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Flutolanil affects circadian rhythm in zebrafish (*Danio rerio*) by disrupting the positive regulators

Yang Yang, Fengshou Dong, Xingang Liu, Jun Xu, Xiaohu Wu, Yongquan Zheng*

State Key Laboratory for Biology of Plant Disease and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, 100193, People's Republic of China

HIGHLIGHTS

- Flutolanil induced abnormal development and behavior of zebrafish.
- Flutolanil significantly enhanced the MT contents.
- Flutolanil significantly up-regulated the clock contents.
- Flutolanil altered the expression levels of the positive genes.

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ABSTRACT

Circadian rhythms are fundamental to behavior and physiology of organisms. Flutolanil as a fungicide is toxic to zebrafish embryos. The aims of this study were to determine whether flutolanil would influence circadian rhythms of zebrafish and the mechanism involved. Zebrafish embryos were exposed to flutolanil (0, 0.125, 0.5 and 2 mg/L) for 4 days. Here we report that flutolanil increased the melatonin levels of zebrafish. The mRNA levels of genes related to circadian rhythms were significantly altered. The clock level was significantly increased, but the content of cry1 showed no apparent changes. Moreover, our findings that the level of GH was significantly decreased were consistent with the abnormal development of zebrafish embryos. The expression levels of genes related to development, behavior and reproduction were significantly altered by flutolanil. These results indicate that flutolanil disturbed circadian rhythms of zebrafish primarily by affecting the positive elements, which were at least in partial responsible for abnormal development and behavior of zebrafish. And we speculate that flutolanil is toxic to zebrafish embryos at least in part via dysregulation of circadian rhythms involving clock.

1. Introduction

Flutolanil as a highly active and low toxic fungicide is used for controlling bunt and smut diseases of cereals (Ito et al., 2004). Also, flutolanil has a long half-life of 90.5 days and is stable under light and heat. Because of its wide use and stability, flutolanil has been detected in the aquatic environments. Flutolanil is observed in river up to 0.273, 1.64, 13, and 230 μg/L in different water areas, respectively (Añasco et al., 2010; Okamura et al., 1999; Tanabe and Kawata, 2009; Yu and Zhu, 1991). Some studies have shown that flutolanil is toxic to aquatic organisms. Reproductive toxicity of flutolanil in *Daphnia magna* was found by Matsumoto et al. (2009). Our previous study has shown that flutolanil can cause abnormal development of zebrafish and symptoms such as pericardial cyst and spinal curvature inhibition are observed in flutolanil treatments (Yang et al., 2016). Importantly, flutolanil is toxic to adult zebrafish under environmental concentration (Li, 2017). There is therefore an urgent need to determine if flutolanil under environmental levels would be a risk to zebrafish embryos and further understand the molecular mechanisms involved in the toxic events.

Notably, circadian rhythm exists widely in organism and is

Abbreviations: MT, melatonin; GH, growth hormone; DA, dopamine; UGT1, UDP glucuronosyltransferase 1; MAO, monoamine oxidase; TH, tamm-horsfall; DBH, dopamine beta-hydroxylase protein; BMAL, brain and muscle ARNT-like; AANAT, arylakylamine N-acetyltransferase; PER, Period; CRY, cryptochrome; IGF, insulin-like growth factor 1; BMP, bone morphogenetic protein; LOX, lysine oxidase; CYP, cytochrome P450 proteins; HE1A, hatching enzyme 1a; DMRT1, double-sex-andmab-3 related transcription factor1; HSD, hydroxysteroid dehydrogenase.

* Corresponding author. Address: No. 2 Yuan mingyuan West Road, Haidian District, Institute of Plant Protection, Chinese Academy of Agricultural Sciences.
E-mail address: zhengyongquan@ippcaas.cn (Y. Zheng).

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fundamental to behavior and development, which indicates a key role of circadian rhythm in many physiological processes such as liver metabolism and hormone secretion (Bass and Takahashi, 2010; Kumar et al., 2004; Wilsbacher and Takahashi, 1998). A disturbance of circadian rhythm can result in a range of diseases, such as metabolic disorders, premature aging and a variety of psychological diseases (Hastings et al., 2003). Therefore, it is of great significance to research into that whether circadian rhythm would play a key role in the toxicity of flutolanil on zebrafish and the mechanisms potential.

The previous findings have shown that the positive and negative elements are involved in circadian system of zebrafish. Briefly, *bmal1a, bmal1b, bmal2, clock1a, clock1b* and *clock2* are the positive elements, whereas p*er1a, per1b, per2, per3, cry1aa, cry1ab, cry1ba, cry1bb, cry2* and *cry3* are the negative elements. The *bmal-clock* can induce negative gene expression and *per-cry* can inhibit block gene expression by combining with bmal-clock (Hao et al., 1997).

To determine whether there are circadian rhythms involved in the risks of flutolanil and the underlying mechanism, we investigated the effects of flutolanil on the development, behavior and reproduction of zebrafish embryos, on the expression levels of the biological clock genes and on the protein levels of MT, clock and cry1.

2. Materials and methods

2.1. Chemicals and reagents

Flutolanil (purity 98.7%, CAS: 66332-96-5) was obtained from the Beijing Huarong Biological Hormone Plant. All other reagents used in the paper were of analytical grade. Standard water was used for the exposure exams (Mu et al., 2013).

2.2. Zebrafish study

Zebrafish (AB strain, *Danio rerio*) were obtained from a local shop (Beijing Hongdagaofeng Aquarium Department) and domesticated in the flow-through feeding equipment for 4 weeks before breeding. Embryo zebrafish were bred with adult zebrafish in the same strain. Adult and embryo zebrafish maintenance were carried out as described by Mu et al. (2013). Embryos at about 1 h post-fertilization (hpf) were randomly exposed in beakers for 4 days with a series of 0.125, 0.50 and 2.0 mg/L concentrations with the test solution renewed daily. The blank and the solvent groups (contained 0.1 ml/L solvent) as the controls were set in this study. Every group was carried out with three repetitions. Each beaker contained 120 embryos with 600 ml solution. The dead embryos were removed daily. The number of spontaneous movements (systemic twist of embryos) at 24 hpf in 20s was counted and the hatching rate at 72 hpf and 96 hpf were investigated. Additionally, the behavior of the hatched larvae at 96 hpf was examined with the Loligo system 4.2.0 (Denmark) and the body length at 96 hpf was measured by an Aigo GE-5 (made by Aigo Corp). All zebrafish experiments carried out in this paper were approved by the Independent Animal Ethics Committee at the Chinese Academy of Agricultural Sciences (CAAS).

2.3. ELISA

Thirty embryos were taken from each beaker for sample preparation at the end of exposure and homogenized in phosphate buffered saline (PBS) with a pH = 7.4. The samples were collected by centrifugation. Melatonin levels were carried out using the enzyme-linked immunosorbent assay kit (RenJie Biotechnology CO., LTD, ShangHai, China). Technically, 50 μL samples were incubated with 100 μL of HRP for 1 h at 37 °C. After washing, the holes were incubated with 50 μL of Detection Reagent A and 50 μL of Detection Reagent B for 15 min at 37 °C. Then, 50 μL of Stop Solution was added, and the reaction was taken with an ELISA analyzer (Multiskan MK3) at 450 nm. GH and DA levels were carried out using assay kits (Jiancheng, Nanjing, China). The level of UGT1 was determined using an ELISA kit (Jianglai Biology, Shanghai, China).

2.4. Western blotting

The method was performed referring to a previously described method (Duan et al., 2014). Technically, proteins were extracted from embryos and separated by running the samples via 10% SDS-PAGE, and then transferred onto polyvinylidene fluoride (PVDF) membranes (ISEQ00010, Millipore, USA). The membranes were blotted with antibodies against cry1 (Aviva Systems Biology, ARP59758_P050, 1:4000), clock (Abbkine, ABP51005, 1:4000) or goat anti-rabbit IgG (H + L) HRP (MDL, MD2141, 1: 3000). Bound antibodies were analyzed using the ChemiDoc MP system (170-8280, Bio-rad, USA), and the density of each band was implemented with Gene Snap software (Syngene, America).

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Thirty embryos in each beaker were pooled to extract total RNA (Tiangen, China). Gene expression was carried out by qRT-PCR and transcripts were quantified by using the $2^{-\Delta\Delta Ct}$ method (Mu et al., 2015). The information about primer sequences is shown in Table S1 *β-actin* was chosen as a house-keeper gene in this study.

2.6. Water analysis

The solution from each beaker was analyzed twice at the beginning and 1 dpe before renewal to determine the actual concentration of flutolanil, as described in Yang et al. (2016). Briefly, the water samples (50 ml) were extracted twice using petroleum ether (10 ml). Afterwards, the organic phase was collected to be evaporated to dryness at 35 °C. The residue was then dissolved in 2 ml methanol and a volume of 20 μL samples was injected into a reversed-phase high −performance liquid chromatography system to detect the actual concentration of flutolanil in each sample. Here are the chromatographic conditions: PAD detector; C18 stainless steel column (250 mm × 46 mm × 5 μm); the mobile phase, 60% aqueous methanol; flow rate, 1 ml/min; the detection wavelength, 254 nm; and the column temperature, 40 °C; the retention time, about 9.34 min.

2.7. Statistical analysis

All values were expressed as the mean ± standard deviation of the mean. Data were analyzed by one-way ANOVA followed by Dunnett's post hoc comparison with the program SigmaStat (SPSS Inc, Chicago, IL, USA). Differences between the control and exposure groups with p value < 0.05 were considered significant.

3. Results

3.1. Chemical analysis and solvent effect

Previous study showed that the deviations were less than 20% between the theoretical and actual concentration (Yang et al., 2016) Thus, the theoretical flutolanil concentration can be used instead of the actual concentration in the present study, referring to the OECD guidelines (OECD, 2013). And there was no significant difference

observed between the solvent control and blank control for any of indexes in the current study (data not shown). Thus, the data of the solvent control could be applied as a control.

3.2. Flutolanil significantly altered the expression levels of clock genes

Here we reported that in the exposure groups, expression of the positive and negative clock genes, were significantly changed by flutolanil (Fig. 1). Flutolanil obviously inhibited the expression levels of *clock1a, bmal1a, bzmal1b, bmal2* and *aanat2* in the all treatment groups. Expression of *clock1a, bmal1a, bmal1b, bmal2* and *aanat2* were decreased by 0.28-fold, 0.35-fold, 0.36-fold, 0.34-fold and 0.29-fold after 0.125 mg/L flutolanil exposure, respectively (Fig. 1A). And the expression levels of *per1b, cy1aa, cry1ab, cry1ba* and *cry1bb* were significantly decreased in the all flutolanil exposure groups. The expression level of *per2* was apparently inhibited by 0.56-fold and 0.67-fold at 0.125 and 2.0 mg/L, respectively. The transcript levels of *per1a* and *per3* were significantly inhibited at 0.125 mg/L, except induction of *per1a* at 2.0 mg/L (Fig. 1B).

3.3. Flutolanil up-regulated the content of clock

The western blotting results showed that clock levels at 0.50 and 2.0 mg/L were significantly increased by 3.64- and 3.63-fold respectively (Fig. 2A–B), whereas the densitometric analysis indicated no apparent change in the content of cry1 in all flutolanil treatment groups compared to the controls (Fig. 2C–D). These matching patterns of the positive genes expression (Fig. 1A) and the clock protein levels (Fig. 2A–B) suggested that the disruption of flutolanil on circadian rhythm of zebrafish might be correlated with the amount of the positive elements.

3.4. Flutolanil up-regulated MT level

The MT level was significantly enhanced at 2.0 mg/L by 1.13-fold, but significance was not reached at 0.125 and 0.50 mg/L (Fig. 3A).

3.5. Flutolanil inhibited development and behavior of zebrafish

The GH levels were significantly reduced at 2.0 mg/L (Fig. 3B). The level of DA was obviously increased at 0.50 mg/L (Fig. 3C). The levels of UGT1 showed no apparent change (Fig. 3D).

Spontaneous movement at 24 hpf was significantly decreased at 0.125 mg/L or higher (Fig. 4A). The hatching rate at 72 hpf was significantly inhibited at 0.50 and 2.0 mg/L and that at 96 hpf was obviously reduced at 2.0 mg/L (Fig. 4B). The body length of the hatched larvae at 96 hpf was dramatically inhibited at 2.0 mg/L (Fig. 4C). Distance moved at 96 hpf in 8 min showed no apparently changes in the all treatment groups compared to the controls (Fig. 4D). But we found less active larvae in the treatment groups.

The expression levels of genes related to behavior, development and reproduction were significantly altered by flutolanil (Fig. 5). The expression levels of *th* were significantly inhibited in all treatments. The transcript levels of *mao* and *dbh* were obviously decreased at 0.125 and 2.0 mg/L, except an induction of *dbh* at 0.50 mg/L (Fig. 5A). In addition, the expression of genes related to development was also significantly altered (Fig. 5B). The transcripts of *gh* and *lox* were obviously inhibited in the all treatment groups. *Bmp2* and *bmp4* expression levels were significantly decreased at 0.125 and 0.50 mg/L, but increased at 2.0 mg/L. And expression of *igf* were significantly decreased at 01.25 and 2.0 mg/L, but increased at 0.50 mg/L. The expression levels of *he1a* were dramatically increased at 0.50 mg/L or higher (Fig. 5B). Moreover, expression of genes related to reproduction were obviously changed as well. The transcripts of *cyp19b* and *dmrt1*were significantly inhibited at 0.125 mg/L or higher. And expression of *17β-hsd* were significantly decreased at 0.125 and 2.0 mg/L (Fig. 5C).

4. Discussion

Circadian rhythms as known exist in different kinds of organisms, from bacteria to humans. Since circadian rhythms regulate many physiological and behavioral processes, its dysregulation by external chemicals can cause lots of health risk (Coldsnow et al., 2017; Kumar et al., 2004; Wilsbacher and Takahashi, 1998). For example, environmental steroid hormones, progestins and corticosteroids can disturb behavior and the circadian rhythm network (Zhao et al., 2018).

Our findings showed that flutolanil significantly altered the expression levels of the positive clock genes and negative clock genes, which might indicate a disruption in circadian rhythm of zebrafish. Notably, since the rhythmic secretion of melatonin is driven by the circadian clock, melatonin is believed to be an endocrine output signal of the endogenous time measuring and keeping system (Pevet, 2002; Pfeffer et al., 2018). The current study

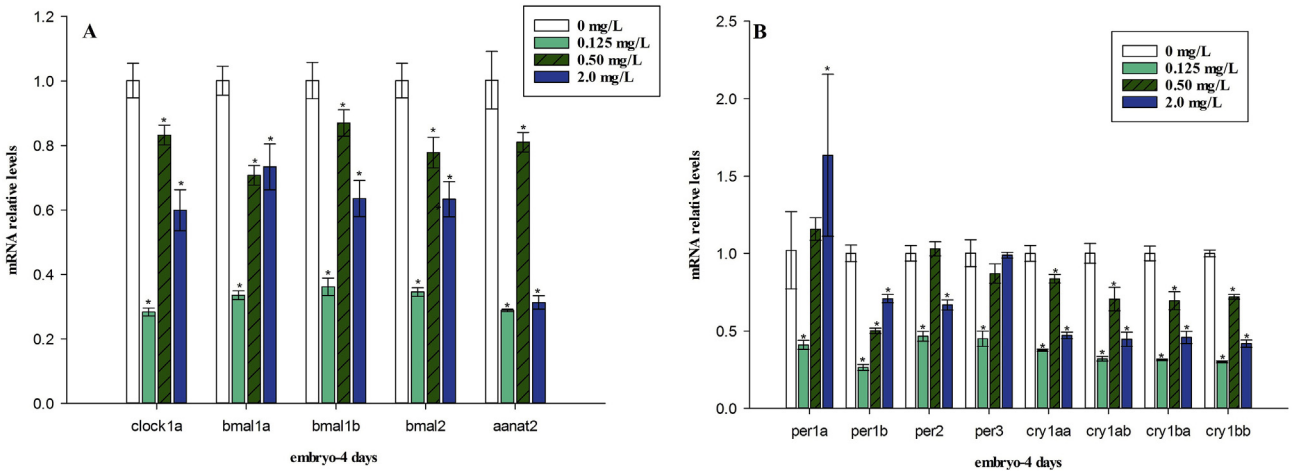

Fig. 1. Expression of the clock genes in zebrafish after exposure to flutolanil for 4 days based on qPCR data. A. The mRNA levels of the positive clock genes and *aanat*; B. The mRNA levels of the negative clock genes. Asterisks indicate significant differences between the controls and exposure groups. Error bars indicate the standard deviation.

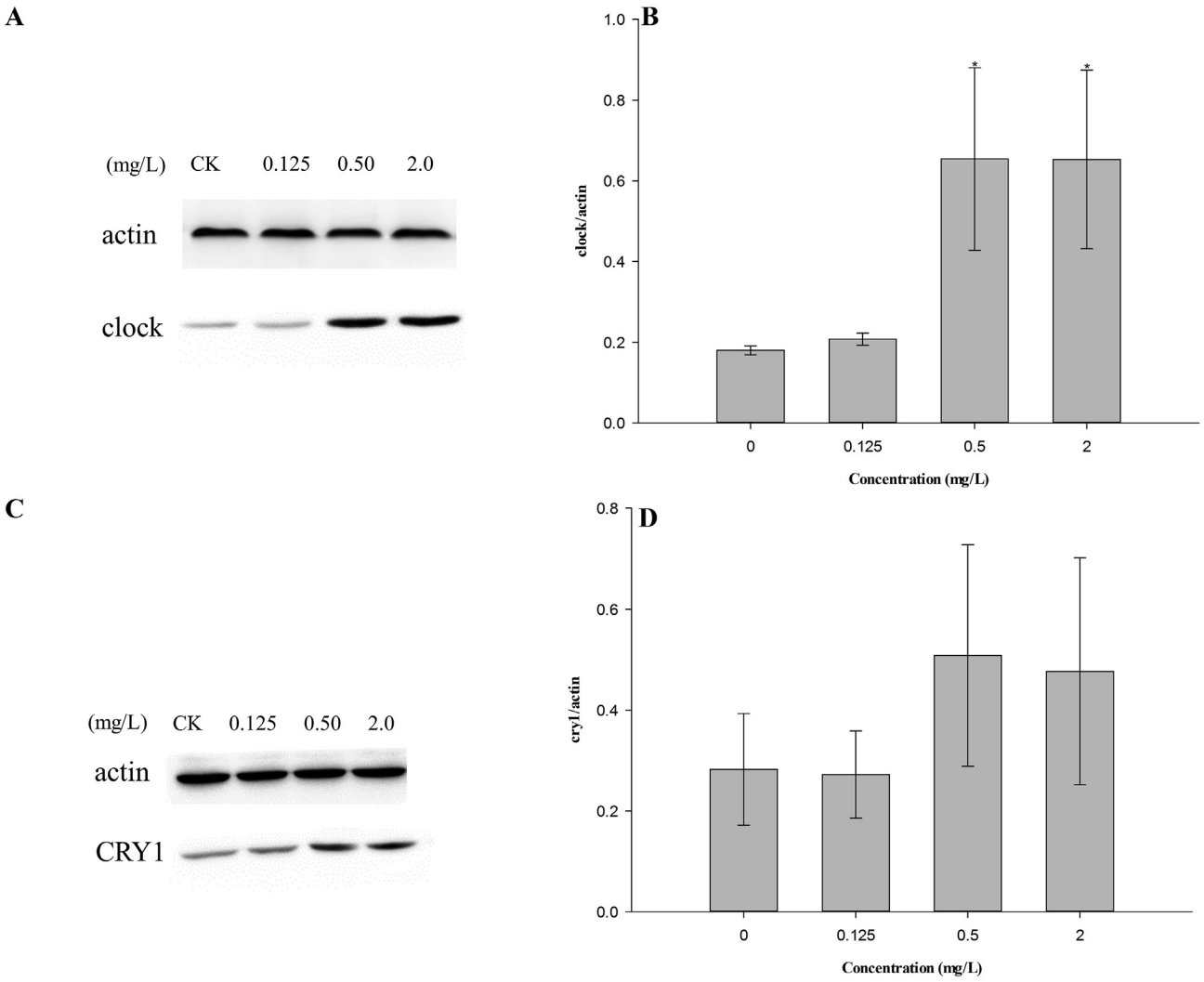

Fig. 2. The contents of cry1 and clock in zebrafish after exposure to flutolanil for 4 days based on western blotting. A/B. Expression of clock in embryos; C/D. Expression of Cry1 in embryos. Variations in protein loading were corrected by actin expression. The symbols indicated three independent biological replicates. $^{*}P < 0.05$ compared to the controls.

determined that flutolanil significantly increased MT level at 2.0 mg/L, which was in line with our previous findings that there was a lack of melanin enrichment or accumulation in zebrafish (Yang et al., 2016). Taken these results together, we suggest that circadian rhythm in zebrafish might be disrupted by flutolanil.

Additionally, we observed that the clock contents were significantly increased at 0.50 and 2.0 mg/L, whereas differences in the cry1 contents between the treatment groups and the control groups did not reach statistical significance. The observations suggest that the significant alteration of the positive elements expression may play a major role in dysregulation of circadian rhythms in zebrafish induced by flutolanil.

Many studies have shown that the circadian clock disruption is a risk to the organism. The developmental and behavioral processes of organisms are regulated by the biological clock (Alleva et al., 1971; McNeil et al., 1998; Zhao et al., 2018; Ziv and Gothilf, 2006). Unsurprisingly, our current study showed that the hatching rate and the body length of the hatching larvae were significantly inhibited by flutolanil. The expression levels of genes related to development was significantly altered. A previous study indicates a fundamental role of GH and IGF-1 in growth and development (Shoba et al., 1999). And an apparent transcription decrease of *GH* and *IGF-1* is involved in a body length decrease of zebrafish (Mu et al., 2016). BMP is the likely regulator in bone formation in vertebrates and the transcription of *BMP2* and *BMP4* is positively associated with development (Mu et al., 2016; Rafael et al., 2006). Moreover, lox has been proved to play an important role in the developmental process of aquatic vertebrate spines and decreased lox expression may be driving abnormal spinal development (van Boxtel et al., 2010; Zhou et al., 2009). In agreement with the above report, the data presented here showed that *GH*, *IGF-1, BMP2, BMP4* and *lox* were expressed lower with decreased body. Zebrafish HE1 is known to be a zinc metalloprotease responsible for hatching (Iuchi and Yasumasu, 2008). Although further investigation is required, the data presented herein supported the increased *he1a* expression to be connected to the inhibition of hatch rate. In line with the gene expression results, the GH levels were significantly inhibited by flutolanil. These observations indicated a significantly developmental inhibition in zebrafish exposed to flutolanil. Notably, clock gene expression is correlated with GH expression (De Leonibus et al., 2016). *Clock1a* alterations can cause embryo defects with shortened body length (Bian et al., 2017). MT can affect the development of organisms (De Pedro et al., 2008; Mustonen et al., 2002; Nieminen et al., 2002). Thus, the developmental

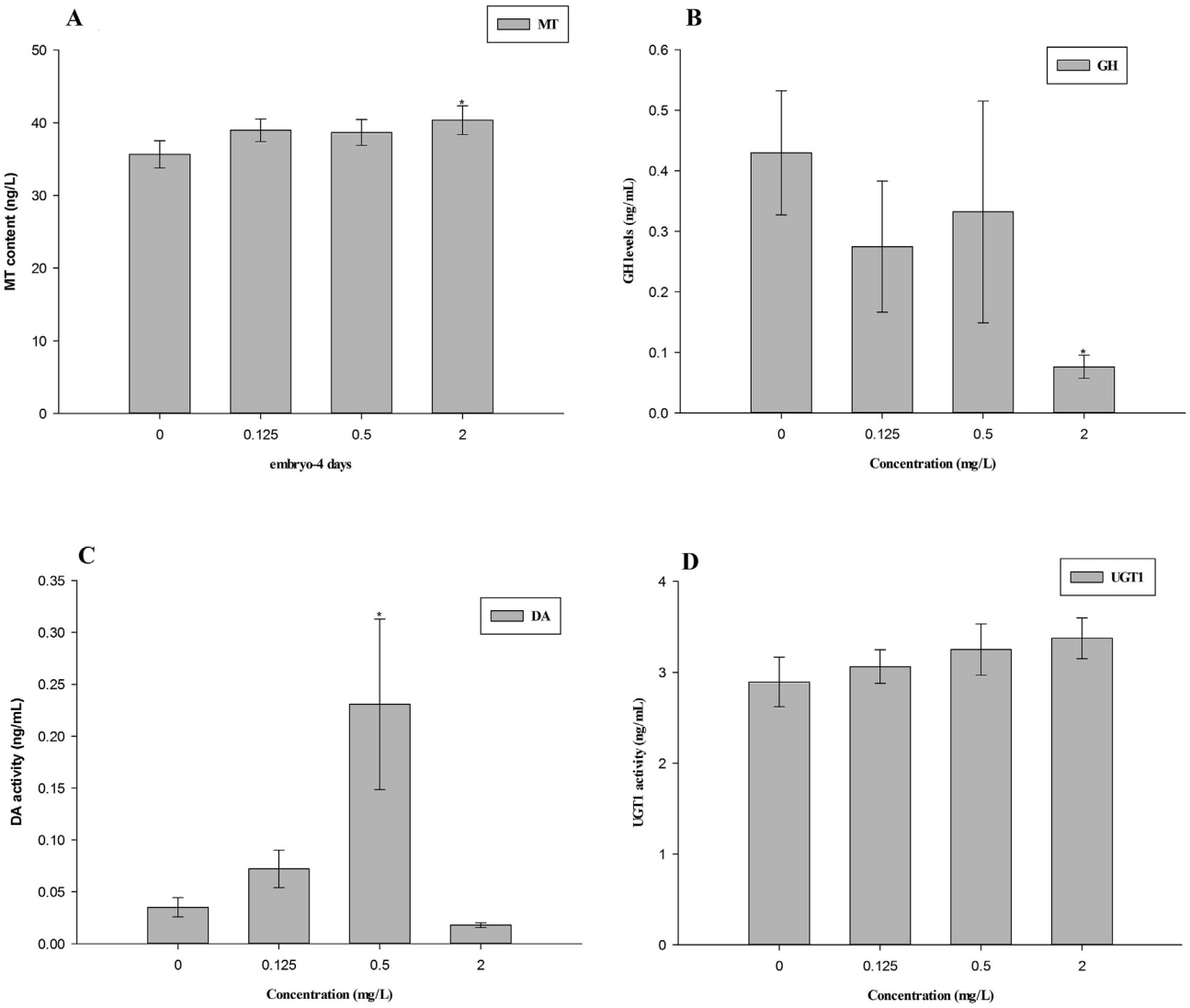

Fig. 3. The levels of MT, GH, DA and UGT1 in zebrafish after exposure to flutolanil for 4 days. Expression of MT (A), GH (B), UGT1 (D) and activity of DA in embryos. Asterisks indicate significant differences between the controls and exposure groups. Error bars indicate the standard deviation.

inhibition of zebrafish is at least in partial due to the significantly altered expression of genes related to the positive elements accompanied by the increased MT levels.

Furthermore, the present study found that the hatched larvae in treatment groups were less active, though the distance moved showed no apparent changes. And, the spontaneous movement was significantly inhibited. These results obtained in the present study indicated that flutolanil was indeed toxic to behavior of zebrafish. Unsurprisingly, the changes in behavioral gene expression and DA contents were observed. Mao play a key role in the decomposition of monoamine neurotransmitters and its reduction indicate an inhibited inactivation of monoamine neurotransmitters (Hussien et al., 2013). Therefore, the decreased *mao* expression might contribute to the inhibited behavior of zebrafish exposed to flutolanil. Previous studies have found that *th* gene is a rate-limiting enzyme in the process of dopamine formation (Postlethwait et al., 2004). DBH can convert DA to norepinephrine (Schmidt et al., 2018). These alterations of *mao*, *th* and *dbh* might be connected to the significant changes of DA. DA can affect the behavior of organisms. A reduction of DA levels is observed companied with an inhibition of behavior of zebrafish (Wu et al., 2017). Interestingly, in the present study, the DA activity was obviously increased, whereas the behavior of zebrafish was inhibited. As known, clock is closely associated with dopamine (McClung et al., 2005; Soria et al., 2010). Loss of clock function can cause increased dopamine release, abnormal dopaminergic transmission and dopamine receptor function (Coque et al., 2011; Spencer et al., 2012). Thus, the suggestion that higher clock levels may rely on other mechanism rather than affecting DA activity to induce behavioral inhibition requires further investigation.

Lastly, there have been a few previous studies about the role of MT in reproduction of organisms. MT is positively associated with fecundity (Carnevali et al., 2010). The observation that the expression of genes related to reproduction was significantly affected, although UGT1 levels showed no apparent change, indicated that the increased MT might affect the reproduction of zebrafish, which requires further research.

5. Conclusion

Flutolanil inhibited development and behavior and disrupted circadian rhythm of zebrafish. The significant changes in expression

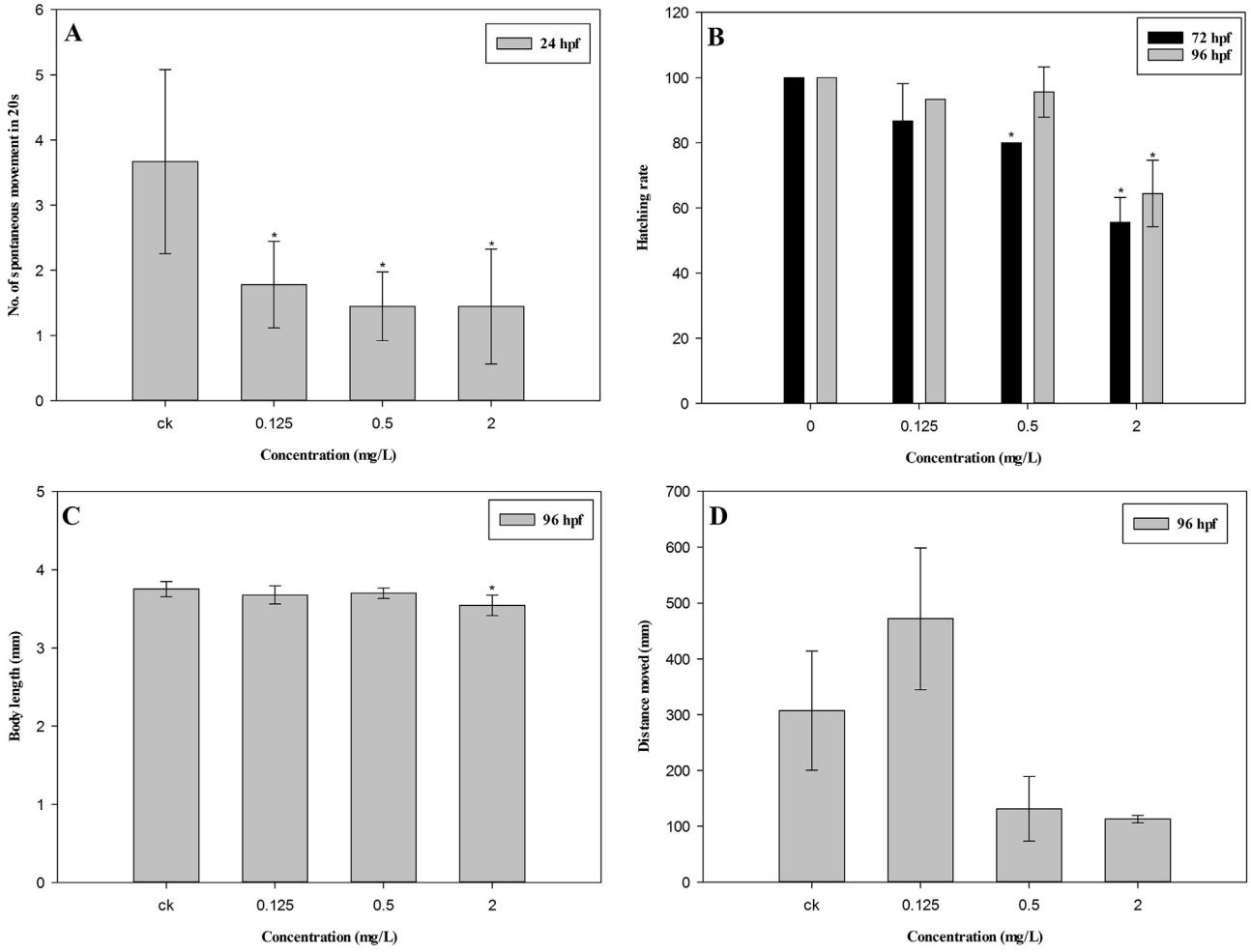

Fig. 4. Abnormal development and behavior in zebrafish after exposure to flutolanil. A. No. of spontaneous movement at 24 hpf; B. Hatching rate of embryos at 72 and 96 hpf; C. Body length of hatched larvae at 96 hpf; D. Distance moved of hatched larvae at 96 hpf. Asterisks indicate significant differences between the controls and exposure groups. Error bars indicate the standard deviation.

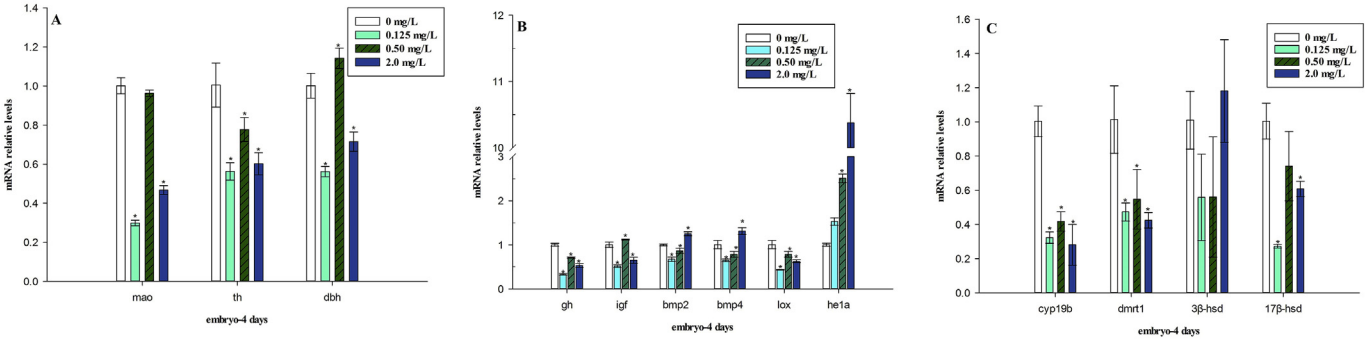

Fig. 5. The transcripts of genes in zebrafish after exposure to flutolanil for 4 days. A. The expression of the behavior related genes; B. The mRNA levels of genes related to development; C. The transcriptions of genes related to reproduction. Asterisks indicate significant differences between the controls and exposure groups. Error bars indicate the standard deviation.

of the positive genes and the increased clock contents might up-regulate MT level, and hence disturb circadian rhythm, which was at least in partial responsible for abnormal development and behavior in flutolanil stressed zebrafish. The positive elements especially clock appeared to be involved in the disruption of circadian rhythm. This study provides a new insight to the toxic mechanism of flutolanil in zebrafish embryos and suggests a lowest toxic concentration (0.125 mg/L).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2019.04.207.

References

Añasco, N., Uno, S., Koyama, J., Matsuoka, T., Kuwahara, N., 2010. Assessment of pesticide residues in freshwater areas affected by rice paddy effluents in southern Japan. Environ. Monit. Assess. 160, 371–383.

Alleva, J.J., Waleski, M.V., Alleva, F.R., 1971. A biological clock controlling the estrous cycle of the hamster. Endocrinology 88, 1368–1379.

Bass, J., Takahashi, J.S., 2010. Circadian integration of metabolism and energetics. Science 330, 1349–1354.

Bian, S.S., Zheng, X.L., Sun, H.Q., Chen, J.H., Lu, Y.L., Liu, Y.Q., Tao, D.C., Ma, Y.X., 2017. Clock1a affects mesoderm development and primitive hematopoiesis by regulating Nodal-Smad3 signalings in the zebrafish embryo. J. Biol. Chem. 292, 14165–14175.

Carnevali, O., Gioacchini, G., Piccinetti, C.C., Maradonna, F., Lombardo, F., Giorgini, E., Tosi, G., 2010. Melatonin control of oogenesis and metabolic resources in Zebrafish. J. Appl. Ichthyol. 26, 826–830.

Coldsnow, K.D., Relyea, R.A., Hurley, J.M., 2017. Evolution to environmental contamination ablates the circadian clock of an aquatic sentinel species. Ecol Evol 7, 10339–10349.

Coque, L., Mukherjee, S., Cao, J.L., Spencer, S., Marvin, M., Falcon, E., Sidor, M.M., Birnbaum, S.G., Graham, A., Neve, R.L., Gordon, E., Ozburn, A.R., Goldberg, M.S., Han, M.H., Cooper, D.C., McClung, C.A., 2011. Specific role of VTA dopamine neuronal firing rates and morphology in the reversal of anxiety-related, but not depression-related behavior in the Clock Delta19 mouse model of mania. Neuropsychopharmacology 36, 1478–1488.

De Leonibus, C., Chatelain, P., Knight, C., Clayton, P., Stevens, A., 2016. Effect of summer daylight exposure and genetic background on growth in growth hormone-deficient children. Pharmacogenomics 16, 540–550.

De Pedro, N., Martinez-Alvarez, R.M., Delgado, M.J., 2008. Melatonin reduces body weight in goldfish (*Carassius auratus*): effects on metabolic resources and some feeding regulators. J. Pineal Res. 45, 32–39.

Duan, J.C., Yu, Y.B., Yu, Y., Wang, J., Geng, W.J., Jiang, L.Z., Li, Q.L., Zhou, X.Q., Sun, Z.W., 2014. Silica nanoparticles induce autophagy and endothelial dysfunction via the PI3K/Akt/Mtor signaling pathway. Int. J. Nanomed. 9, 5131–5141.

Hao, H., Allen, D.L., Hardin, P.E., 1997. A circadian enhancer mediates PER-dependent mRNA cycling in Drosophila melanogaster. Mol. Cell Biol. 17, 3687–3693.

Hastings, M.H., Reddy, A.B., Maywood, E.S., 2003. A clockwork web: circadian timing in brain and periphery, in health and disease. Nat. Rev. Neurosci. 4, 649–661.

Hussien, H.M., Abdou, H.M., Yousef, M.I., 2013. Cypermethrin induced damage in genomic DNA and histopathological changes in brain and haematotoxicity in rats: the protective effect of sesame oil. Brain Res. Bull. 92, 76–83.

Ito, Y., Muraguchi, H., Seshime, Y., Oita, S., Yanagi, S.O., 2004. Flutolanil and carboxin resistance in Coprinus cinereus conferred by a mutation in the cytochrome b560 subunit of succinate dehydrogenase complex (complex II). Mol. Genet. Genom. 272, 328–335.

Iuchi, I., Yasumasu, S., 2008. Purification and characterization of zebrafish hatching enzyme-an evolutionary aspect of the mechanism of egg envelope digestion. FEBS J. 275, 5934–5946.

Kumar, V., Singh, B.P., Rani, S., 2004. The bird clock: a complex, multi-oscillatory and highly diversified system. Biol. Rhythm Res. 35, 121–144.

Li, C.L., 2017. The Studies on the Toxic Mechanism of Flutolanil in Zebrafish. Master Degree Thesis of China. Agricultural University, 2017.

Matsumoto, K.I., Hosokawa, M., Kuroda, K., Endo, G., 2009. Toxicity of agricultural chemicals in Daphnia magna. Osaka City Med. J. 55, 89–97.

McClung, C.A., Sidiropoulou, K., Vitaterna, M., Takahashi, J.S., White, F.J., Cooper, D.C., Nestler, E.J., 2005. Regulation of dopaminergic transmission and cocaine reward by the Clock gene. Proc. Natl. Acad. Sci. U.S.A. 102, 9377–9381.

McNeil, G.P., Zhang, X., Genova, G., Jackson, F.R., 1998. A molecular rhythm mediating circadian clock output in Drosophila. Neuron 20, 297–303.

Mu, X.Y., Chai, T.T., Wang, K., Zhang, J., Zhu, L.Z., Li, X.F., Wang, C.J., 2015. Occurrence and origin of sensitivity toward difenoconazole in zebrafish (*Danio reio*) during different life stages. Aquat. Toxicol. 160, 57–68.

Mu, X.Y., Chai, T.T., Wang, K., Zhu, L.Z., Huang, Y., Shen, G.M., Li, Y.R., Li, X.F., Wang, C.J., 2016. The developmental effect of difenoconazole on zebrafish embryos: a mechanism research. Environ. Pollut. 2012, 18–26.

Mu, X., Pang, S., Sun, X., Gao, J., Chen, J., Chen, X., Li, X., Wang, C., 2013. Evaluation of acute and developmental effects of difenoconazole via multiple stage zebrafish assays. Environ. Pollut. 175, 147–157.

Mustonen, A.M., Nieminen, P., Hyvarinen, H., 2002. Melatonin and the wintering strategy of the tundra vole, *Microtus oeconomus*. Zool. Sci. 19, 683–687, 2002.

Nieminen, P., Mustonen, A.M., Asikainen, J., Hyvarinen, H., 2002. Seasonal weight regulation of the raccoon dog (*Nyctereutes procyonoides*): interactions between melatonin, leptin, ghrelin, and growth hormone. J. Biol. Rhythm. 17, 155–163.

OECD, 2013. OECD Guidelines for the Testing of Chemicals. No. 210: Fish Early-Life Stage Toxicity Test. Organization for Economic Cooperation and Development, Paris, France.

Okamura, H., Omori, M., Luo, R., Aoyama, I., Liu, D., 1999. Application of short-term bioassay guided chemical analysis for water quality of agricultural land run-off. Sci. Total Environ. 234, 223–231.

Pevet, P., 2002. Melatonin. Dialogues Clin. Neurosci. 4, 57–72.

Pfeffer, M., Korf, H.W., Wicht, H., 2018. Synchronizing effects of melatonin on diurnal and circadian rhythms. Gen. Comp. Endocrinol. 258, 215–221.

Postlethwait, J., Amores, A., Cresko, W., Singer, A., Yan, Y.L., 2004. Subfunction partitioning, the teleost radiation and the annotation of the human genome. Trends Genet. 20, 481–490.

Rafael, M.S., Laizé, V., Cancela, M.L., 2006. Identification of Sparus aurata bone morphogenetic protein 2: molecular cloning, gene expression and in silico analysis of protein conserved features in vertebrates. Bone 39, 1373–1381.

Schmidt, K., Ralle, M., Schaffer, T., Jayakanthan, S., Bari, B., Muchenditsi, A., Lutsenko, S., 2018. ATP7A and ATP7B copper transporters have distinct functions in the regulation of neuronaldopamine-hydroxylase. J. Biol. Chem. 293, 20085–20098.

Shoba, L., An, M.R., Frank, S.J., Lowe Jr., W.L., 1999. Developmental regulation of insulin-like growth factor-I and growth hormone receptor gene expression. Mol. Cell. Endocrinol. 152, 125–136.

Soria, V., Martinez-Amoros, E., Escaramis, G., Valero, J., Perez-Egea, R., Garcia, C., Gutierrez-Zotes, A., Puigdemont, D., Bayes, M., Crespo, J.M., Martorell, L., Vilella, E., Labad, A., Vallejo, J., Pérez, V., Menchón, J.M., Estivill, X., Gratacòs, M., Urretavizcaya, M., 2010. Differential association of circadian genes with mood disorders: CRY1 and NPAS2 are associated with unipolar major depression and CLOCK and VIP with bipolar disorder. Neuropsychopharmacology 35, 1279–1289.

Spencer, S., Torres-Altoro, M.I., Falcon, E., Arey, R., Marvin, M., Goldberg, M., Bibb, J.A., McClung, C.A., 2012. A mutation in CLOCK leads to altered dopamine receptor function. J. Neurochem. 123, 124–134.

Tanabe, A., Kawata, K., 2009. Daily variation of pesticides in surface water of a small river flowing through paddy field area. Bull. Environ. Contam. Toxicol. 82, 705–710.

van Boxtel, A.L., Kamstra, J.H., Fluitsma, D.M., Legler, J., 2010. Dithiocarbamates are teratogenic to developing zebrafish through inhibition of lysyl oxidase activity. Toxicol. Appl. Pharmacol. 244, 156–161.

Wilsbacher, L.D., Takahashi, J.S., 1998. Circadian rhythms: molecular basis of the clock. Curr. Opin. Genet. Dev. 8, 595–602.

Wu, Q., Yan, W., Cheng, H.C., Liu, C.S., Hung, T.C., Guo, X.C., Li, G.Y., 2017. Parental transfer of microcystin-LR induced transgenerational effects of developmental neurotoxicity in zebrafish offspring. Environ. Pollut. 231, 471–478.

Yang, Y., Qi, S.Z., Chen, J.Y., Liu, Y., Teng, M., Wang, C.J., 2016. Toxic effects of bromothalonil and flutolanil on multiple developmental stages in zebrafish. Bull. Environ. Contam. Toxicol. 97, 91–97.

Yu, Z.G., Zhu, Y.H., 1991. Depositional amount of flutolanil in rice and environment and its digestion dynamics. Nong Yao 30, 11–13.

Zhao, Y.B., Zhang, K., Fent, K., 2018. Regulation of zebrafish (*Danio rerio*) locomotor behavior and circadian rhythm network by environmental steroid hormones. Environ. Pollut. 232, 422–429.

Zhou, S., Dong, Q., Li, S., Guo, J., Wang, X., Zhu, G., 2009. Developmental toxicity of cartap on zebrafish embryos. Aquat. Toxicol. 95, 339–346.

Ziv, L., Gothilf, Y., 2006. Circadian time-keeping during early stages of development. Proc. Natl. Acad. Sci. U.S.A. 103, 4146–4151.