three replicates per experimental group. One group was fed commercial food, while the other one was fed commercial food containing 0.5 mg per feed of hydrocortisone. Zebrafish mothers received approximately 25 μg cortisol/g body mass. Fish were fed twice per day for 5 days. (2) On day 3 post-treatment, five females from each group were transferred to breeding tanks with untreated males. (3) Fertilized eggs were collected in Petri dishes containing embryo medium. (4) Eggs, embryos, and larvae were collected at 0 to 144 hpf for survival rate and phenotype characterization, such as length and pericardial edema. The eggs, embryos, and early larvae from 0 to 72 hpf were counted and pools were collected for gene expression analysis. Embryos at 48 hpf from the control and cortisol groups were taken to calculate heartbeat rate per minute ($n \sim 20$ /condition). (5) Juveniles at 30 dpf were also collected for the histological analysis of muscle fiber in both groups. The illustration is available online: <https://BioRender.com> (accessed on 1 November 2024).

2.3. Breeding

On day 3, female fish from each experimental group (control and cortisol) ($n = 5$ /group) were placed for reproduction. The females were individually placed in separate tanks ($n = 1$ per tank) with unexposed males, following the usual methods for zebrafish reproduction [34]. At 18 h, the daily breeding setup began, and fish were transferred into breeding traps in the same tank. Eggs were collected within the first light (at 9 h), after mating, the following morning. Fertilized eggs were collected in Petri dishes containing embryo medium (Hank's medium, https://zfin.org/zf_info/zfbook/chapt10.html#wptohtml16 (accessed on 6 March 2023)). The eggs, embryos, and larvae from each group were collected and evaluated at 0, 24, 48, 72, 96, 120, and 144 h post-fertilization (hpf). In the period from 0 to 72 hpf, the eggs, embryos, and early larvae were counted and pools of 20 ($n = 5$ pools of 20 individuals for each pool) were snap-frozen on liquid nitrogen and stored at $-80\text{ }^{\circ}\text{C}$ for gene expression analysis. Furthermore, eggs, embryos, and larvae from 0 to 144 hpf were sampled for phenotype characterization and larval morphology. Juveniles at 30 days post-fertilization (dpf) were also collected for muscle fiber analysis in both groups, as described above (Figure 1).

2.4. Transcript Levels of the Somatotrophic Axis by Quantitative Real-Time PCR (qPCR)

The mRNA abundance of key genes involved in the somatotrophic axis was measured by qPCR in embryos from 0 to 72 hpf. Embryos were pooled into 20 ($n = 5$ pools of 20 individuals for each pool) per experimental group (control and cortisol treatment). Total RNA was extracted from the pools using a commercial kit (PureLinkTM RNA mini kit, Ambion, Life Technologies, Carlsbad, CA, USA). After RNA extraction, the usual downstream methods were followed according to the manufacturer's instructions. cDNA synthesis was performed using a commercial kit (iScriptTM cDNA Synthesis Kit, Bio-Rad) (<https://www.bio-rad.com/pt-br/product/iscript-cdna-synthesis-kit?ID=M87EWZESH> (accessed on 10 April 2023)) according to the manufacturer's instructions and the procedure described by Nóbrega et al. (2010) [35]. qPCR reactions were conducted using 5 μL of 2X SYBR-Green Universal Master Mix (Applied Biosystems), 1 μL of forward primer (1.125 nM), 1 μL of reverse primer (1.125 nM), 0.5 μL of DEPC water, and 2.5 μL of cDNA. The relative mRNA levels of intracellular genes related to growth regulation through syntheses, such as *mtor* (mammalian target of rapamycin), and protein degradation, including *foxo3a*, *mafbx*, and *murfl* (forkhead box O3, muscle atrophy F-box, and muscle ring-finger protein-1, respectively), were quantified. Additionally, genes involved in the systemic or hormonal control of anabolic pathways, such as *gh*, *igf1*, *igf2a*, and *igf2b* (growth hormone, insulin-like growth factor 1, and insulin-like growth factor 2a and 2b, respectively), as well as *mstna* (myostatin), which is related to the regulation of catabolism, were also measured. Furthermore, the relative mRNA levels of *hsd11b2* (hydroxysteroid 11-beta dehydrogenase 2) and *fkbp5* (glucocorticoid receptor chaperone protein) were evaluated to denote changes in the Hypothalamic–Pituitary–Interrenal (HPI) axis [21,36]. mRNA levels of the target expressions (Cts) were normalized by the transcript levels of the β -actin gene as a reference. Results were expressed as relative values to the control group (as fold induction) using the $2^{-(\Delta\Delta\text{CT})}$ method. Primers were designed according to zebrafish genetic sequences available in GenBank (NCBI, <https://www.ncbi.nlm.nih.gov/genbank/> (accessed on 10 April 2023)) (Table S1). Each primer was validated using a melting curve (Figure S1).

2.5. Embryo and Larvae Phenotypes

Embryos and larvae at 48 to 72 hpf from both the control and cortisol-treated groups were collected from spawning tanks and evaluated for morphological changes. The selected period corresponds to the post-hatching stage, during which larvae are presumed to experience the most significant influence of maternal cortisol deposition, as *de novo* synthesis of this steroid begins only after hatching. A total of 30 larvae were randomly chosen from each group, and 30 bright-field images from each specimen were captured using an Olympus MVX10 MacroView stereomicroscope. These images were subsequently utilized to measure morphometric parameters in developing larvae with annotation performed using the Olympus cellSens dimensions software 1.6 (<https://evidentscientific.com/en/software/cellsens> (accessed on 10 April 2023)) and analysis conducted via ImageJ software 2.1.0/1.54f (<http://imagej.nih.gov/ij/index.html> (accessed on 10 April 2023)). The developmental differences between the control and treatment groups were assessed through morphometric evaluations. The total length of larvae was measured, and abnormal developments were identified, particularly pericardial edema. The survival rate of zebrafish embryos and larvae in the range of 0 to 144 hpf was evaluated for each group using the Kaplan–Meier method. Survival was assessed based on the percentage of viable larvae relative to the total number of specimens collected post-fertilization (0 hpf). Embryos and larvae found at the bottom of the Petri dish with opaque appearance and abnormal coloration, along with hyporesponsiveness to mechanical stress, were considered dead.

2.6. Heart Rate Measurement in Embryos at 48 Hpf

Videos of zebrafish embryos at 48 hpf from both maternal control ($n = 12$) and cortisol-treated groups ($n = 18$) were recorded to calculate heart rate per minute. The non-anesthetized embryos were immobilized using excavated slide blanks containing sufficient embryonic medium to ensure survival during filming. The setup consisted of an Olympus MVX10 MacroView stereomicroscope linked to Olympus cellSens dimensions software 1.6 (<https://evidentscientific.com/en/software/cellsens> (accessed on 10 April 2023)) for video acquisition. The video files (AVI) were obtained at 30 frames per second (fps), totaling 60 s of recording, with a resolution of 4080×3072 pixels. Heart rate quantification was performed using a Python script with CardiOT2[®] software 1.0, which employs the Fast Fourier (FFT) methodology to calculate heart rate contraction cycles.

2.7. Histological Analysis of Muscle Tissue

Five fish at 30 days post-fertilization (dpf) were collected from each experimental group (control and cortisol) for cross-sectional analysis, while three larvae per group were used for longitudinal analysis. The specimens were anesthetized with 0.1% benzocaine and sacrificed for skeletal muscle tissue collection. Samples were immediately fixed in Karnovsky solution (glutaraldehyde 2.5%, paraformaldehyde 2%, 0.1 M phosphate buffer, pH 7.2) and preserved in 70% alcohol. Samples were dehydrated using graded ethanol concentrations (80, 95, and 95%) and embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany), according to the manufacturer's protocol. Longitudinal and cross-section histological areas ($4 \mu\text{m}$) of muscle fibers were prepared through a microtome equipped with a glass knife. Sections were stained with 0.1% toluidine blue in 1% sodium borate. Fiber diameter and frequency were determined using a Leica DMI6000 microscope (Leica, Heidelberg, Germany) coupled with an image analyzer. Muscle fibers were classified based on the criteria previously described [37], with fibers smaller than $9 \mu\text{m}$ categorized as thin fibers, and those larger than $9 \mu\text{m}$ as thick fibers.

2.8. Statistical Analysis

The data were subjected to the mean $\pm$ SEM (Standard Error of Mean). Significant differences between the two groups were identified using non-parametric (unpaired) Student's *t*-tests to compare two groups, the treated group versus the control group. The significance level (*p*) was considered ≤ 0.05 in both cases. The normal distribution of the samples was assessed using the Kolmogorov–Smirnov test, which was performed for all genes used in this study. All analysis was carried out using Graph Pad Prism software 8.0.1 (Graph Pad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Maternal Cortisol Influences the mRNA Expression Levels of Genes Associated with the Somatotrophic and HPI Axes in Zebrafish Offspring

Samples were collected at 0, 24, 48, and 72 hpf for both the control and cortisol-treated groups for gene expression analysis by qPCR (Figure 1). The expression levels of essential components involved in the somatotrophic and HPI axes, such as *mtor*, *foxo3a*, *mafbx*, *murf1*, *mstna*, *gh*, *igf1*, *igf2a*, *igf2b*, *fbp5*, and *11hsdb2*, were significantly altered in the zebrafish progeny from females fed with a cortisol-enriched diet when compared to the control (Figure 2). Our analysis revealed maternal cortisol treatment up-regulated *mtor* mRNA levels in embryos at 24 hpf (Figure 2A). We also evaluated the genes involved in muscle protein degradation. Embryos and larvae from the maternal cortisol-treated group exhibited increased levels of *foxo3a* at 24, 48, and 72 hpf compared to the control (Figure 2B). The mRNA expression levels of *mafbx* and *murf1*, genes involved in the catabolic pathway, were significantly altered in the treated group. While *mafbx* levels were significantly elevated at 48 and 72 hpf (Figure 2C), *murf1* increased expression at 0 and 48 hpf (Figure 2D). Regarding the expression of *mstna*, a protein that inhibits myogenesis, a significant decrease in its expression was observed in the treated group at 0 hpf, followed by a significant increase at 24 hpf (Figure 2E). Maternal cortisol treatment also significantly increased the expression of *gh* in embryos at 0 hpf (Figure 2F). However, a substantial decrease in primary *gh* transcripts was observed in early larvae at 72 hpf from females fed with a cortisol-treated diet (Figure 2F). The expression levels of *igf1*, *igf2a*, and *igf2b* were measured. *igf1* was up-regulated from 0 to 48 hpf in the cortisol-treated group (Figure 2G), while *igf2b* expression increased only at 0 hpf (Figure 2I). On the other hand, the mRNA levels of *igf2a* remained unchanged across the experimental groups at the sampled time points (Figure 2H). Finally, we evaluated the expression of genes responsive to the HPI axis. A significant decrease in *fbp5* expression levels was observed exclusively at 0 hpf (Figure 2J). However, the mRNA levels of the enzyme 11 β -HSD2 (*11hsdb2*), responsible for converting cortisol into its inactive form, cortisone, showed no significant differences between the experimental groups (Figure 2K).

3.2. Maternal Cortisol Treatment Affected Different Stages of Offspring Development in Different Parameters of Evaluation

We investigated the impact of maternal cortisol on embryos and larvae survival and phenotype. Embryos and larvae were collected at intervals from 0 to 144 hpf in both the control and cortisol-treated groups for survival analysis. The survival rate was conducted using the Kaplan–Meier method. The results showed a higher mortality rate among offspring from females fed a cortisol-enriched diet compared to the control group (Figure 3A). The log-rank test (Mantel–Cox) indicated a significant relative risk of death between the groups during the study period (chi-square = 64; *p* < 0.0001). The control group maintained a survival rate of 90.5% at 24 hpf, with no decline observed up to 144 hpf.

However, the offspring from the cortisol group showed survival rates of 79.6%, 78.6%, 73.5%, and 70.9% at 24, 48, 72, and 144 hpf, respectively.

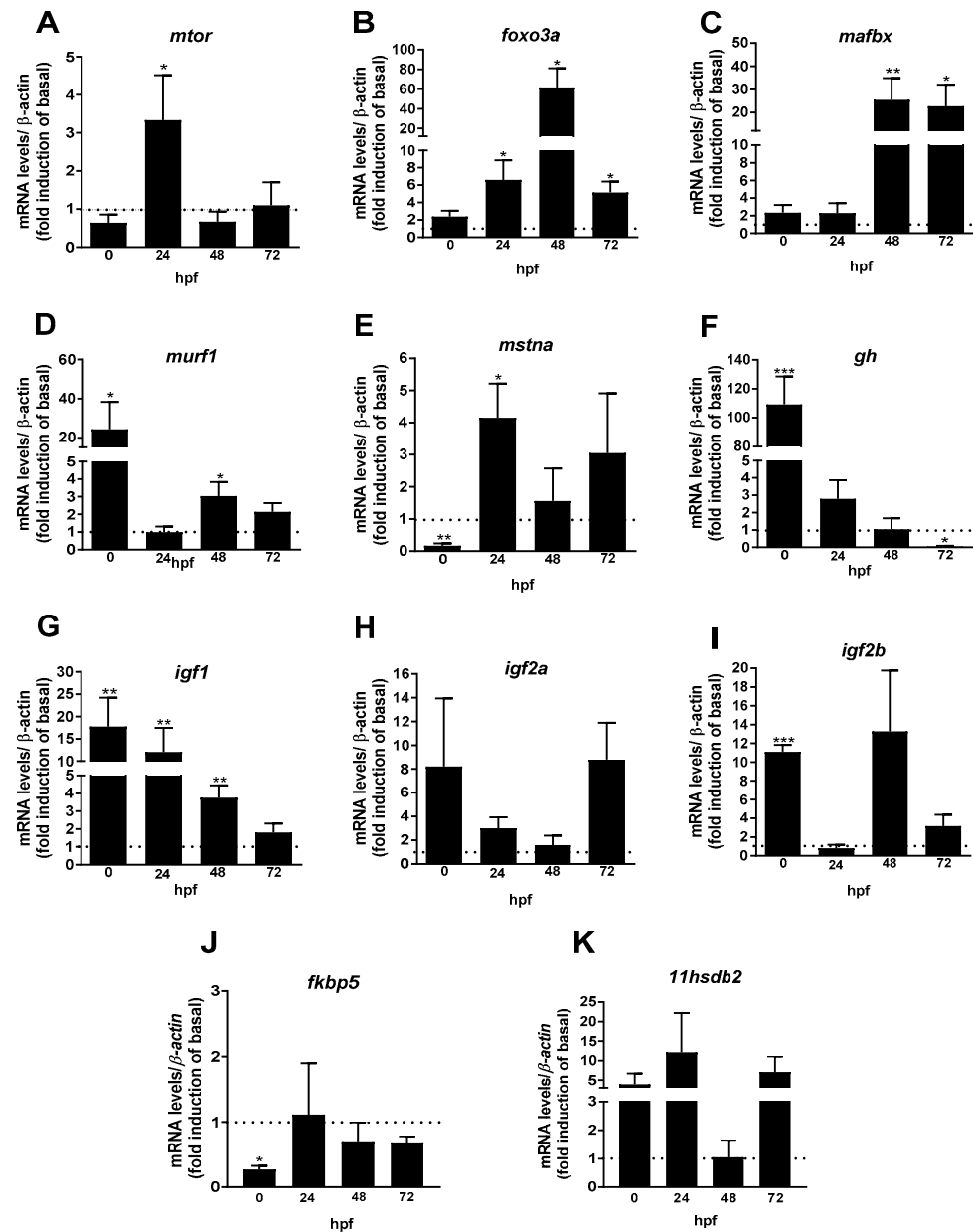


Figure 2. Somatotrophic and HPI-related genes in embryos and early larvae at 0 to 72 hpf from mothers fed with a hydrocortisone enriched diet (0.5 mg per g feed) compared to the untreated group (control) (dotted black line set at 1). The selected genes *mtor* (mammalian target of rapamycin) (A); *foxo3a* (forkhead box O3) (B); *mafbx* (muscle atrophy F-box) (C); *murf1* (muscle ring-finger protein-1) (D); *mstna* (myostatin) (E); *gh* (growth hormone) (F); *igf1* (insulin-like growth factor 1) (G); *igf2a* (insulin-like growth factor 2a) (H); *igf2b* (insulin-like growth factor 2b) (I); *fkbp5* (glucocorticoid receptor chaperone protein) (J); and *11hsdb2* (hydroxysteroid 11-beta dehydrogenase 2) (K) were evaluated. Ct values were normalized with β -actin and expressed as relative values of the control (untreated fish) levels of expression. Bars represent the mean $\pm$ SEM fold change ($n = 5$ pools of 20 individuals for each pool) relative to the control. Student's unpaired *t*-test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

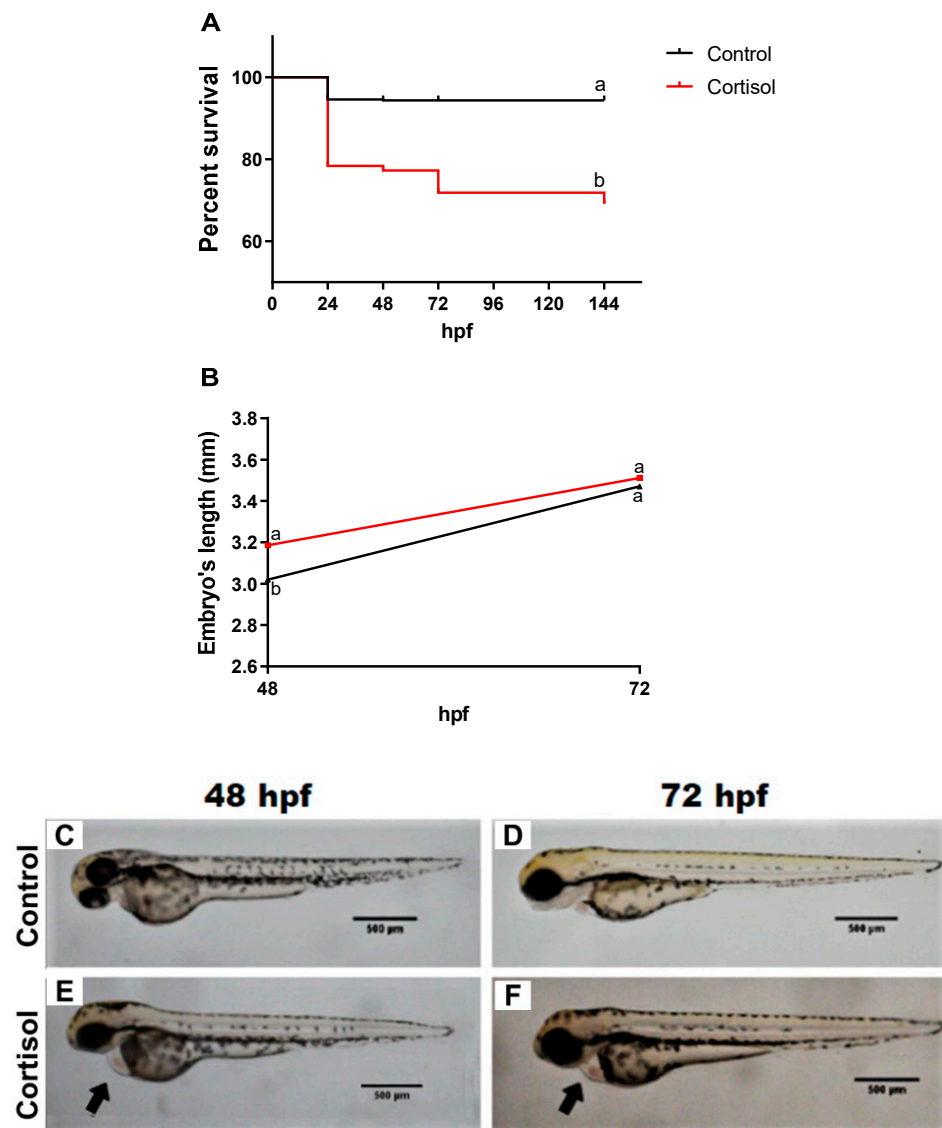


Figure 3. The survival rate of embryos and larvae at 0 to 144 hpf (A) was demonstrated in both control and treated animals. Embryo and larvae length at 48 to 72 hpf (B) were measured (bars represent the mean $\pm$ SEM; ANOVA followed by Tukey's multiple comparison tests or Student's unpaired *t*-test). Distinct letters denote significant differences; $p \leq 0.05$). The phenotype of embryos and early larvae from control females (C,D) was compared with those of progeny impacted by maternal transfer of corticosteroid (E,F). Control animals showed normal pericardium morphology (C,D). However, embryos from cortisol-treated females were affected. Arrows highlight the presence of pericardial edema (E,F). Scale bar = 500 μ m ($n = 30$, basal and treatment).

The body lengths of larvae at 48 and 72 hpf were measured. Maternal cortisol exposure significantly increased larval length at 48 hpf compared to the control group (Figure 3B). The morphological evaluation indicated that offspring from females fed a cortisol-enriched diet exhibited moderate cardiac edema, as classified previously [16]. Specifically, 63.3% and 36.6% of larvae displayed moderate pericardial edema at 48 and 72 hpf, respectively (Figure 3C,D). No morphological abnormalities were noted in the control group (Figure 3E,F).

Additionally, heart rate measurements at 48 hpf showed no significant difference between the offspring of cortisol-treated and control females (Figure 4).

At 30 dpf, a histological analysis of muscle fibers in the progeny of cortisol-treated females revealed distinct changes. Longitudinal sections revealed that juveniles from the

maternal cortisol-treated group had significantly longer muscle bundles compared to the control group (mean $387.6 \pm 6.71 \mu\text{m}$ and $287.6 \pm 24.81 \mu\text{m}$, respectively) (Figure 5A–C).

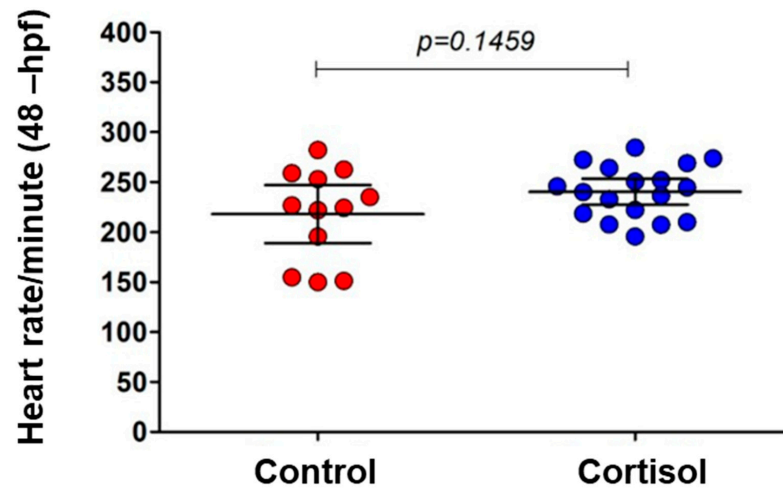


Figure 4. The heartbeats of embryos at 48 hpf for the control (red circles) ($n = 12$) and maternal cortisol (blue circles) ($n = 18$) groups were demonstrated. No significant heartbeat difference was found between the control and treatment embryos. Student's unpaired t -test, $p = 0.1459$.

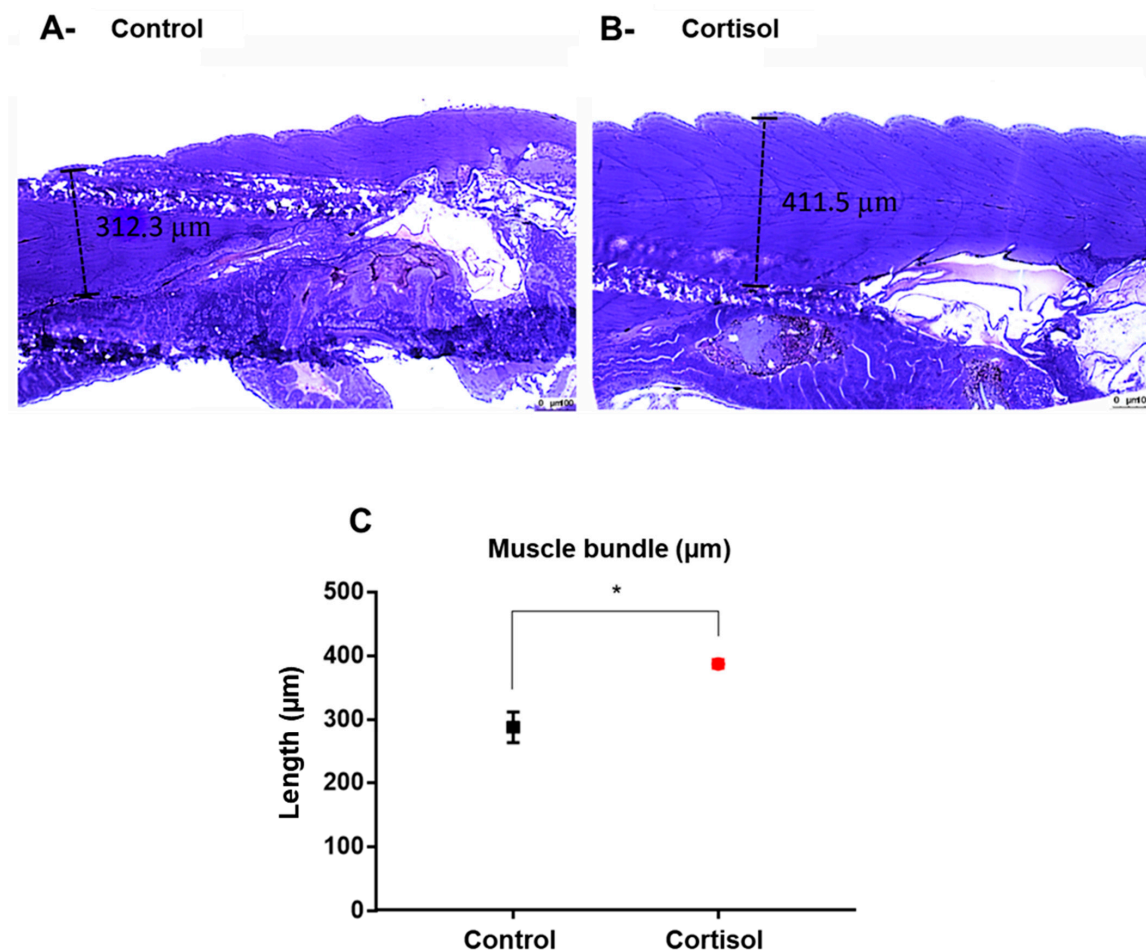


Figure 5. Longitudinal histological sections of muscle from 30 dpf zebrafish juveniles from females fed with a commercial diet (A) or a diet supplemented with cortisol (B). Muscle bundle lengths at 30 dpf for juveniles from control (black) and cortisol-treated (red) females (C). (Bars sampled in each group represent the mean $\pm$ SEM; Student's unpaired t -test, * $p < 0.05$). Scale bar = 100 μm . Staining: Toluidine blue ($n = 3$, basal and treatment).

Furthermore, cross-section analysis indicated that the total muscle diameter was larger in juveniles from mothers treated with cortisol ($254 \pm 9.27 \mu\text{m}$) compared to those from control females ($187.7 \pm 6.44 \mu\text{m}$) (Figure 6A,B,E). Furthermore, cross-sections showed a higher percentage of thick muscle fiber ($>9 \mu\text{m}$) in the juveniles from cortisol-treated females, averaging 72.5%, compared to 35.7% in the control group. However, the progeny from the control group exhibited a higher proportion of thin muscle fibers ($<9 \mu\text{m}$), averaging 63.7% compared to 27.5% in the progeny from the maternal cortisol group (Figure 6C,D,F).

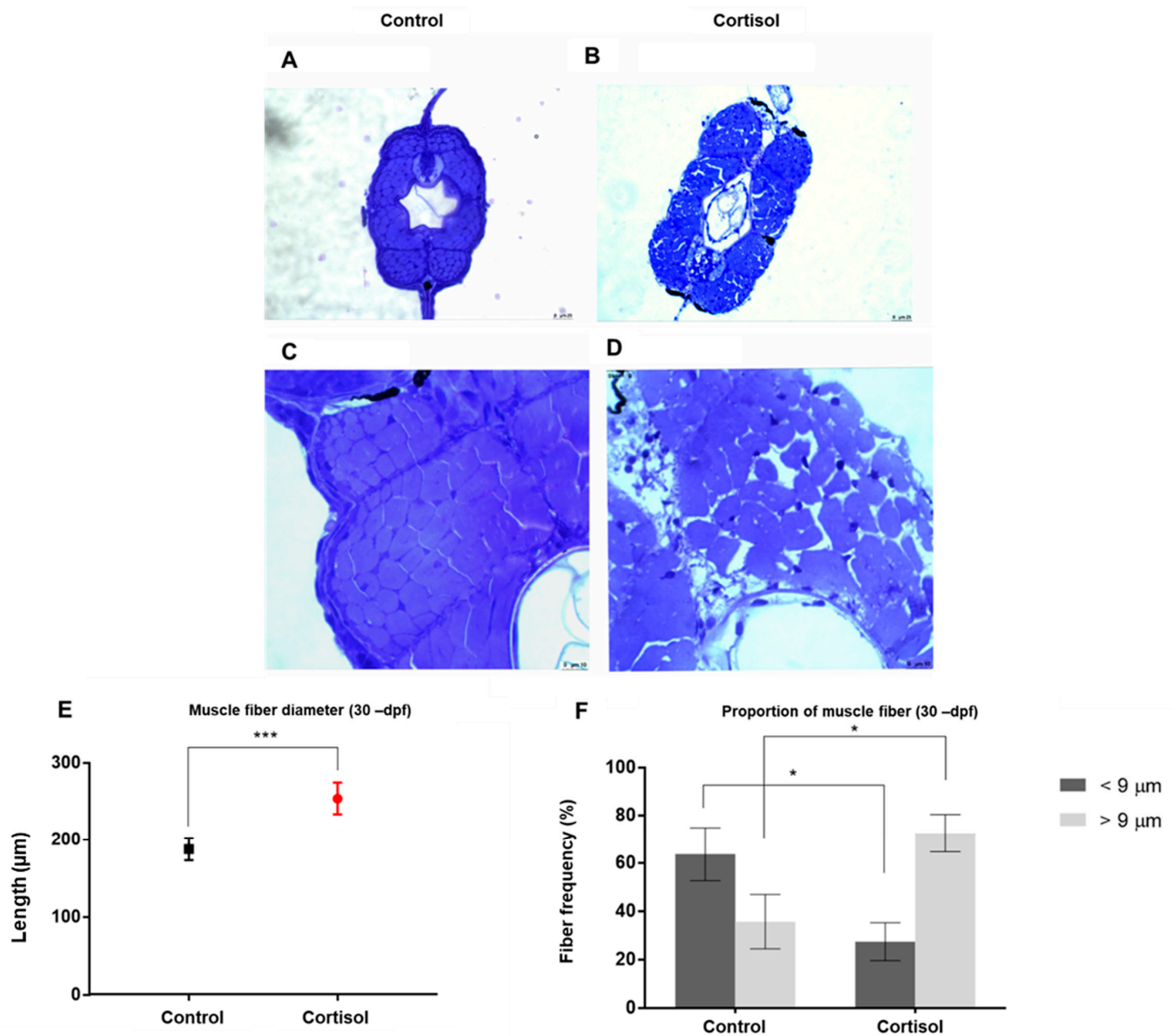


Figure 6. Histological cross-sections of muscle in zebrafish juveniles at 30 dpf from females fed a commercial diet (control) (A,C) or a diet supplemented with cortisol (B,D). The cross-sectional diameters of muscle fibers in both progeny from non-treated and cortisol-treated females were measured in offspring at 30 dpf (E). The proportion of thin ($<9 \mu\text{m}$) and thick ($>9 \mu\text{m}$) muscle fibers in zebrafish juveniles at 30 dpf from control and cortisol-treated females were measured (F). (Symbols or bars represent the mean $\pm$ SEM; Student's unpaired *t*-test, * $p < 0.05$; *** $p < 0.001$). Scale bar = $25 \mu\text{m}$ (A,B); $10 \mu\text{m}$ (C,D). Staining: Toluidine blue ($n = 5$; 75 muscle fibers per animal, basal and treatment).

4. Discussion

Zebrafish (*Danio rerio*) are an excellent model for investigating the early developmental effects of cortisol signaling, as de novo synthesis of cortisol begins only after hatching (48–72 h post-fertilization, hpf). Consequently, maternal cortisol plays a crucial role in early developmental programming [26]. Previous research has highlighted the critical role of maternal cortisol in embryogenesis [16,32]. Studies employing morpholino oligonucleotides to reduce glucocorticoid receptor expression in zebrafish embryos showed mesodermal malformations, including bent tails and deformed somites, as well as reduced growth and decreased survival rates [16,32].

On the other hand, several experiments have been conducted in fish to investigate the impact of increased maternal cortisol deposition on embryos, particularly concerning phenotypic changes in offspring and the somatotrophic axis [38]. In zebrafish, Nesan and Vijayan (2016) [27] demonstrated that microinjecting 32 pg of cortisol into newly fertilized embryos—a technique used to mimic maternal cortisol transfer and deposition—resulted in increased embryo size at 48 and 72 hpf. Similarly, our study observed that adding cortisol to the maternal diet resulted in the maternal transfer of cortisol, which increased offspring size at 48 hpf. Interestingly, the same researchers [27] also demonstrated that the injection of cortisol-specific antibodies resulted in decreased embryo length and abnormal morphologies, including moderate tail twist and deformed body curvature. These findings demonstrated the crucial role of cortisol in mesoderm formation and muscle development [16], prompting further investigation into whether a positive adjustment of cortisol levels could be beneficial for embryogenesis and myogenesis.

Another critical aspect to consider is the cardiac structure. Offspring from females fed a cortisol-enriched diet exhibit pericardial edema, a finding that is particularly relevant to our study, as the heart is composed of muscle tissue and derived from the mesodermal layer during embryogenesis [39]. Interestingly, Nesan and Vijayan (2012) [16] demonstrated that increasing cortisol levels in newly fertilized zygotes through microinjection disrupted cardiogenesis, leading to pericardial edema and cardiac chamber malformations. Similar findings support the hypothesis that maternal cortisol programs embryogenesis, with elevated levels of this steroid in the embryo having adverse effects. Moreover, the morphometric data from our study align with the literature, confirming that a cortisol-enriched diet effectively simulates maternal stress and facilitates cortisol transfer to offspring [21]. Nesan and Vijayan (2012) [16] also observed a reduction in heart rate in 72 hpf larvae exposed to elevated cortisol levels during pre-hatch. On the other hand, our study showed no alteration in heart rate at 48 hpf in larvae, despite the presence of pericardial edema. We hypothesize that the discrepancy between the results may be attributed to differences in the treatment methods—such as cortisol microinjection into embryos versus maternal cortisol diet supplementation—or to the specific time points at which heart rate was measured.

To gain a deeper understanding of how cortisol influences zebrafish embryonic development, we focused on gene expression analysis. We examined key developmental genes involved in the somatotrophic axis, including *gh*, *igf1*, *igf2a*, and *igf2b*. In vertebrates, GH functions directly by binding to receptors on the muscle sarcolemma and indirectly by stimulating the production and release of insulin-like growth factors (Igf) in the liver and peripheral tissues [40]. In zebrafish, the Igf system includes *igf1*, *igf2*, and *igf3*, with *igf3* being specific to gonadal tissue [41]. *igf2* and *igf3* are involved in midline development. Of particular relevance to our study is *igf1*, which acts through the PI3K-Akt-TOR anabolic pathways by binding to its receptor, thereby being essential for normal embryonic development and myogenesis [28]. Indeed, reduced *igf1* signaling in zebrafish has been associated with decreased synthesis of major muscle proteins, leading to a reduction in muscle fiber diameter [37]. The gene expression data reveal a significant increase in *gh* mRNA levels

after fertilization, suggesting that this transcript is maternally inherited and positively regulated by maternal cortisol. As development progresses, relative *gh* levels normalize at 24 and 48 hpf. However, there was a marked decline of *gh* levels at 72 hpf in the offspring of cortisol-treated females. Consistent with this, *igf1* mRNA levels increased at 0, 24, and 48 hpf in embryos and larvae from cortisol-treated females. Notably, the increase in larval size at 48 hpf brings aligns with the elevated levels of the growth factor. In our study, the elevated transfer of maternal cortisol resulted in a coincident increase in *gh* and *igf1* levels in the first hour post-fertilization, followed by a subsequent downregulation during embryogenesis. This suggests a direct relationship between the HPI and somatotrophic axes. Our findings are consistent with the literature, which emphasizes the crucial role of cortisol in the proper morphological development of zebrafish [25,42].

Additionally, we analyzed the mRNA expression levels of genes related to skeletal muscle development, including *mtor*, *foxo3a*, and *murf1*. The regulation of muscle fiber volume and quantity involves a complex interplay between anabolic and catabolic proteins, which promote protein synthesis and degradation, respectively [28]. The IGF-Akt pathway plays a crucial role in this process, functioning through two key mechanisms in fish. In the anabolic process, the *mtor* protein is phosphorylated and activated, facilitating cell growth and proliferation, which drives hypertrophy and hyperplasia. On the other hand, muscle atrophy is prevented by the phosphorylation and inactivation of the *foxo3a* protein, leading to the downregulation of the ubiquitin ligases *murf1* and *mafbx*, which target proteins for degradation by the proteasome complex [43]. In this study, we observed an upregulation of *mtor* and *foxo3a* expression in embryos at 24 hpf in the maternal cortisol-treated group, indicating an increase in muscle protein turnover. This phenomenon may explain the increased embryo length observed in larvae at 48 hpf in the treated group. Additionally, we observed the upregulation of the mRNA expression of protein degradation markers, such as *foxo3a*, *murf1*, and *mafbx*, in embryos at 48 and 72 hpf, accompanied by the stabilization of *mtor* expression, which may have prevented exacerbated growth post-fertilization. Consequently, no significant difference in embryo length was observed at 72 hpf between the maternal cortisol-treated and control groups.

On the other hand, myostatin, a member of the tumor growth factor- β (TGF- β) superfamily, is a well-established negative regulator of myogenesis in vertebrates. In zebrafish, the inhibition of myostatin expression results in a significant increase in muscle mass [44,45]. Furthermore, two myostatin gene variants, *mstna* and *mstnb*, can be found in zebrafish. *mstna* mRNA is maternally inherited and highly expressed in embryos at 48 hpf [46–48]. Our study revealed that *mstna* mRNA levels were suppressed in embryos after fertilization in the maternal cortisol-treated group. This suggests that the observed increase in larval length at 48 hpf is related to the inhibition of *mstna* expression, leading to enhanced muscle growth, as indicated by elevated *mtor* expression. However, this interpretation became more complex when we observed the suppression of mRNA transcripts of genes involved in protein degradation, such as *murf1* and *mafbx*. Furthermore, there was a significant increase in *mstna* expression in embryos of the treated group at 24 hpf, which could be directly related to the elevated expression of *foxo3a*, *murf1*, and *mafbx* at 48 and 72 hpf. Furthermore, additional studies are required to deepen our understanding of the regulatory mechanisms involved in this process, particularly during the post-fertilization period.

Finally, we also evaluated the expression of cortisol-responsive genes *hsd11b2* and *fkbp5* [21,36,42]. FK506-binding protein 5 (Fkbp5) is a glucocorticoid receptor chaperone protein that responds to elevated glucocorticoid levels in zebrafish. This observation was confirmed in Fdx1b-deficient zebrafish males, in which its expression was decreased in response to decreased plasma cortisol levels [49]. In the present study, *fkbp5* expression was reduced in 0 hpf embryos from cortisol-treated females, suggesting either that maternal

cortisol transfer did not occur as expected, or that there are counter-regulatory mechanisms in response to excess glucocorticoids in the offspring. The low response to cortisol receptors activating during the early stages of embryogenesis could have influenced the increased larval size observed at 48 hpf in the treated group, which indicates crosstalk between the HPI and somatotrophic axes.

The literature indicates that ovarian follicles, eggs, and embryos are not passive recipients of maternal steroids [38]; rather, they possess mechanisms to either transport cortisol out of the embryo or metabolize it into its inactive form. Consequently, the transfer of maternal cortisol to the offspring and its subsequent effects involves passive and counter-regulatory mechanisms. Faught et al. (2016) [21] demonstrated an increase in the *hsd11b2* transcript in zebrafish ovarian follicles following ex vivo cortisol incubation, indicating that maternal stress may lead to the activation of ovarian enzymes that mitigate cortisol levels in oocytes. However, our findings suggest that cortisol deposition is transient due to the asynchronous nature of zebrafish reproduction, in which vitellogenic oocytes might be susceptible to excessive steroid uptake during a specific window before the upregulation of *hsd11b2* activity in ovarian follicles in response to increased cortisol levels. Notably, no significant differences in *hsd11b2* expression were observed between experimental groups up to 72 hpf, supporting our hypothesis. Although *hsd11b2* mRNA levels were unchanged, the expression of *fkbp5* was reduced post-fertilization. Therefore, the mechanism behind the increased cortisol levels in embryos remains unclear, and further research is required to better understand the mechanism of maternal cortisol transfer in fish. We propose that maternal cortisol transfer occurred without a counter-regulatory effect on *fkbp5* mRNA levels, as previously demonstrated by Tovo-Neto et al. (2020) [36], who showed that high cortisol concentrations increase *fkbp5* expression. The morphological and histological results obtained in this study support these findings. While cortisol levels were not directly measured in treated mothers or their offspring in our study, we adhered to the cortisol-stimulated female zebrafish model established by Faught et al. (2016) [21]. Following this framework, we hypothesize that feeding females a cortisol-enriched diet promotes the maternal transfer of cortisol to their offspring.

Histological analysis of muscle fiber in larvae at 30 dpf from a mother fed a cortisol-enriched diet revealed a significantly larger muscle bundle size and an increased cross-sectional diameter compared to larvae from the control group. These findings are consistent with previous studies. Wilson et al. (2016) [42] showed that treating fertilized embryos with the corticosteroid dexamethasone for 5 days resulted in increased larval size at 84 dpf. This suggests that changes in corticosteroid levels, either in ovarian follicles or directly in embryos, can induce long-term morphological alterations. The same study [42] also investigated both the acute and chronic effects of the synthetic glucocorticoid receptor (GR) agonist dexamethasone. At 120 hpf, treated larvae exhibited reduced cortisol levels compared to control larvae. Additionally, Nesan and Vijayan (2016) [27] demonstrated that alterations in maternal cortisol availability affect the cortisol response in zebrafish embryos post-hatching. Specifically, reducing cortisol levels in embryos via microinjection of specific antibodies resulted in an enhanced cortisol response. In contrast, larvae from embryos microinjected with cortisol did not show a cortisol response following an acute physical stressor, indicating a potential disruption of HPI axis activity. In vertebrates, muscle growth occurs through a combination of two main processes: hypertrophy, which is the increase in the size and length of existing muscle fibers, and hyperplasia, which involves the formation of new muscle fibers from the differentiation of muscle stem cells. Research on zebrafish has demonstrated that the cross-section area (CSA) of muscle fibers correlates directly with the fish's age [30]. Additionally, newly formed muscle fibers through hyperplasia in older zebrafish exhibit increased CSA and length compared to those in younger fish [29]. Our

finding showed that juveniles at 30 dpf from cortisol-fed females had a higher percentage of thicker fibers compared to the control group. These findings suggest that maternal cortisol treatment may have accelerated the metabolic rate and developmental progression of the embryos, as evidenced by changes in larval length. Another hypothesis is that the treatment induced greater hypertrophy than muscle hyperplasia.

5. Conclusions

This study demonstrates that maternal stress, associated with cortisol deposition, significantly impacts the survival and morphological and cardiac development of offspring during the post-fertilization period. Embryos from cortisol-treated females showed altered expression of the genes involved in protein synthesis, suggesting accelerated growth in the first hour post-fertilization. In juveniles, significant changes were observed in muscle fiber size and cross-sectional diameter. These findings underscore the critical influence of maternal treatment on the regulation of the Hypothalamic–Pituitary–Interrenal (HPI) axis in offspring, thereby impacting their growth, development, and overall viability.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/fishes10020037/s1>. Figure S1: (A) Primer specificity: The primer specificity was verified according to the melt curve temperature (dissociation temperature). The dissociation temperature in all qPCR reactions is demonstrated for each primer. Each dot represents the melt curve temperature for one sample. (B) A scatter plot was generated to assess the stability of β -actin as a reference gene in samples of embryo/early larvae collected at 0 to 72 hpf from females fed only with commercial food (control group) and fed with a diet laced with 0.5 mg hydrocortisone per gram of feed. No significant differences ($p > 0.05$; ANOVA following Tukey's multiple comparison test) were observed in β -actin Ct values between the control and treated samples across the experimental groups. Table S1: primers used for gene expression studies (qPCR) (FW = Forward; RV = Reverse).

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher. The data are available from the author, M.S.R., upon request.

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