

# Leptin-Mediated Modulation of Steroidogenic Gene Expression in Hypoxic Zebrafish Embryos: Implications for the Disruption of Sex Steroids

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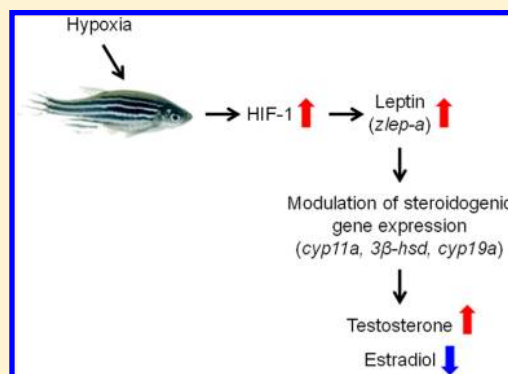
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**ABSTRACT:** Hypoxia can impair reproduction of fishes through the disruption of sex steroids. Here, using zebrafish (*Danio rerio*) embryos, we investigated (i) whether hypoxia can directly affect steroidogenesis independent of pituitary regulation via modulation of steroidogenic gene expression, and (ii) the role of leptin in hypoxia-induced disruption of steroidogenesis. Exposure of fertilized zebrafish embryos to hypoxia (1.0 mg O<sub>2</sub> L<sup>-1</sup>) from 0–72 h postfertilization (hpf), a developmental window when steroidogenesis is unregulated by pituitary influence, resulted in the up-regulation of *cyp11a*, *cyp17*, and *3β-hsd* and the down-regulation of *cyp19a*. Similar gene expression patterns were observed for embryos exposed to 10 mM cobalt chloride (CoCl<sub>2</sub>, a chemical inducer of hypoxia-inducible factor 1, HIF-1), suggesting a regulatory role of HIF-1 in steroidogenesis. Testosterone (T) and estradiol (E2) concentrations in hypoxic embryos were greater and lesser, respectively, relative to the normoxic control, thus leading to an increased T/E2 ratio. Expression of the leptin-*a* gene (*zlep-a*) was up-regulated upon both hypoxia and CoCl<sub>2</sub> treatments. Functional assays suggested that under hypoxia, elevated *zlep-a* expression might activate *cyp11a* and *3β-hsd* and inhibit *cyp19a*. Overall, this study indicates that hypoxia, possibly via HIF-1-induced leptin expression, modulates sex steroid synthesis by acting directly on steroidogenic gene expression.



## INTRODUCTION

Hypoxia arising as a result of eutrophication is now one of the most pressing problems in aquatic ecosystems worldwide, and this problem is likely to be exacerbated by climate change in the coming years.<sup>1,2</sup> Chronic, sublethal hypoxia can impair reproduction and sexual development of fish through disruption of sex steroids.<sup>3</sup> Previously, we demonstrated that hypoxia can alter both the absolute and relative concentrations of estradiol (E2) and testosterone (T), which resulted in a male-biased F1 generation in zebrafish (*Danio rerio*).<sup>4</sup> Other forms of impairment of reproductive fitness, such as retarded gonadal growth and gametogenesis, ovarian masculinization, and reduced fecundity with concomitant perturbation of E2 and T were similarly reported in several other teleosts exposed to chronic hypoxia.<sup>5–9</sup> Nonetheless, there is limited information on the molecular mechanisms of the hypoxia-induced disruption of sex steroids.

Steroidogenesis is generally under the control of various regulators, principally pituitary peptides, which interact with specific cell surface receptors which are expressed in steroidogenic tissues. In the case of gonadal and interrenal tissues, the synthesis of sex steroids is controlled by the pituitary-derived gonadotropins, follicle-stimulating hormone (FSH), and luteinizing hormone (LH), via the hypothalamic-pituitary-steroidogenic (HPS) feedback circuit.<sup>3,10</sup> Recent evidence suggests that hypoxia can act on multiple targets of this circuit to disrupt synthesis of sex steroids. In Atlantic croakers (*Micropogonias undulatus*), hypoxia has been shown to suppress secretion of LH by inhibiting synthesis of the hypothalamic neurotransmitter serotonin (5-HT) and expression of gonadotropin-releasing hormone-I (GnRH-I) mRNA.<sup>7</sup>

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Table 1. Sequences of Real-Time PCR Primers

gene	primer sequence (5'→3') forward	reverse
<i>star</i>	CTGAGAATGGACCCACCTGT	CACCTGGGTTTGTGAAAGT
<i>cyp11a</i>	AAAATCTGCTGCAGGTCAAGGT	TGCCCACTCCTCTCAATCTGTT
<i>cyp17</i>	TGGAGCTCTTTGCATGTTTG	CAGCACTGTTTTGGCTTTCA
<i>3β-hsd</i>	CAGGAGAGGTGTGTGGGTG	ATGAGCTCTGAGCGGATGTT
<i>17β-hsd</i>	GTGAATTCCTCGGCAGTGT	CTTTTATGCGCCATAACCT
<i>cyp19a</i>	CCATCAGTCTGTTCTTCATGC	CTTGGACAGATGCGAGTGCTG
<i>cyp19b</i>	GGCAGTCTCTGGAGGATGAC	CAGTGTCTCGAAGTTCTCCA
<i>igfbp-1</i>	GTCAATGAAGGCAGCTCCAC	TCTTGCATCGCGTTGACT
<i>vegf</i>	CCAAAGGCAGAAGTCAAAGC	TGCAGGACATTTACAGGTG
<i>zlep-a</i>	CAGGGAACACATTGACGGCA	ATGGAGCCGAGCCCTTGATG
<i>β-actin</i>	CGAGCAGGAGATGGGAACC	CAACGAAACGCTCATTAC

Furthermore, other studies have revealed that expression of genes encoding key transporters and enzymes involved in steroidogenesis is modulated by hypoxia,<sup>4,9,11</sup> which implies that hypoxia can also exert a direct effect on the steroidogenesis pathway.

Leptin is a peptide hormone secreted primarily by adipocytes and functions to regulate intake of food, metabolism, and reproduction.<sup>12</sup> In humans and rodents, concentrations of leptin in blood plasma are significantly correlated with the body mass index (BMI) or percent body fat.<sup>13</sup> Although leptin is a permissive signal for reproductive function, greater concentrations of leptin can directly inhibit steroidogenesis, likely by altering expression of genes involved in the steroidogenesis pathway.<sup>14–18</sup> This altered expression of steroidogenic genes might, in part, account for the poor fertility observed in obese humans.<sup>19</sup> In zebrafish, we recently demonstrated that one of the leptin genes, *zlep-a*, is activated by hypoxia in both embryos and adults.<sup>20</sup> This transcriptional response to hypoxia is mediated by the hypoxia-inducible factor 1 (HIF-1), a transcription factor that functions as a central regulator of oxygen homeostasis.<sup>21</sup> On the basis of the induction of *zlep-a* expression by hypoxia and the ability of leptin to modulate mammalian steroidogenesis, we hypothesize that the effects of hypoxia on modulating expression of steroidogenic genes and concentrations of sex steroids in fish are, at least in part, due to HIF-1-enhanced leptin expression.

The primary objectives of this study were to investigate the direct effect of hypoxia on expression of steroidogenic genes and to elucidate the underlying mechanisms in relation to HIF-1 and leptin. To ascertain that the observed effects were independent of the hypothalamic-pituitary axis, zebrafish embryos at a stage when steroidogenic activity is unregulated by the pituitary (i.e., 0–72 h postfertilization, hpf)<sup>22</sup> were used for treatments and subsequent analyses. Changes in expression of seven key genes that are involved in the steroidogenesis pathway (steroidogenic acute regulatory protein *star*, cholesterol side-chain cleavage enzyme *cyp11a*, 17 $\alpha$ -hydroxylase *cyp17*, 3 $\beta$ -hydroxysteroid dehydrogenase *3β-hsd*, 17 $\beta$ -hydroxysteroid dehydrogenase *17β-hsd*, and aromatase *cyp19a* and *cyp19b*) and the endogenous concentrations of E2 and T were quantified in zebrafish embryos under hypoxia and after treatment with cobalt chloride (CoCl<sub>2</sub>, a HIF-1 inducer). Also, these changes were assessed following knockdown and overexpression of *zlep-a* under hypoxia and normoxia, respectively. Our results indicate that hypoxia, possibly via HIF-1, can directly alter expression of steroidogenic genes and affect the absolute and relative concentrations of E2 and T and

suggest that leptin is involved in hypoxia-induced disruption of steroidogenesis.

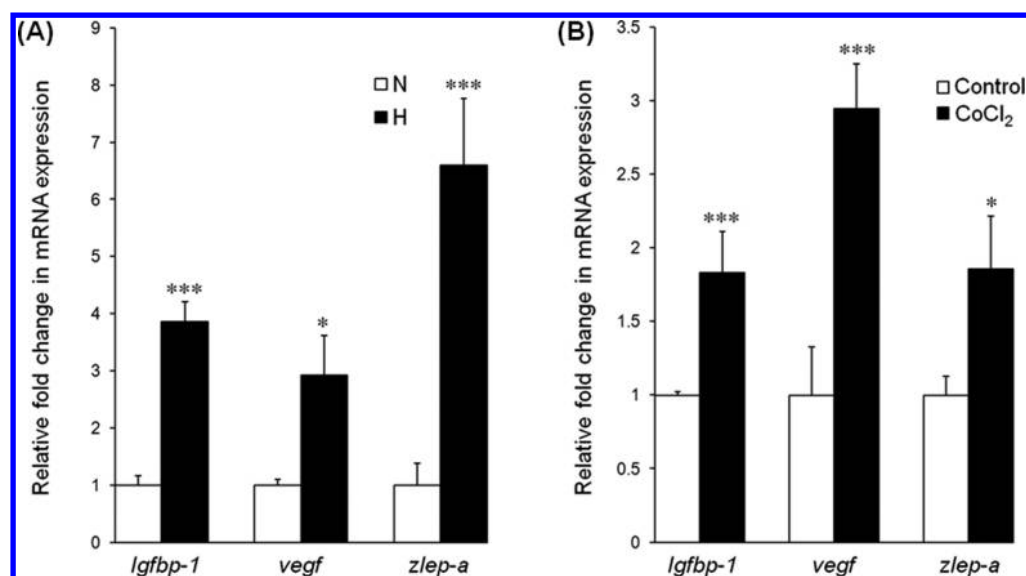
## MATERIALS AND METHODS

**Zebrafish Maintenance.** Wild-type adult zebrafish (*Danio rerio*) breeding colonies were obtained from a local supplier and were maintained in tanks supplied with well-aerated water filtered by reverse osmosis (RO) at 28.5 °C. Fish were maintained under a constant 14-h light/10-h dark photoperiod. Fertilized eggs were collected at the first hour of the light period. Embryos were collected and incubated in standard zebrafish E3 embryo medium (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L CaCl<sub>2</sub>, 0.33 mmol/L MgSO<sub>4</sub>, 10<sup>-5</sup>% methylene blue) at 28.5 °C.

**Hypoxia and CoCl<sub>2</sub> Exposure Experiments.** Tanks used for hypoxia exposure were set up as previously described.<sup>4</sup> Dissolved oxygen (DO) concentrations used for normoxic and hypoxic exposure were 7.0 ± 0.2 and 1.0 ± 0.2 mg O<sub>2</sub> L<sup>-1</sup>, respectively. Fertilized embryos (0 hpf) were placed in net cages and allowed to develop until 72 hpf. For CoCl<sub>2</sub> exposure, embryos were cultured in Petri dishes containing E3 medium at 28.5 °C. At 24 hpf, embryos were transferred to either 10 mM CoCl<sub>2</sub> in E3 medium or E3 medium alone (control) and further incubated until 72 hpf. In both experiments, each treatment consisted of five replicates of 60 embryos each. At the end of the experiments, embryos were snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction or hormone analysis.

**RNA Extraction and Reverse Transcription.** RNA was extracted from zebrafish embryos using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. Before reverse transcription, contaminating genomic DNA was removed using RQ1 RNase-free DNase (Promega). First-strand cDNA was synthesized using 1 μg total RNA, 1.25 μL dNTP (10 mM), 2.4 μL random hexamer (50 ng/μL), 1 μL RNaseOUT (40 U; Invitrogen), and 1 μL M-MLVRT (H-) (200 U/μL; Promega) in a total volume of 25 μL in 1× M-MLVRT reaction buffer at 42 °C for 50 min. The reaction was terminated by incubation at 70 °C for 15 min.

**Real-Time PCR.** Real-time PCR, used for gene expression quantification, was performed as described previously.<sup>23</sup> PCR assays were conducted using a StepOnePlus Real-time PCR System (Applied Biosystems) with the SYBR Green-based detection method. Primer sequences used for real-time PCR are listed in Table 1. Melting curve analysis was performed at the end of each PCR thermal profile to assess amplification specificity. The identity of PCR amplicons was confirmed by DNA sequencing. Real-time PCR reactions for all samples were



**Figure 1.** Activation of hypoxia markers and *zlep-a* by hypoxic and  $\text{CoCl}_2$  treatments. (A) Fertilized zebrafish embryos were exposed to either normoxic (N;  $7.0 \pm 0.2 \text{ mg O}_2 \text{ L}^{-1}$ ) or hypoxic (H;  $1.0 \pm 0.2 \text{ mg O}_2 \text{ L}^{-1}$ ) conditions until 72 hpf. (B) Zebrafish embryos at 24 hpf were transferred to either embryo medium (control) or  $\text{CoCl}_2$  (10 mM) in embryo medium and reared until 72 hpf. For both treatments, the relative expression levels of the hypoxia markers, *igfbp-1* and *veg*, and *zlep-a* were quantified by real-time PCR followed by normalization to  $\beta$ -actin expression. Five replicates ( $n = 5$ ) of 60 pooled embryos each were used. The data are presented as the mean relative fold change  $\pm$  SD with respect to the gene expression level in normoxic control (A) or embryo medium control (B) (they were arbitrarily set to 1). Expression levels that are significantly different from those of the control are indicated by asterisks (Student's *t*-test, \* $p < 0.05$ , \*\*\* $p < 0.001$ ).

performed in triplicate. Rates of expression of genes were normalized to expression of  $\beta$ -actin expression according to the algorithm described by Simon.<sup>24</sup> The data are reported as the mean relative fold change  $\pm$  SD with respect to the control level (arbitrarily set to 1) in each experiment.

**Hormone (E2 and T) Measurement.** Sixty embryos from each replicate were homogenized in 300  $\mu\text{L}$  ELISA (EIA) buffer. Samples were centrifuged for 5 min at  $5000 \times g$  at room temperature and the supernatant was then collected for hormone extraction. All samples were spiked with 10  $\mu\text{L}$  1,2,6,7-<sup>3</sup>H-labeled T (0.0002  $\mu\text{Ci}/\mu\text{L}$ ) (PerkinElmer) prior to extraction to determine extraction recovery. The hormones were extracted twice with 2.5 mL diethyl ether, and phase separation was achieved by centrifugation at  $2000 \times g$  for 10 min. The solvent phase containing the target hormones was then evaporated under a stream of nitrogen. Residues containing hormones were reconstituted in 300  $\mu\text{L}$  EIA buffer (Cayman Chemical) and diluted to 1:10 or 1:50 for T and E2 analysis, respectively. Hormone levels were measured using a commercial competitive ELISA kit (Cayman Chemical).

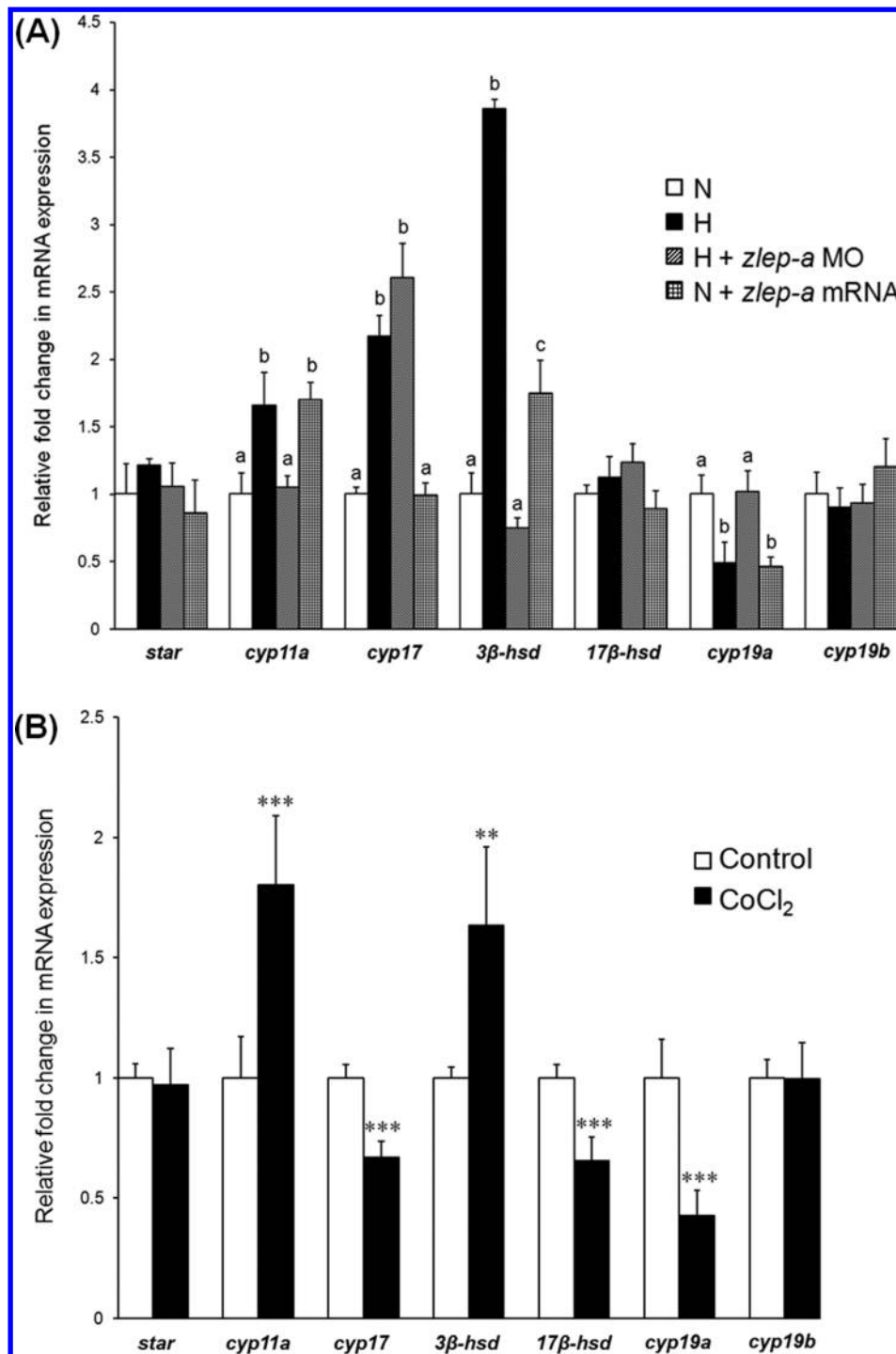
***zlep-a* Knockdown.** Antisense morpholino oligonucleotides (MOs) were obtained from Gene Tools (Philomath, OR). An MO targeted against the translation initiation region of *zlep-a* (*zlep-a* MO, 5'-CGGAGAGCTGGAAAACGCATACTTC-3') was used to knockdown the *zlep-a* protein. The standard control MO (5'-CCTCTTACCTCAGTTACAATTTATA-3') was used as an injection control. The MOs were dissolved in Hanks buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM  $\text{MgSO}_4$ , 0.6 mM  $\text{Ca}(\text{NO}_3)_2$  and 5.0 mM HEPES (pH 7.6)] containing 0.1% phenol red (visualizing indicator) and injected (6 ng MO) into 1–2-cell-staged embryos using a Femojet microinjection system (Eppendorf). Five replicates of 60 fertilized embryos each were used for both treatments. Injected embryos were reared until 72 hpf under the same hypoxic conditions as described above.

***zlep-a* Overexpression.** In vitro synthesis of *zlep-a* mRNA (GenBank accession no. NM\_001128576) and microinjection were conducted according to Kajimura et al.<sup>25</sup> Briefly, *zlep-a* mRNA was synthesized using a SP6 mMessage mMachine kit (Ambion) and diluted to a final concentration of 3 ng/nL in Hanks buffer containing 0.1% phenol red. At the 1–2-cell stage, embryos were injected with either *zlep-a* mRNA (3 ng) or Hanks buffer alone (injection control) using a Femojet microinjection system (Eppendorf). Five replicates of 60 fertilized embryos each were used for both treatments. Injected embryos were reared until 72 hpf under the same normoxic conditions as described above.

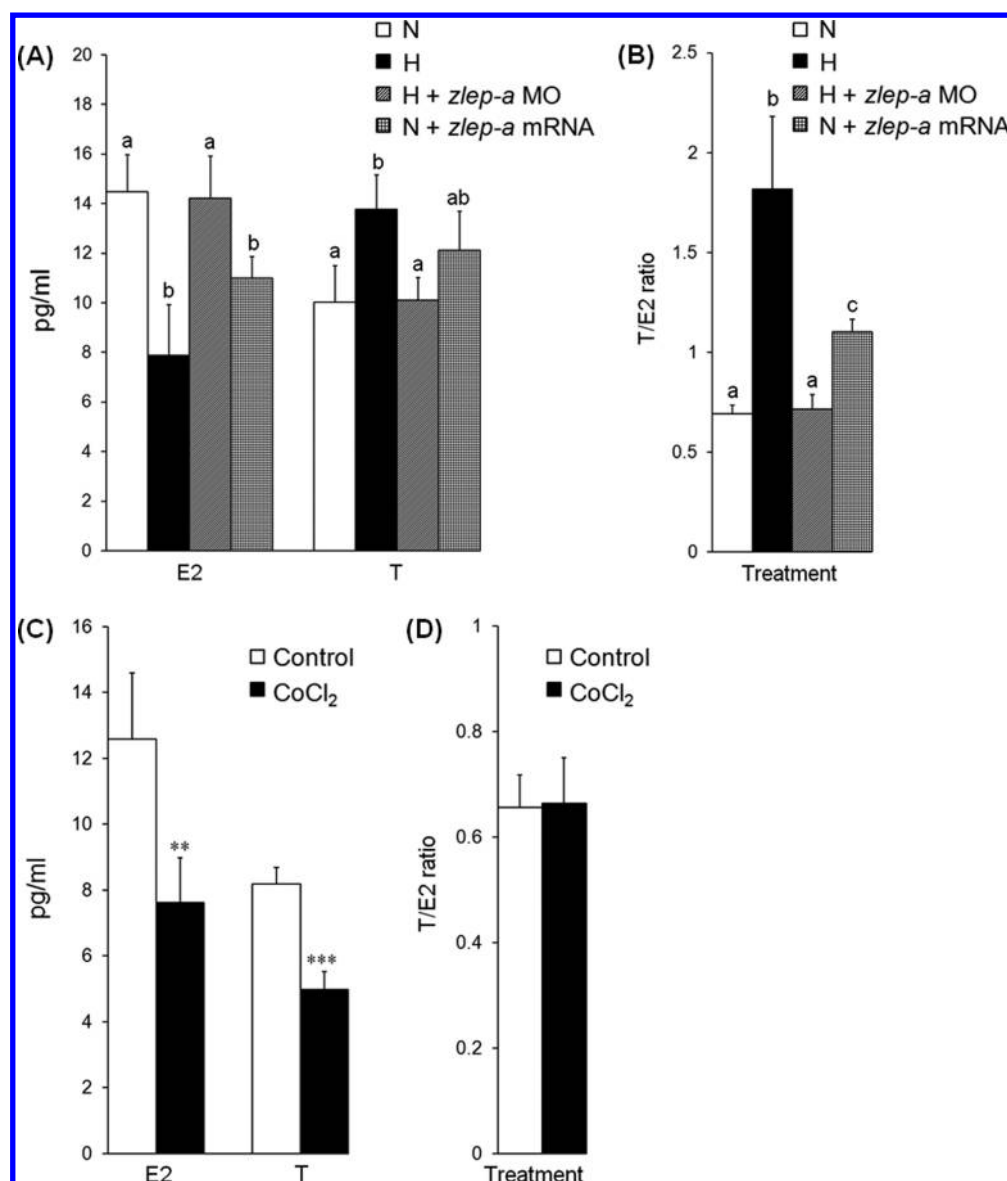
**Statistical Analysis.** A two-tailed Student's *t* test or one-way ANOVA followed by Student–Newman–Keuls posthoc test was used for comparing the means between treatment groups. All statistical analyses were conducted using Prism 3.02 (GraphPad, San Diego, CA). The data are expressed as means  $\pm$  SD, and a *p*-value  $< 0.05$  was considered statistically significant.

## RESULTS

**Activation of the HIF-1 Pathway and *zlep-a* by Hypoxia and  $\text{CoCl}_2$ .** To confirm activation of the HIF-1 signaling pathway in zebrafish embryos raised under our experimental hypoxic or  $\text{CoCl}_2$  conditions, real-time PCR was used to quantify the expression levels of the HIF-1 target genes, *igfbp-1* and *veg*, in embryos at 72 hpf. The results demonstrated that expressions of *igfbp-1* and *veg* were significantly increased by approximately 4- and 3-fold, respectively, upon exposure to hypoxia ( $1.0 \text{ mg O}_2 \text{ L}^{-1}$ ) (Figure 1A). Similar patterns of up-regulation of *igfbp-1* (2-fold) and *veg* (3-fold) were also observed for  $\text{CoCl}_2$  (10 mM)-treated embryos (Figure 1B). These results indicated that under our experimental conditions, the HIF-1 pathway is activated in zebrafish embryos by both hypoxia and cobalt



**Figure 2.** Effects of hypoxia, *zlep-a* expression and  $\text{CoCl}_2$  on steroidogenic gene expression. (A) Effects of hypoxia and *zlep-a* expression on steroidogenic gene expression. Fertilized zebrafish embryos were exposed to either normoxic (N) or hypoxic (H) conditions (as described in Figure 1) until 72 hpf. To study the effect of *zlep-a* expression on steroidogenic gene expression, 1–2-cell stage zebrafish embryos were injected with *zlep-a* MO (knockdown) or *zlep-a* mRNA (overexpression) and reared under hypoxic or normoxic conditions, respectively, until 72 hpf. Concurrently, embryos were injected with a standard control MO or Hanks buffer as injection controls for knockdown and overexpression, respectively. As there was no statistically significant difference between the data sets of injection control and no-injection control (one-way ANOVA with a  $p < 0.05$  threshold), only the data from the no-injection controls are graphically illustrated. (B) Effects of  $\text{CoCl}_2$  on steroidogenic gene expression. Methods for  $\text{CoCl}_2$  exposure are detailed in Figure 2. For all treatments, the relative expression levels of *star*, *cyp11a*, *cyp17*, *3β-hsd*, *17β-hsd*, *cyp19a*, and *cyp19b* were quantified by real-time PCR followed by normalization to  $\beta$ -actin expression. Five replicates ( $n = 5$ ) of 60 pooled embryos each were used. The data are presented as the mean relative fold change  $\pm$  SD with respect to the gene expression level in the normoxic control (A) or embryo medium control (B) (they were arbitrarily set to 1). Different letters above the bars in (A) indicate statistically significant differences between the indicated groups for the same gene ( $p < 0.05$ , Student–Newman–Keuls) while expression levels that are significantly different from those of the control in (B) are indicated by asterisks (Student's *t*-test, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ).



**Figure 3.** Effects of hypoxia, *zlep-a* expression and  $\text{CoCl}_2$  on E2 and T levels in zebrafish embryos. (A) Effects of hypoxia and *zlep-a* expression on E2 and T levels. Methods for hypoxic exposure and *zlep-a* knockdown and overexpression are detailed in Figures 1 and 2, respectively. Hormone levels in whole zebrafish embryos (5 replicates of 60 pooled embryos each) were measured by the ELISA method. The data are presented as the mean concentration of hormone (in picograms per milliliter of embryo homogenate)  $\pm$  SD. Different letters above the bars indicate statistically significant differences between the indicated groups for the same hormone ( $p < 0.05$ , Student–Newman–Keuls). (B) Effects of hypoxia and *zlep-a* expression on T/E2 ratios. Data are presented as mean  $\pm$  SD ( $p < 0.05$ , Student–Newman–Keuls). Different letters above the bars indicate statistically significant differences between the indicated groups. (C) Effects of  $\text{CoCl}_2$  on E2 and T levels. Methods for  $\text{CoCl}_2$  exposure are described in Figure 1. Hormone levels were measured as described above for hypoxia treatment and data presented in picograms per milliliter of embryo homogenate)  $\pm$  SD (Student's *t*-test, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). (D) Effects of  $\text{CoCl}_2$  on T/E2 ratios. Data are presented as mean  $\pm$  SD (no statistical significance was found, Student's *t*-test  $p < 0.05$ ).

chloride. In our previous study, *zlep-a* was identified as a hypoxia (or HIF-1)-inducible gene in zebrafish.<sup>20</sup> In the study upon which we report here, we showed that *zlep-a* expression in 72-hpf zebrafish embryos was significantly up-regulated by approximately 7- and 2-fold upon exposure to hypoxia (Figure 1A) and  $\text{CoCl}_2$  (Figure 1B), respectively.

**Expression of Steroidogenic Genes.** After exposure of fertilized embryos to hypoxia for 72 h, *cyp11a*, *cyp17*, and *3 $\beta$ -hsd* expression was significantly up-regulated by 1.7-, 2.2-, and 3.9-fold, respectively, while *cyp19a* expression was down-regulated by 2-fold; the expression of the other steroidogenic genes, *star*, *17 $\beta$ -hsd*, and *cyp19b*, was unaffected (Figure 2A).

Overexpression and knockdown of *zlep-a* (by mRNA and MO injection, respectively) selectively affected the expression of *cyp11a*, *3 $\beta$ -hsd*, and *cyp19a* mRNA but not the expression of the other steroidogenic genes (Figure 2A). Upon *zlep-a* knockdown in hypoxic embryos, the expression of both *cyp11a* and *3 $\beta$ -hsd* was decreased to normoxic levels, while *cyp19a* expression was restored to normoxic levels. In contrast, *zlep-a* overexpression in normoxic embryos resulted in a 1.7-fold up-regulation in the expression of both *cyp11a* and *3 $\beta$ -hsd* and a 2-fold down-regulation of *cyp19a* expression. Altogether, the results suggest that elevated *zlep-a* expression stimulates *cyp11a* and *3 $\beta$ -hsd* expression, but inhibits *cyp19a* expression in

hypoxic zebrafish embryos, whereas the hypoxic up-regulation of *cyp17* seems to not be dependent on *zlep-a* expression.

CoCl<sub>2</sub>-treated embryos exhibited a partially overlapping pattern of expression compared to that obtained under hypoxia; *cyp11a* and *3β-hsd* expression was up-regulated by 1.8- and 1.6-fold, respectively, while *cyp19a* expression was down-regulated by 2.3-fold; *star* and *cyp19b* expression remained unaffected (Figure 2B). In contrast to the hypoxic embryos (in which expression of *cyp17* and *17β-hsd* were up-regulated and unaffected, respectively), the expression of both *cyp17* and *17β-hsd* was down-regulated by 1.5-fold in CoCl<sub>2</sub>-treated embryos.

**E2 and T Concentrations.** Upon hypoxia treatment, concentrations of E2 and T in whole embryos were 1.8-fold less, and 1.4-fold greater than those of the normoxic controls (Figure 3A), which resulted in a 2.6-fold greater T/E2 ratio (from  $0.7 \pm 0.04$  (normoxia) to  $1.8 \pm 0.37$  (hypoxia); Figure 3B). Knockdown of *zlep-a* in hypoxic embryos restored both E2 and T levels (Figure 3A) and their ratio (Figure 3B) to that observed for the normoxic control. In contrast, *zlep-a* overexpression under normoxia resulted in concentrations of E2 and T that were 1.3-fold less, and 1.2-fold greater than the controls, respectively (Figure 3A). However, the difference for T was not statistically significant ( $p = 0.062$ ). These changes resulted in a 1.5-fold greater T/E2 ratio compared to the normoxic control (Figure 3B). Overall, these findings suggest that greater expression of *zlep-a* under hypoxia results in less production of E2 but more production of T, which results in the observed greater T/E2 ratio.

CoCl<sub>2</sub>-treated embryos displayed a slightly different pattern from that observed for hypoxic embryos; E2 and T concentrations were 1.7- and 1.4-fold, respectively, less than that of the controls (Figure 3C) and the T/E2 ratio was unaffected (Figure 3D). These results might indicate that hypoxia and CoCl<sub>2</sub> modulate E2 and T synthesis in slightly different ways.

## DISCUSSION

There is emerging evidence that hypoxia impairs reproduction and sexual development of fish through perturbation at multiple points in the HPS axis, affecting gonadotropins and hypothalamic neurotransmitters<sup>7,26</sup> as well as certain enzymes controlling steroidogenesis.<sup>4,6</sup> Steroidogenic enzymes are primarily regulated at the level of transcription, which is under control of pituitary tropic hormones.<sup>27</sup> In the gonad, FSH and LH regulate steroidogenesis through interactions with membrane-bound receptors on the surface of gonadal somatic cells. Subsequent regulation of cAMP-dependent transcription is mediated by the transcription factor steroidogenic factor 1 (SF-1). Similar mechanisms also exist in the control of adrenal steroidogenesis.<sup>28</sup> However, whether hypoxia influences steroidogenic enzymes through other mechanisms, in particular those occurring in steroidogenic tissues, remains unresolved. The results of the study upon which we report here provide novel evidence that hypoxia directly alters steroidogenic gene expression independent of pituitary influence and affects the production of sex steroids in zebrafish embryos.

Among the seven steroidogenic genes examined, significant differential expression was observed for four genes under hypoxia in 72-hpf zebrafish embryos, whereby *cyp11a*, *cyp17*, and *3β-hsd* were up-regulated and *cyp19a* was down-regulated compared with the normoxic control (Figure 2A). This differential expression pattern was not likely a result of delayed

or accelerated development of steroidogenic tissues (primarily consisting of interrenal tissues at 72 hpf) because expression of *ff1b* (the zebrafish *SF-1* ortholog, which also serves as a marker for early interrenal development) was found to be unaffected by hypoxia (real-time PCR data not shown). Differential expression of steroidogenic genes under hypoxia has been previously described in both fish<sup>4,9,11</sup> and mammalian<sup>29,30</sup> models. The regulatory mechanisms that have been proposed to date include the involvement of LH and HIF-1. In Atlantic croakers, lesser pituitary secretion of LH and down-regulation of ovarian expression of *cyp19a* were observed after chronic laboratory exposure to hypoxia.<sup>7,9</sup> However, this mechanism cannot explain the differential steroidogenic gene expression observed here for hypoxic zebrafish embryos because steroidogenesis in zebrafish embryos is unregulated by pituitary influence from 0–72 hpf.<sup>22</sup> At the organ level, HIF-1 was shown to be constitutively expressed in Leydig cells of murine testis and have the ability to activate the *3β-hsd* promoter.<sup>30</sup> In our present study, treatment of zebrafish embryos with the HIF-1 inducer CoCl<sub>2</sub> resulted in greater expression of *cyp11a* and *3β-hsd* and lesser expression of *cyp19a* (Figure 2B) in a manner comparable to that observed in the hypoxia-treated embryos. These parallel observations suggest that HIF-1 might act as a transcriptional regulator (direct and/or indirect) of certain steroidogenic genes in both mammals and fish.

There is increasing evidence that inhibition of estrogen synthesis by hypoxia critically affects reproduction and sexual development in fish by causing retarded gonadal growth and masculinization of the ovary.<sup>5–9</sup> Moreover, significantly lower concentrations of E2 in females under hypoxic conditions have been reported in multiple fish species.<sup>4,6,7,9,31</sup> However, in those studies, the T concentrations in hypoxic fish were usually comparable if not greater than those measured in the normoxic controls, which excludes the possibility that the lesser production of estrogen under hypoxia is due to a central suppression of the HPS axis. Our results indicated that under hypoxia, concentrations of E2 in embryos were 1.8-fold lesser whereas concentrations of T were 1.4-fold greater than those measured in embryos exposed to normoxic conditions (Figure 3A). This then led to a 2.5-fold greater T/E2 ratio compared to that of the normoxic control (Figure 3B). This result is consistent with our previous observation of lesser concentrations of E2 and greater concentrations of T in female zebrafish at sexually mature and spawning stages when exposed to hypoxic conditions.<sup>4</sup> Taken together, these observations suggest that the direct effects of hypoxia on steroidogenesis might occur at different life stages in fish and could contribute, at least in part, to the control of sex steroid production in zebrafish.

Aromatase is the enzyme responsible for the aromatization of T into E2. The concomitant decrease of *cyp19a* expression (Figure 2A,B) and E2 concentration (Figure 3A,C) in both hypoxic and CoCl<sub>2</sub>-treated zebrafish embryos, therefore, suggests that inhibition of *cyp19a* expression and related aromatase availability could be an important cause of the reduction in E2 levels in fish under hypoxia. Regrettably, the correlation between the levels of *cyp19a* expression and aromatase protein/activity could not be verified in this study due to the low expression level of the latter in zebrafish embryos. Further in vitro studies using high estrogen- or aromatase-producing tissues (such as the ovary) could help decipher the link between expression of *cyp19a* mRNA and aromatase under hypoxic conditions.

The observation of elevated concentrations of T in hypoxic zebrafish embryos could be due to less conversion of T to E2 caused by less aromatase. Another possible explanation might be an increased synthesis of T as a result of the up-regulation of the steroidogenic genes that act upstream of T aromatization, including *cyp11a*, *cyp17*, and *3 $\beta$ -hsd*, in hypoxic embryos (Figure 2A). Unlike the effect of hypoxia, exposure of embryos to CoCl<sub>2</sub> resulted in a moderate decrease in T concentrations (Figure 3C). While the reason for the observed difference is unclear, possible explanations include the down-regulation of *cyp17* and *17 $\beta$ -hsd* in CoCl<sub>2</sub>-treated embryos (Figure 2B; which was not observed in hypoxic embryos) and the potential inhibition of certain steroidogenic enzymes (such as 3 $\beta$ -HSD) by the pro-oxidative effect of CoCl<sub>2</sub>.<sup>32,33</sup> The possibility of retarded development of steroidogenic tissues in CoCl<sub>2</sub>-treated embryos could be excluded as *ff1b* expression was unaffected by CoCl<sub>2</sub> (real-time PCR data not shown).

Leptin regulates steroidogenesis through its receptors, expressed both centrally (at the hypothalamic-pituitary level) and peripherally (at the level of the steroidogenic organs themselves, including the ovary, testis, and adrenal gland).<sup>34</sup> It has been demonstrated in a number of in vitro studies that leptin inhibits the production of E2 by human ovarian granulosa cells under various conditions.<sup>35–39</sup> Consistent with this finding, the present study supports a causative relationship between elevated leptin gene expression and inhibited E2 synthesis under hypoxic conditions. Evidence supporting this mechanism includes: (i) *zlep-a* expression was greater and E2 concentrations were less in zebrafish embryos exposed to hypoxic conditions (Figures 1A and 3A, respectively), (ii) knocking down *zlep-a* restored normal E2 concentrations under hypoxia (Figure 3A), and (iii) overexpression of *zlep-a* resulted in lesser E2 concentrations under normoxia (Figure 3A). Since the changes in E2 concentrations occurred concomitantly with the changes in *cyp19a* expression (Figure 2A) under all of these conditions, it is possible that the inhibition of E2 synthesis during hypoxia is associated with leptin-dependent inhibition of *cyp19a* expression. Similarly, the effects of leptin on T levels were comparable to those induced by hypoxia; leptin appeared to increase T levels (Figure 3A), which were accompanied by the up-regulation of *cyp11a* and *3 $\beta$ -hsd* (Figure 2A).

Interestingly, the observed association between leptin and sex steroid levels and the changes in steroidogenic gene expression has been similarly described in polycystic ovary syndrome (PCOS) in humans. PCOS is an endocrine disorder in which there is an imbalance of sex hormones (with a high androgen-to-estrogen ratio) and one of the major causes of infertility in women. The fact that many PCOS patients (approximately 50%) are obese or overweight<sup>40</sup> suggests the role of adipose tissue in the pathophysiology of PCOS. A recent study has shown that leptin mRNA expression in subcutaneous adipose tissue (SAT) of PCOS patients is 2-fold higher than that of matched controls.<sup>18</sup> The levels of E2 and *cyp19* mRNA were lower, while the levels of testosterone and *3 $\beta$ -hsd* mRNA were higher in SAT of PCOS patients compared to controls. In another study, human recombinant leptin was shown to down-regulate aromatase activity in primary cultured intra-abdominal preadipocytes from women,<sup>16</sup> indicating an inhibitory effect of leptin on estrogen synthesis through aromatase regulation. Altogether, these findings raise the intriguing possibility that leptin may work in hypoxic fish to cause imbalance of sex steroid levels via a mechanism similar to PCOS. To clarify this possibility, further investigation of the correlation between the

concentrations of leptin and sex steroids in blood plasma and the effects of leptin administration on the in vivo production of sex steroids is needed. Although the findings reported here provide a perspective on the functional relevance of leptin in regulating steroidogenesis in developing embryos of fish, whether this finding can be extrapolated to steroidogenesis in adult fish, in which gonadal steroidogenesis contributes to the major sources of sex steroids, is unknown. Further research on this question will help decipher the role of leptin in causing endocrine disruption and reproductive impairment in hypoxic adult fish. In conclusion, the results of the study that are presented here indicate that hypoxia (possibly via HIF-1) modulates synthesis of sex steroid hormones by affecting expression of specific steroidogenic genes and further suggest that leptin is involved in the regulation of some of these genes.

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### Notes

The authors declare no competing financial interest.

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