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Research article

Sex-specific responses of the reproductive system of zebrafish (*Danio rerio*) to electrolysis

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ABSTRACT

Adult zebrafish (*Danio rerio*) were electrolyzed at different current densities to explore the effects of electrolysis on their reproductive system, especially on embryo production, and to uncover the molecular mechanism of changes in sex hormone and vitellogenin (VTG) levels. The results showed that embryo reproduction of zebrafish was reduced at a current density of 0.64 A/m^2 after 28 days of exposure. In addition, the 17β-estradiol concentration significantly decreased and the testosterone concentration increased in female zebrafish above 0.53 A/m^2. However, opposite trends were observed in male zebrafish. The VTG concentration was reduced considerably in the livers of female zebrafish in the 0.64 A/m^2 electrolysis group ($p < 0.05$). In addition, the mRNA expression of hormone-regulating genes was significantly altered in female and male zebrafish when the current density was greater than 0.53 A/m^2, and their change trends were sex-dependent. The genes expression levels of *vtg1* and *esr1* were downregulated in female zebrafish. However, the gene expression of esr1 and *cyp19a* was upregulated in male zebrafish. These changes were related to disruption in the hormone balance and VTG levels of adult zebrafish. Thus, electrolysis could cause masculinization of female zebrafish and feminization of male zebrafish. Nonetheless, there were few influences on the hormone levels and reproduction rate of adult zebrafish at the threshold of 0.26 A/m^2. Thus, the current density of electrolysis needs to be controlled within a specific range to reduce its harmful effects on the reproductive system of aquatic animals.

1. Introduction

With the process of industrialization, environmental pollution is becoming severe, especially water pollution, which poses a significant threat to aquatic ecosystems and humans (Schwarzenbach et al., 2010). Electrolysis technology, as an environmentally friendly and efficient wastewater treatment method, is becoming known and is being widely studied (Chaplin, 2019). Numerous studies have reported that electrolysis methods coupled with ecological floating beds or constructed wetlands with a low current density (0.02–0.37 mA/cm^2) have become increasingly adopted for water remediation applications due to their various and highly efficient ways of eliminating contaminants (Gao et al., 2018; Yan et al., 2021). However, ecological floating beds are used in the river for more extended periods, and adverse effects of the electric field on the fish near the electrodes have been observed, even though the fish were not in direct contact with the electrodes (Bohl et al., 2010; Dwyer et al., 1993). Thus, electrolysis technology is of great concern to aquatic organisms in river water.

As far as we know, previous studies have confirmed that with enough electric field intensity and duration of exposure, any type of current can be lethal; in addition, electric fishing conditions at a peak output of 260 V DC, 60 pulses/s, and a duty cycle of 25% has been shown to induce hemorrhages and fractured vertebrae in fish (Snyder, 2003). Moreover, Schreer et al. (2004) found that electroshocking for 32 s at 100 V, with a pulse width of 2 ms and a frequency of 30 Hz, causes cardiac arrest in fish. Henry and Grizzle (2003) demonstrated that exposing to 60 Hz pulsed DC with a voltage gradient of 16 V/cm electroshock in water with an ambient conductivity of 100 mS/cm for 20 s would cause necrotic skeletal muscle in fish. Therefore, electric fishing may have some impact on fish populations (Schill and Beland, 1995), since fish mortality and fish reproduction affect fish populations. To our knowledge, although studies have demonstrated injuries and mortality due to electrofishing, the effects of electrolysis on the growth and reproduction of fish have not been thoroughly investigated. Stewart (2014) reported that the interclutch time interval increased and the proportion of viable eggs decreased if Red Shiner was exposed to electroshock. Demski and Dulka

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(1984) found that sperm release was evoked by electrical stimulation (thresholds of 5–20 μA) of the olfactory tracts in male goldfish. Severe 100-V electric shocking had no effect on the males but did increase egg mortality in pink salmon females (Marriott, 1973). The motility of sperm did not change upon electrical stimulation at 50 V/10 mm at 60 s (Saito et al., 1999). The above studies suggest that electrolysis changes the number of eggs and sperm of fish, but studies have not yet explored the mechanism of the egg decrease.

The gonad is an organ that performs reproductive functions, and its developmental process determines whether the organism has a normal reproductive function. Assessing its physiological status could help to further evaluate the endocrine-disrupting effects of the external environment (Dang et al., 2018). Sex steroid hormones (e.g., 17β-estradiol (E2) and testosterone (T)) are essential regulators of reproductive processes and play direct roles during gametogenesis and reproductive maturation (Devlin and Nagahama, 2002; Sofikitis et al., 2008). Vitellogenin (VTG) is a sensitive and mature biomarker of estrogenic endocrine disruption and is generally produced in response to stimulation by estrogen (e.g., E2) binding to specific estrogen receptors (ERs), which activates VTG gene expression (Bao et al., 2020). Furthermore, assessing transcription levels of genes is expected to be an appropriate and sensible approach to identify gene targets and modulation pathways affected by endocrine-disrupting compounds (Schiller et al., 2012), which can be used as biomarkers or indicators of endocrine activity after exposure to electrolysis.

Zebrafish is an attractive model organism for evaluating the reproductive toxicity and endocrine-disrupting effects of pollutants because of its small size, ease of culture, and prolific egg production with high fertilization and hatching rates (Segner, 2009). Here, adult zebrafish were selected as the test animal. The guidelines of the Organization for Economic Co-operation and Development (OECD) indicated that 28 days was a reasonable electrolysis exposure time (OECD, 2013). By 28-day electrolysis exposure to adult zebrafish, the main aims of the research work were (1) to determine the sex-specific effects of electrolysis on reproductive capability, such as growth, gonadosomatic index (GSI), reproduction, and hatching rate of fish eggs of adult zebrafish, (2) to explore the possible sex-specific molecular mechanisms by evaluating the influence of electrolysis on the mRNA expression levels of hormone-regulating genes, gonad sex hormones, and VTG level of adult zebrafish at various electric current densities, and (3) to determine the electric current density that has no endocrine-disrupting potential in adult zebrafish and to facilitate a systematic understanding of the biosecurity of electrolysis in aquatic animals.

2. Materials and methods

2.1. Zebrafish culture and experimental design

In this study, adult zebrafish (3 months old, AB wild-type) were purchased from the China Zebrafish Resource Center (Wuhan, China) and maintained in a recirculating zebrafish cultivation system (ESEN, Beijing, China) over 30 days. Adult zebrafish were separated according to sex and transferred to fish culture water (temperature 27 ± 1 °C, pH 7.2–7.6, dissolved oxygen 7.6–7.8 mg/L, electrical conductivity 518–520 μS/cm) with a photoperiod of 14 h: 10 h (light: dark). They were fed newly hatched brine shrimp (*Artemia sinica*) twice daily.

Adult zebrafish were placed in a testing apparatus that contained 6 L fish culture water as mentioned previously (Fig. 1). Ti mesh as an anode and $Ti/IrO_2/RuO_2$ mesh as a cathode were inserted vertically at 30 cm apart, and the electrodes were connected by using wiring alligator clips with a diameter of 1 mm to a Zhaoxin Model KXN-3020D DC regulated power supply (Zhaoxin Electronic Instruments and Equipment Co., Ltd., Shenzhen, China), with a voltage range between 0 V and 30 V and an amperage from 0 A to 5 A. The DC regulated power provided a series of current densities by adjusting the voltage during the whole experimental process.

To explore the effect of current densities on adult zebrafish mortality rate, various current densities were used. Then, the dose-effect mortality curve of the adult zebrafish (3 months old) was fitted by using a variable slope model (GraphPad Prism 7, San Diego, CA, USA). The electrolysis used in most studies was defined in terms of the required current density rather than voltage that affected aquatic organisms (Jung-Schroers et al., 2020; Lambooij et al., 2008). No electrolysis reactions were performed on the control groups. All electrolysis groups and control groups had three replicates.

At 0, 7, 14, 21 and 28 days of the electrolysis reaction, all fish were paired one by one. Specifically, one male and one female fish were selected randomly from the same electric current density group and placed in 3 L mating chambers containing 2 L fish culture water as mentioned previously. Fertilized embryos were recorded as embryo production after collection from the natural spawning of the paired breeding zebrafish in the morning once the lights were turned on at 8:00 am. After 2 h of light, all fish were reintroduced to their respective exposure apparatus (Song et al., 2020; Rice et al., 2011). In addition, all fertilized embryos were washed three times and incubated in fish cultured water. All fertilized embryos were examined under a stereomicroscope (Leica, Solms, Germany), healthy embryos were selected, and hatchability was determined at 96 h postfertilization (hpf). After electrolysis exposure for 28 days, all fish were anesthetized on ice, and the body weights and lengths were measured. GSI (GSI, % = 100 × [gonad weight (g)/body weight (g)]) values were calculated (Lei et al., 2013). Each replicate group of fish gonads was excised, snap-frozen over

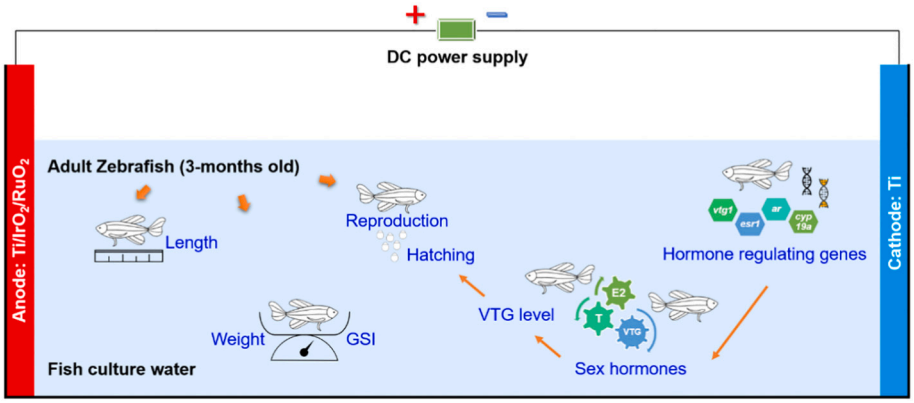

Fig. 1. Schematic diagram of the experimental design apparatus.

liquid nitrogen, and stored at −80 °C until further analysis.

After 28 days of exposure, one fish per testing apparatus was randomly selected from each treatment group by placing it in ice water and removing it if the fish did not respond to external stimuli. The ovaries of the fish were first fixed in a paraformaldehyde solution (4%, Yuan Ye Biotech. Co., Ltd., Shanghai, China) for 24 h, transferred to 70% ethanol (Yuan Ye Biotech. Co., Ltd., Shanghai, China), and then stored at 4 °C. Then, the sections were sent to Servicebio (Wuhan, China) for a series of experimental procedures, and photomicrographs of hematoxylin and eosin-stained ovaries and testes sections from zebrafish after 28 days of exposure to electrolysis were finally obtained.

2.2. RNA extraction and qRT–PCR analysis

Total RNA was extracted from the gonad, brain, and liver pools of zebrafish (4 pooled fish per sex/tissue/treatment) using TRIzol reagent (TaKaRa Bio Inc., Japan) according to the manufacturer's instructions. RNase-free DNase was used (TaKaRa Bio Inc., Japan) according to the manufacturer's protocols to remove DNA contamination. Total RNA templates were reverse transcribed to cDNA and stored at −20 °C.

Quantitative real-time fluorescence PCR (qRT–PCR) of mRNA was performed by using the CFX Connect Real-Time System (Bio–Rad, USA). The conditions of the qRT–PCR were as follows: initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 18 s. Melting curves were used to determine the specificity of the cDNA reaction products. All primers used were previously reported by Blüthgen et al. (2013) and Chen et al. (2018), which were synthesized by Sangon Biotech (Shanghai, China) for synthesis. The primer sequences of the hormone-regulating genes used in this study are listed in Table 1. The housekeeping gene *RpL13a* was used as an internal reference when performing gene expression analysis. The relative mRNA expression level calculation was performed by using relative expression software (Bio–Rad, USA) based on the $2^{-\Delta\Delta Ct}$ method (Arocho et al., 2006).

2.3. Sex hormones and vitellogenin measurements

Gonad sex steroid hormones (E2 and T), plasma cortisol, and liver VTG levels were measured in both male and female zebrafish by using enzyme-linked immunosorbent assay (ELISA) kits (Jiangsu Meimian Industrial Co. Ltd., Yancheng, China) according to the manufacturer's instructions. The detection limits of E2, T, cortisol, and VTG were 4 pmol/L, 0.6 nmol/L, 60 ng/L, and 16 μg/L, respectively. All samples and standards were run in triplicate. The coefficients of variation for these assays were less than 10%.

2.4. Statistical analysis

IBM SPSS 16.0 (SPSS, Chicago, IL, USA) and Graphpad Prism 7 (Graphpad Software, San Diego, CA, USA) were used for statistical analyses. Differences between groups were tested using one–way analysis of variance (ANOVA) and Tukey test. Results were shown as the mean ± standard error (*SE*). $p < 0.05$ was considered statistically significant.

Table 1
The primers of significantly expressed genes in adult zebrafish.

Gene	Forward Primer Sequence (5′-3′)	Reverse Primer Sequence (5′-3′)
RpL13a	AGCTCAAGATGGCAACACAG	AAGTTCTTCTCGTCCTCC
vtg1	AGCTGCTGAGAGGCTTGTTA	GTCCAGGATTTCCCTCAGT
esr1	TGAGCAACAAAGGAATGGAG	GTGGGTGTAGATGGAGGGTTT
ar	CACTACGGAGCCCTCACTTGCGGA	GCCCTGAACTGCTCCGACCTC
cyp19a	CTGAAAGGGCTCAGGACAA	TGGTCGATGGTGTCTGATG
crh	TTCCACCGCCGTATGAATGT	CGAGCCGGATGAAGTACTCC
mc2r	CCTCTTGGTGATTGTGGCTGTG	AAAGGGCCGCGTAAGTTCAG
gr	CTACGTTGAACAGGCTGGGT	AGGTCTGGAGCGAAACACAG
mr	ATTGGGCCTAGTGCAAAATG	TCTCTGTTTGGCTCGGTCTT

3. Results and discussion

3.1. Sex-specific tissue somatic indices and stress status of zebrafish

According to the lethal curve of current density to adult zebrafish (Fig. S1), the 10% lethal electric current density was 0.64 A/m^2, the 0% lethal electric current density was 0.53 A/m^2, and half of the 0% lethal electric current density was 0.26 A/m^2. The above three different current densities were used as the electrolysis groups. No mortality was observed in any electrolysis group during the experiment time of 96 h to 28 d. As the results shown in Table 2, there were some reductions in the body weights and lengths of female and male zebrafish. However, there is no significant difference ($p > 0.05$) in the electrolysis groups compared with the control group, indicating that the adult zebrafish were not unduly stressed. Specifically, the mean body weight of adult female zebrafish in the 0.64 A/m^2 electrolysis group (0.61 ± 0.09 g) was lower than that in the control group (0.64 ± 0.06 g). In addition, the mean length of adult female zebrafish in the 0.64 A/m^2 electrolysis group was 3.83 ± 0.22 cm, which was shorter than that in the control group (3.93 ± 0.13 cm). Moreover, we observed that the mean body weight of adult male zebrafish was lower in the 0.64 A/m^2 electrolysis group (0.43 ± 0.07 g) than that in the control group (0.47 ± 0.07 g). Meanwhile, the mean length of male adult zebrafish in the 0.64 A/m^2 electrolysis group (3.66 ± 0.30 cm) was also shorter than that in the control group (3.78 ± 0.15 cm). The nonsignificant difference in adult zebrafish body mass among the different electrolysis groups (0, 0.26, 0.53, and 0.64 A/m^2) might be related to the fact that the test was conducted on adult zebrafish that have fully developed in body mass and body length and were invulnerable to external induction than the juvenile fish. Thus, there was no effect on the external phenotype of zebrafish at a current density of 0.64 A/m^2.

Studies have shown that electrolysis can affect the stress system of fish (Barton and Dwyer, 1997; Barton and Grosh, 1996; Schreck et al., 1976). Hormones associated with stress clearly affect the reproductive characteristics of fish (Schreck, 2010). Stress effects primarily on hormones of the hypothalamic pituitary interrenal (HPI) axis and possibly on those of the hypothalamic pituitary gonadal (HPG) axis were reviewed by Mosconi et al. (2006). Therefore, several critical stress-related genes (*crh*, *mr*, *mc2r*, *gr*) and hormones (cortisol) associated with electrolysis in zebrafish were measured in this study (Fig. S2). The results showed that the cortisol level of the adult females in the 0.64 A/m^2 electrolysis group (2353.90 ± 59.30 ng/g) was significantly higher than that in the control group (1794.15 ± 31.31 ng/g), and similar trend was found in the male zebrafish. In addition, treatment with electrolysis also altered the expression of stress-related genes (*crh*, *mr*, *mc2r*, *gr*), but only the expression of the *mr* gene was significantly upregulated in the adult female zebrafish in the 0.64 A/m^2 electrolysis group. The overall results showed that electrolysis significantly affected the cortisol level and the expression of stress-related genes along the HPI axes in both female and male zebrafish, which suggested that positive or negative feedback may be one possible mechanism for zebrafish to maintain a relatively stable physiological status if exposed to electrolysis. Liu et al.

Table 2
Growth index of female and male zebrafish after electrolysis for 28 days ($n = 3$).

Gender	Electric current density (A/m^2)	Weight (g)	Length (cm)
Female	0	0.64 ± 0.06 [a]	3.93 ± 0.13 [a]
	0.26	0.64 ± 0.08 [a]	3.95 ± 0.20 [a]
	0.53	0.63 ± 0.09 [a]	3.90 ± 0.21 [a]
	0.64	0.61 ± 0.09 [a]	3.83 ± 0.22 [a]
Male	0	0.47 ± 0.07 [a]	3.78 ± 0.15 [a]
	0.26	0.46 ± 0.08 [a]	3.76 ± 0.23 [a]
	0.53	0.45 ± 0.08 [a]	3.65 ± 0.24 [a]
	0.64	0.43 ± 0.07 [a]	3.66 ± 0.30 [a]

Notes: Lowercase letter "a" in the same column indicate no significant difference between treatments ($p > 0.05$).

(2011) reported the effects of chemicals on cross-talk among the HPI and HPG axes in zebrafish. Therefore, exposure to electrolysis may also disrupt the HPG axis, which could lead to changes in sex hormones.

The GSI is a highly comprehensive physiological index that reflects the level of sex hormones and reveals the impact of exogenous estrogen on fish (Ankley et al., 2001). The GSIs of adult female and male zebrafish exposed to electrolysis for 28 days are shown in Fig. 2. Compared with the control group, in which the GSI value was 11.39 ± 1.64%, a decreasing trend was observed in adult female zebrafish in the electrolysis group. The GSI significantly decreased to 7.27 ± 0.43% in the 0.64 A/m^2 electrolysis group ($p < 0.05$). Moreover, there was a slight GSI reduction in adult male zebrafish in the electrolysis groups compared with the control group. The GSI changes presented here were similar to those observed in other studies (Dang et al., 2018; Guo et al., 2021; Zhang et al., 2008). The current results showed that an electric current density of lower than 0.53 A/m^2 had little negative effect on the GSI of zebrafish gonads.

Based on the GSI changes in females, the histological development of the ovary was further investigated after electrolysis at different current densities for 28 days (Fig. S3). Quantitative analysis of oogenesis confirmed that a reduced number of vitellogenic oocytes (VOs) and mature oocytes (MOs) were found in the ovary with increasing current density, while perinucleolar oocytes (POs) and cortical alveolar oocytes (COs) increased. Therefore, electrolysis inhibited the transformation of POs to MOs. It is known that the sizes of POs and COs are smaller than those of VOs and MOs. In addition, only MOs can be fertilized normally (Zhang et al., 2016). Consequently, the small egg size and low fecundity of oocytes in the ovaries led to the low GSI in the females in the electrolysis group. Gonad development is highly complex, and Nagahama and Yamashita (2008) proved that E2 regulated ovarian growth by controlling VTG synthesis in the liver throughout oocyte growth. A decreased VTG might delay the development of the gonad and reduce the quality of the egg (Hou et al., 2016). In this study, the decreased GSI and percentage of MOs in the female zebrafish in the electrolysis group were likely caused by the induction of VTG (Zhang et al., 2016). This hypothesis was further validated by our subsequent analysis of embryo production, *vtg1* mRNA expression, and VTG concentration in the gonad.

3.2. Effects of electrolysis on reproduction and hatching in zebrafish

The fecundity of adult zebrafish in the electrolysis groups was measured (Fig. 3A), which showed that there was a significantly reduced embryo production in the adult zebrafish in the 0.64 A/m^2 electrolysis group at 28 days compared with the control group ($p < 0.05$). It was assumed that electrolysis affected their spawning ability to a certain degree. The reduced fertilization rate of zebrafish eggs was probably caused by the decreased MOs (Fig. S3), which could further represent a potential threat to the zebrafish population. It indicated no apparent electrolysis-induced reproductive damage in zebrafish of the F1 generation when the current density was lower than 0.64 A/m^2 due to no statistical significance (Fig. 3B).

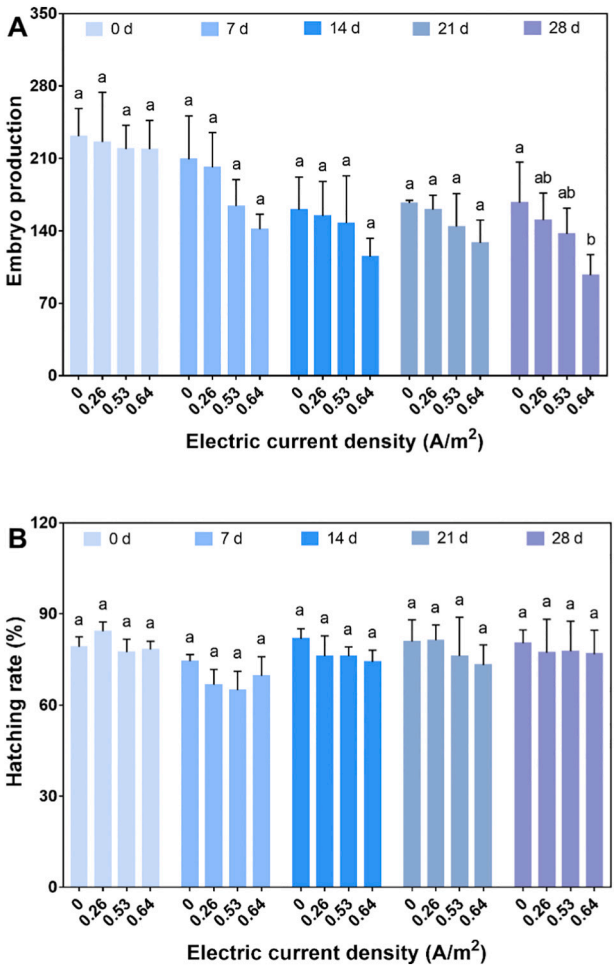

Fig. 3. Effects of electrolysis on embryo production (A) and hatching rate (B) in breeding pairs of zebrafish. Data are expressed as the mean ± *SE* ($n = 6$). Different letters on the same day indicate statistically significant differences ($p < 0.05$).

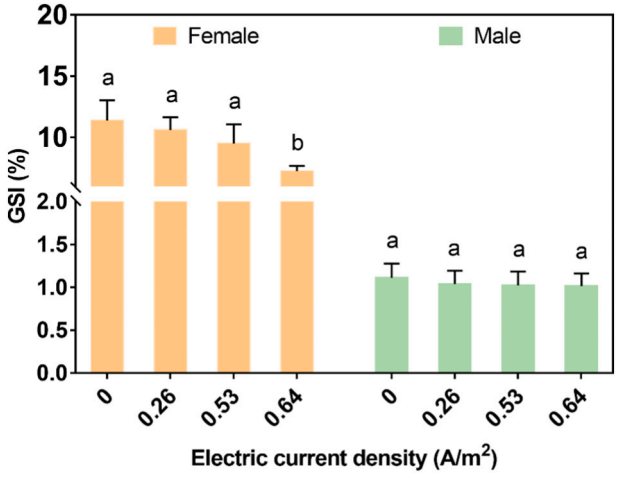

Fig. 2. Gonadosomatic index (GSI) of adult male and female zebrafish under different electric current densities after 28 days of exposure. Data are expressed as the mean ± *SE* ($n = 3$). Different letters indicate statistically significant differences ($p < 0.05$).

3.3. Sex-specific sex hormones and VTG levels in zebrafish

Sex hormones are crucial for reproduction by controlling some of the most important factors, such as fecundity, fertilization, hatching, and survival of the embryo (Ma et al., 2018). Therefore, the quantities of steroid hormones are the most important parameters for understanding the adverse impacts on the reproduction of adult zebrafish.

As the electric current density of electrolysis increased from 0.26 A/m^2 to 0.64 A/m^2, the E2 concentration in the ovaries of the female zebrafish significantly decreased in the 0.64 A/m^2 electrolysis group compared with the control group ($p < 0.05$, Fig. 4A), with a fold change of 0.81 ± 0.02. However, the E2 concentration in the testicles of the male zebrafish significantly increased in the 0.64 A/m^2 electrolysis group compared with the control group ($p < 0.05$, Fig. 4A), with a fold change of 1.59 ± 0.03.

In addition, the T concentration in the ovaries of the female zebrafish

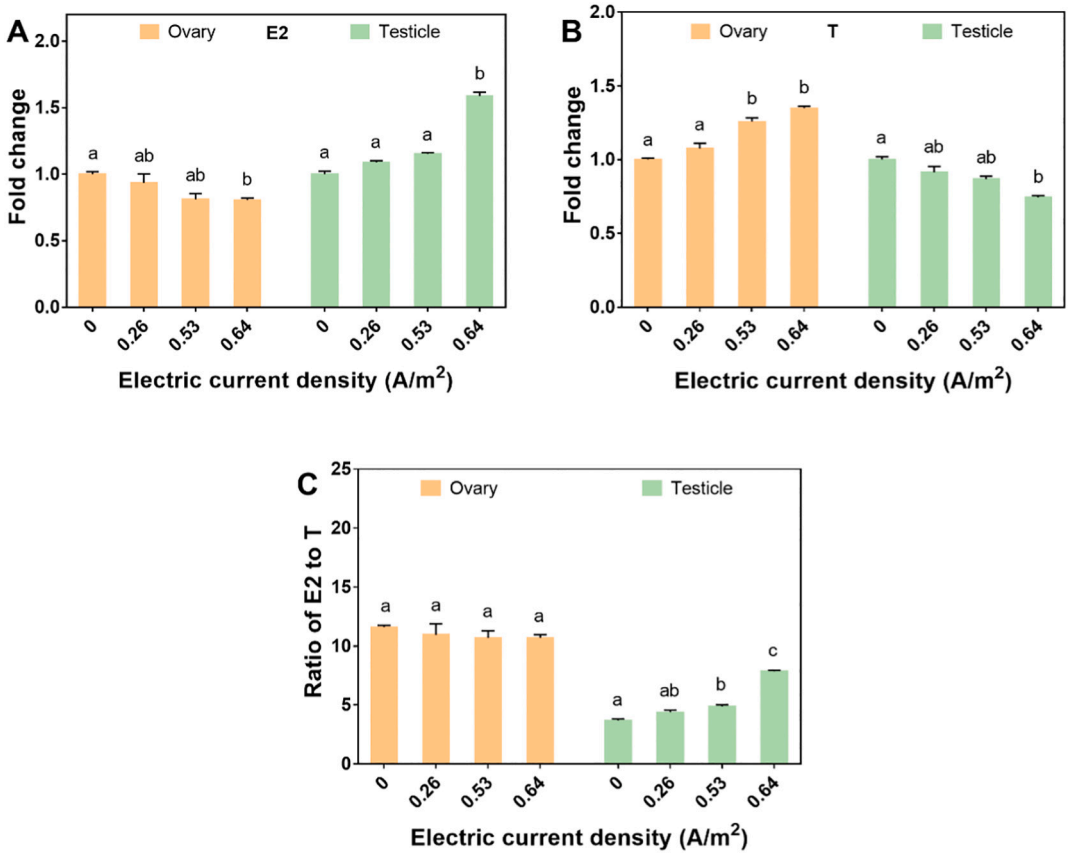

Fig. 4. Effects of electrolysis on hormone levels of E2 (A) and T (B) and the E2/T ratio (C) in adult female and male zebrafish after 28 days of electrolysis exposure. Data are expressed as the mean ± *SE* ($n = 6$). Different letters indicate statistically significant differences ($p < 0.05$).

significantly increased in the electrolysis groups with current densities of 0.53 and 0.64 A/m^2. Compared with the control group, the fold changes could reach 1.26 ± 0.02 and 1.35 ± 0.01, respectively ($p < 0.05$, Fig. 4B). In contrast, the T concentration in the testicles of the male zebrafish significantly decreased in the 0.64 A/m^2 electrolysis group compared with the control group, with a fold change of 0.74 ± 0.01 ($p < 0.05$, Fig. 4B). These changes in sex hormone concentrations in the testicles of the male zebrafish led to a significant increase in E2/T ratios (7.83 ± 0.10) in the 0.64 A/m^2 electrolysis group compared with the control group (3.67 ± 0.14) ($p < 0.05$, Fig. 4C). This was mainly caused by the significant increase in E2 concentration and the significant decrease in T concentration in the testicles of the male zebrafish. The E2/T ratio could be used as a sensitive biomarker of abnormal sex hormones in fish (Orlando et al., 2004). Moreover, the gene expression of *cyp19a* catalyzes the conversion of T to E2 (Liu et al., 2012). Upregulation of *cyp19a* transcription and an increased E2/T ratio suggest that exposure to electrolysis could result in an estrogenic response by enhancing the production of and conversion to endogenous E2. Sex steroid hormones play crucial roles in various parameters associated with fish reproduction (Devlin and Nagahama, 2002). Sex hormonal changes indicated that electrolysis could disturb sex steroid hormone production and eventually disrupt of reproductive performance in adult female and male zebrafish. Furthermore, electrolysis could cause masculinization of female zebrafish and feminization of male zebrafish. As shown in Fig. 3A, electrolysis did not affect spawning ability when the current density was lower than 0.53 A/m^2. However, at the sex hormone level, the response of the zebrafish sex hormones to electrolysis was more sensitive. There was no negative effect of electrolysis on the gonads of zebrafish when the current density was lower than 0.26 A/m^2.

VTG is a vital biomarker related to fish production, and VTG synthesis in fish mainly depends on plasma E2 levels and is directly induced by E2 (Takemura and Kim, 2001). The VTG concentrations in the livers of adult female and male zebrafish are shown in Fig. 5. The mean VTG concentration in the livers of the female zebrafish in the 0.64 A/m^2 electrolysis group was significantly lower than that in the control group ($p < 0.05$), with a fold change of 0.79 ± 0.05. The mean VTG concentration in the livers of the male zebrafish showed an increasing trend, but no statistical significance ($p > 0.05$). The VTG concentration in female fish could therefore indirectly reflect egg production (Miller et al.,

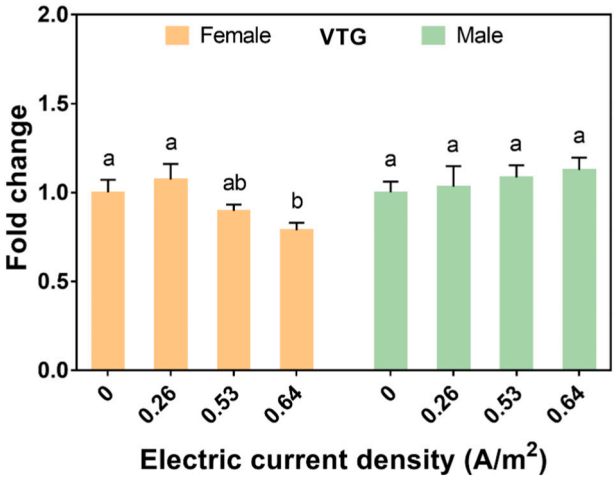

Fig. 5. Effects of electrolysis on vitellogenin (VTG) levels in the livers of female and male zebrafish after 28 days of exposure. Data were expressed as the mean ± *SE* ($n = 3$). Different letters indicated statistically significant differences ($p < 0.05$).

2010). In addition, the decreased GSI in the adult female zebrafish mentioned above was directly caused by the suppression of VTG. Hence, the lower embryo production (Fig. 3A) might be due to the decrease in both VTG concentration and GSI in the female zebrafish in the 0.64 A/m^2 electrolysis group. VTG is a very important factor in the embryo production of zebrafish, but it demonstrated a lower sensitivity to electrolysis. The effect of electrolysis on VTG of zebrafish was negligible when the current density was lower than 0.53A/m^2.

3.4. Sex-specific mRNA expression of sex hormone-regulating genes in zebrafish

The gonadal development of zebrafish is influenced by both sex hormone-regulating gene regulation and environmental factors. In addition, environmental factors may also affect gene regulation and thus affect changes in hormone levels (Alejandro et al., 2019). Therefore, it was very important to understand the key hormone-regulating gene expression changes in zebrafish. We further analyzed the transcription levels of key genetic markers (*vtg1*, *esr1*, and *ar*) in the livers of adult female and male zebrafish that are involved in the synthesis of VTG and sex hormones. Different forms of the *cyp19* gene are expressed in the gonads and the brain, and the gonadal form, *cyp19a*, has been found to be more abundant than the brain form, *cyp19b* (Fenske and Segner, 2004). Therefore, the expression of the *cyp19a* gene in the gonads was examined in this study.

VTG is an egg yolk precursor protein that is synthesized primarily in the liver of females, and alterations in *vtg1* expression have been associated with severe reproductive effects (Filby et al., 2007). After female zebrafish were exposed to 28 days of electrolysis, there was a significant downregulation of *vtg1* in the livers in the electrolysis groups (0.53–0.64 A/m^2) compared with the control group ($p < 0.05$). However, a slight increasing trend in *vtg1* gene expression in the livers of male zebrafish was observed, but the increase was not significant ($p > 0.05$) (Fig. 6A).

Sex hormones must bind to their receptors to function, and thus altered expression of *estrogen receptor* (*esr1*) and *androgen receptor* (*ar*) might adversely affect gonadal development (Filby et al., 2007). ERs transduce hormone signals into various physiological responses in various organs, such as the reproductive organs (Ascenzi et al., 2006; Jørgensen et al., 2007). The *esr1* and *ar* mRNA levels in zebrafish exposed to electrolysis are presented in Fig. 6B and C, respectively. A downregulation tendency of *esr1* expression was observed in the livers of female zebrafish in the electrolysis groups; in particular, there was a significant decrease in the 0.53 and 0.64 A/m^2 electrolysis groups compared with the control group ($p < 0.05$), with fold changes of 0.23 ± 0.07 and 0.21 ± 0.07, respectively. In contrast, the gene expression of *esr1* was upregulated in the livers of male zebrafish in the 0.64 A/m^2 electrolysis group compared with the control group ($p < 0.05$), with a fold change of 2.10 ± 0.21. The change in *esr1* expression may lead to changes in the reproductive system of adult zebrafish by interacting with E2. Moreover, *esr1* plays a key role as a regulatory mediator of the *vtg* gene (Zhu et al., 2017). Consequently, the lower transcription levels of the *vtg1* gene in female zebrafish in this study may be in response to inhibition by weakened E2 binding to *esr1* (Fig. 6A and B). Moreover, the expression of *ar* in the livers of adult female zebrafish showed an upregulation tendency, but the difference was not significant ($p > 0.05$). However, the opposite change was observed in the livers of male zebrafish.

The transcription levels of *cyp19a* were significantly upregulated in the testicles of adult male zebrafish in the electrolysis groups (0.53–0.64

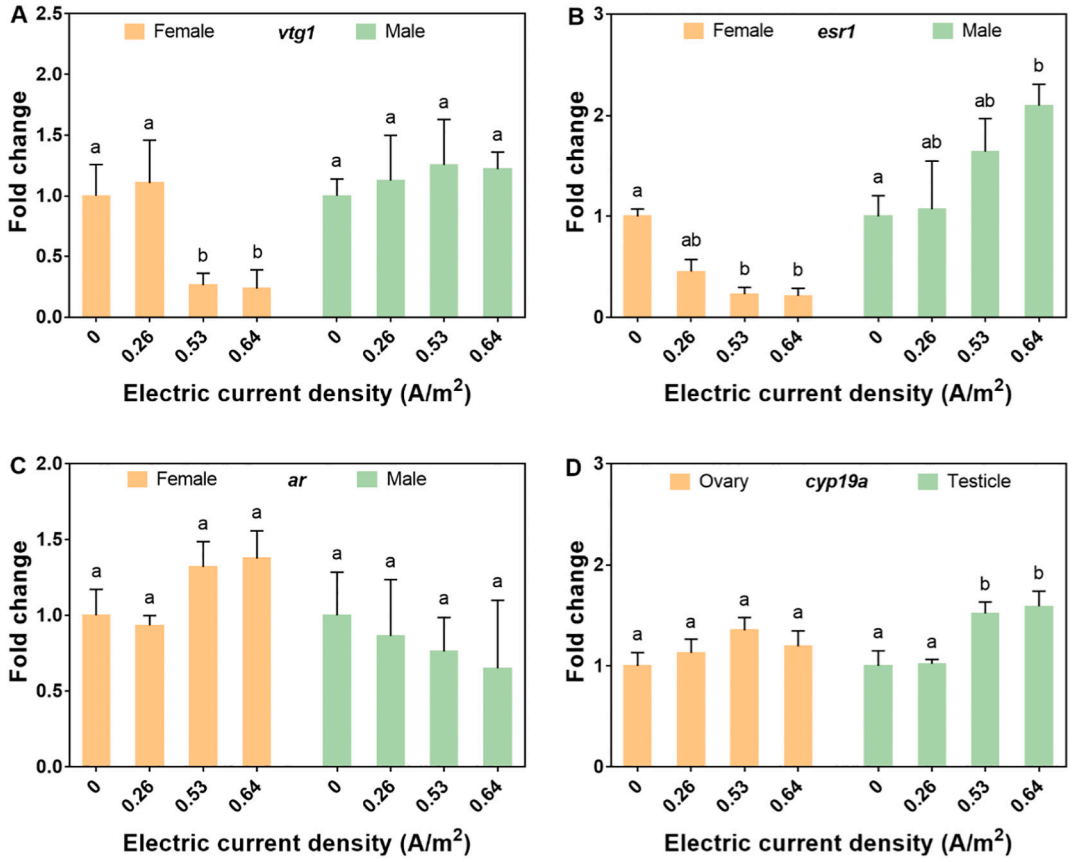

Fig. 6. Relative genes expression in gonads of adult female and male zebrafish after 28 days of exposure to different electric current densities. Transcripts of the following target genes are shown: (A) *vtg1*; (B) *esr1*; (C) *ar*; (D) *cyp19a*. Data are expressed as the mean ± *SE* ($n = 6$). Different letters indicate statistically significant differences ($p < 0.05$).

A/m^2) compared with the control group ($p < 0.05$, Fig. 6D). The aromatase cyp19a, which catalyzes the conversion of T to E2, is a key enzyme in the process of estrogen synthesis (Blüthgen et al., 2013; Liu et al., 2013). The high expression of *cyp19a* induced a significantly higher E2 concentration and lower T concentration in adult male zebrafish in the electrolysis groups than in the control group (Fig. 4A and B). VTG synthesis is produced by the binding of estrogen to a specific ER (Bao et al., 2020; Ma et al., 2018); hence, higher E2 in male zebrafish, which interacts with the ER in the liver, initiates more VTG synthesis in the livers of male zebrafish. Moreover, VTG is the egg yolk precursor in fish livers. This indicated an opposite change in VTG and muscularization in male zebrafish. In addition, Christen et al. (2011) pointed out that potential changes in the transcription of hormone receptors might be partially responsible for the histological changes in the testes and ovaries of exposed fish. Upregulated *vtg1* mRNA expression can accelerate ovarian development (Zhang et al., 2016). In this study, the histomorphological changes (reduced numbers of VOs and MOs) in the ovaries might result from the downregulated *vtg1* and *esr1* mRNA expression in adult female zebrafish. As mentioned above, the responses of the gene levels of zebrafish to electrolysis were also sensitive. However, a current density of lower than 0.26 A/m^2 had few impacts on the zebrafish.

As described in schematic in Fig. 7, the present study delineated the sex-specific responses of the reproductive system of female and male zebrafish to electrolysis. In female zebrafish, electrolysis caused the downregulated expression of *esr1* and E2, along with decreased expression of *vtg1* and generation of VTG. Simultaneously, increased T was observed. Downregulated *vtg1* expression retarded ovarian development, and thus the GSI decreased, and reduced numbers of VOs and MOs were found in the ovaries. In male zebrafish, electrolysis resulted in the upregulated expression of *cyp19a*, which was responsible for converting T to E2, and therefore decreased T and increased E2 were found in the testicles. In addition, increased E2 might lead to the increased expression of *vtg1* and the generation of VTG. Opposite trends in the assessed indicators from the gene level to the organismal level were found between female and male zebrafish. Taken together, the above results clearly indicated that electrolysis could interfere with the endocrine system and that a decrease in embryo production of zebrafish was observed. Moreover, sex hormone changes indicated that electrolysis could cause masculinization of female zebrafish and feminization of male zebrafish at a high current density. Electrolysis had a negligible negative effect on the reproduction of adult female and male zebrafish when the current density was lower than 0.26 A/m^2.

4. Conclusion

Electrolysis is highly effective for wastewater treatment, but overwhelming electric current density hurts the reproduction of zebrafish. This study demonstrated that electrolysis with a current density greater than 0.53 A/m^2, especially at 0.64 A/m^2, in this apparatus imposed significant adverse effects on the adult female and male zebrafish after 28 d of exposure. It was suggested that 0.26 A/m^2, the current density at which no effect was observed, was promising for few adverse effects on the reproductive system of adult female and male zebrafish. In the future, the effect of electrolysis on other organisms in the river, such as native fish, macrophytes, benthic animals, and microorganisms, needs to be studied in depth.

Ethical approval

The care and use of experimental animals complied with Animal Ethical and Welfare Committee's animal welfare laws, and animal handling was conducted following the guidelines of the Animal Ethical and Welfare Committee of Nanjing University, Nanjing, China.

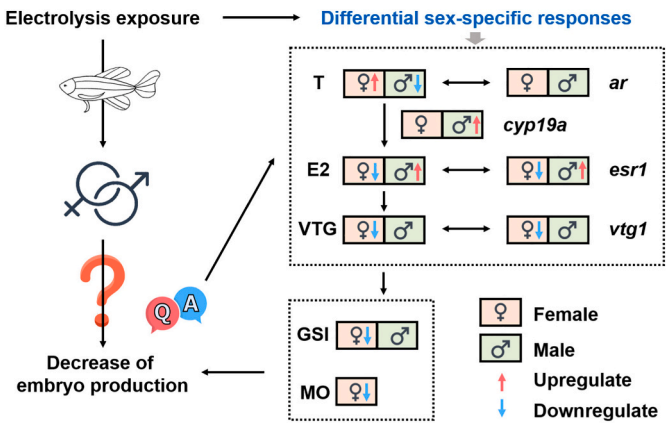

Fig. 7. Sex-specific responses of the reproductive system of female and male zebrafish to electrolysis. Sex hormones, VTG, hormone-regulating gene expression, GSI and MOs of adult female and male zebrafish after electrolysis exposure to an excess current density for 28 days are shown by arrows of different colors. Red arrow: significantly upregulated; blue arrow: significantly downregulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CRediT authorship contribution statement

Chaoqun Zheng: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Cheng Chen:** Conceptualization, Methodology, Formal analysis. **Yan Gao:** Funding acquisition, Conceptualization, Methodology, Formal analysis. **Lin Gan:** Conceptualization, Methodology, Formal analysis. **Wen Zhang:** Conceptualization, Methodology, Formal analysis. **Liuyan Yang:** Funding acquisition, Conceptualization, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2022.109294.

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