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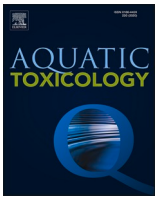

Phenytoin causes behavioral abnormalities and suppresses kisspeptin expression, reducing reproductive performance in Japanese medaka

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ABSTRACT

Phenytoin, an antiepileptic drug, induces neurotoxicity and abnormal embryonic development and reduces spontaneous locomotor activity in fish. However, its effects on other endpoints remain unclear. Therefore, we investigated the effects of phenytoin on the swimming behavior and reproductive ability of Japanese medaka. Abnormalities in swimming behavior, such as imbalance, rotation, rollover, and vertical swimming, were observed. However, when phenytoin exposure was discontinued, the behavioral abnormality rates decreased. Phenytoin exposure also significantly reduced reproductive ability. By investigating reproduction-related gene expression of *gnrh1, gnrh2, fshb*, and *lhb* remained unchanged in males and females. In contrast, *kiss1* expression was significantly suppressed due to phenytoin exposure in males and females. *kiss2* expression was also significantly suppressed in females but not in males. We filmed videos to examine phenytoin exposure effects on sexual behavior. Females showed no interest in the male's courtship. As the kisspeptin 1 system controls sexual behavior in Japanese medaka, phenytoin exposure may have decreased *kiss1* expression, which decreased female reproductive motivation; hence, they did not spawn eggs. This is the first study to show that phenytoin exposure induces behavioral abnormalities, and suppresses *kiss1* expression and reproductive performance in Japanese medaka.

1. Introduction

In epilepsy, abnormal neuronal excitation in the brain causes recurrent seizures, such as ataxia, numbness in limbs, and convulsions; as of 2018, approximately 70 million people worldwide have been diagnosed with epilepsy (Espinosa-Jovel et al., 2018). The term "antiepileptic drugs" is generic and refers to a class of drugs used to control epileptic seizures. They primarily act on the central nervous system, which consists of the brain and spinal cord, and suppress "epileptic seizures" via inhibiting the abnormal excitation of brain nerve cells (Patocka et al., 2020). Phenytoin is a typical example of an antiepileptic drug despite their wide variety and is often the treatment of choice among antiepileptic drugs, particularly because of its low price. In 2020, 1616,629 prescriptions of phenytoin were recorded in the USA (ClinCalc, 2020), placing it among the top 300, which is the highest among all drug classes prescribed in the USA in 2020.

Phenytoin has been detected in surface water (United States of America, 150 ng/L; Laws et al., 2011), urban watersheds (United States of America, 145 ng/L; Bai et al., 2018), hospital effluent (United States of America, 60–100 ng/L; Oliveira et al., 2015), river (Japan, 3.1–26 ng/L; (Simazaki et al., 2015), WWTP (Spain, 31–2375 ng/L; Mijangos et al., 2018), estuary (Spain, 3–1401 ng/L; Mijangos et al., 2018). To date, several studies have reported on the effects of phenytoin on fish. For example, zebrafish exposure to phenytoin results in neurotoxicity and oxidative stress (Cardoso-Vera et al., 2022a) and zebrafish embryo exposure causes malformations (Cardoso-Vera et al., 2022b). Brandão et al. (2013) reported that exposure to phenytoin caused adaptive responses in catalase and glutathione S-transferases (in the liver)

Abbreviations: HPG axis, hypothalamus, pituitary glands, and gonads; NIES, National Institute for Environmental Studies; PPCPs, pharmaceutical and personal care products; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WWTPs, wastewater treatment plants.

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activities, but without any behavioral alterations. Furthermore, Sawada et al. (2024) reported that phenytoin exposure induced abnormal swimming behavior in Japanese aucha perch (*Coreoperca kawamebari*), but not in silver pike characin (*Ctenolucius hujeta*). However, the effects on fish reproduction have not been reported. As reproduction is directly related to species conservation, investigating the effects of chemicals on reproduction is important to assess their risk (OECD, 2012).

The Japanese medaka (*Oryzias latipes*) is used worldwide as a model organism because it is easy to maintain and can be bred successively. In addition, its reproductive mode is oviparous and *in vitro* fertilization, which enables easy confirmation of spawning. Hence, it is suitable for studying the effects of chemical substances on reproduction; it is designated as a test fish by the OECD Test Guidelines. In general, fertilized eggs of Japanese medaka are obtained through the following processes: (1) males court females, (2) mating, and (3) egg-laying (Ono and Uematsu. 1957). Abnormal swimming behavior induced by chemical exposure is more likely observed at an early stage than physiological abnormalities, such as a decrease in fertilized eggs (Scott et al., 2004).

Therefore, in this study, we examined the behavioral abnormalities, followed by the reproductive effects of phenytoin exposure. To clarify the mechanism by which phenytoin exposure suppressed fertility, we investigated changes in the gonad-related gene expression. Although the exposure concentrations of behavioral abnormalities and reproductive inhibition induced by phenytoin exposure were significantly higher than those detected in the natural water environment, this study is the first to show that phenytoin exposure induces behavioral abnormalities, and suppresses *kiss1* expression and reproductive performance in Japanese medaka.

The significance of this study lies in its elucidation of the adverse effects of phenytoin exposure on Japanese medaka. Specifically, it reveals abnormal swimming behavior, reduced reproductive ability, and expression suppression of crucial reproductive genes, such as *kisspeptin 1*. By highlighting the potential mechanisms by which phenytoin exposure impairs reproductive ability, these findings not only contribute to our understanding of the impact of antiepileptic drugs on fish, but also underscore the importance of considering broader ecological impacts in antiepileptic drugs development and environmental management strategies.

2. Material and methods

2.1. Fish and chemical reagents

The Japanese medaka (*Oryzias latipes*) used in this study was an NIES-R strain transferred from the National Institute for Environmental Studies (NIES). Phenytoin (CAS No. 57–41–0, $>$ 99 % purity) was purchased from FUJIFILM Wako Pure Chemicals Corporation (Tokyo, Japan).

All experiments involving fish were performed in strict compliance with the applicable national guidelines, including the Act on Welfare and Management of Animals from the Ministry of the Environment in Japan and the ARRIVE guidelines 2.0. Fish were handled according to the animal care and use guidelines established by Kobe University. In addition, the Institutional Animal Care and Use Committee of the Research Center for Inland Seas at Kobe University approved all experiments (permission number: 2021–04).

2.2. Abnormal swimming behavior after phenytoin exposure

Phenytoin exposure concentrations in the abnormal swimming behavior test were set to control, 2.25 mg/L, and 9 mg/L. The nominal concentrations were set to those at which a reduction in reproductive capacity was observed; however, exposure concentration was much higher than environmental-related concentration. The experimental procedure is illustrated in Supplementary Figure 1. First, 15 Japanese medaka were exposed to each concentration group for 24 h in a 5 L glass aquarium (20 $\times$ 20 $\times$ 20 cm) containing 4 L of each phenytoin concentration or control. After 24 h, five fish were placed in a 2 L glass aquarium (9 $\times$ 18 $\times$ 13 cm) containing the exposure solution of each phenytoin concentration or control and recorded for 1 h from the aquarium front with a video camera (LUMIX DC-GH5S; Panasonic, Osaka, Japan). After recording, the fish were moved to a 5 L glass aquarium (20 $\times$ 20 $\times$ 20 cm) containing 4 L of phenytoin-free dechlorinated water and left for 24 h to allow the phenytoin to drain out of their bodies. After 24 h, five fish were moved to a 2 L glass aquarium containing 2 L of phenytoin-free dechlorinated water and were again recorded for 1 h with a video camera (LUMIX DC-GH5S).

The following abnormal swimming behaviors were observed: equilibrium abnormalities (Video 1), rotational behavior (Video 1), vertical swimming (Video 1), rolling over (Video 1), and aggression (Video 1). The number of test repetitions was 20; 100 fish were used for each concentration group. The number of fish with abnormal swimming behavior was determined via observing videos taken 24 h after phenytoin exposure, and 24 h after phenytoin exposure was stopped. For aggression, the number of times they attacked was recorded.

2.3. Reproduction success after phenytoin exposure

Phenytoin exposure concentrations in the reproduction test were set to control, 2.25 mg/L, and 9 mg/L. The experimental procedure is illustrated in Supplementary Figure 2. First, four 1 L plastic tanks were prepared (8 $\times$ 16 $\times$ 9 cm): one for the control, one for the 2.25 mg/L exposure group, and two for the 9 mg/L exposure group. Each tank was filled with 1 L of the reagent at the concentration of each test group. One male-female pair was placed in each tank. The fish were paired beforehand, and only pairs that were confirmed to have spawned continuously for at least three days were used. After 24 h exposure, video cameras were set up in front of the four tanks to record videos (60 fps, LUMIX DC-GH5S). In general, the Japanese medaka oviposit at night, after which they mate and spawn in the morning. Therefore, the shooting time was set to 10 h, from 22:00 pm to 8:00 am. After the recordings were completed, spawning occurrence was confirmed via observing the tank. In the recorded video, the presence or absence of courtship was confirmed via observing the behavior of the medaka after the lights were turned on. Courtship was defined as the male circling horizontally under the female body for more than one rotation (Video 2). In pairs that were confirmed to have spawned, the spawning time was identified and then courtship was checked around the spawning time. For pairs that were not confirmed to have spawned, courtship was checked directly. The test was repeated nine times, 10 pairs were observed in the control and 2.25 mg/L exposure groups, and 20 pairs were observed in the 9 mg/L exposure group.

2.4. Gene expression using reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

The results of the “2.3 Reproduction success after phenytoin exposure” section showed a reduction in reproductive performance in the 9 mg/L concentration group; therefore, we investigated the adverse mechanism by which reproductive performance was reduced. The phenytoin exposure concentration was 9 mg/L and 12 female and male medaka pairs were used in the control and 9 mg/L exposure groups. These pairs were pre-paired, and stable spawning (for three or more consecutive days) was observed.

The pairs were placed in a 500 mL beaker containing 500 mL of 9 mg/L phenytoin solution for at least 24 h The fish were then removed from the beaker at 9:00 am and immediately euthanized by placing them in ice-water. The fish head was dissected, and the brain and pituitary gland were removed and stored in RNAlater (Sigma-Aldrich, MO, USA).

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer’s instructions. The RNA concentration was determined using a NanoDrop One Microvolume UV–Vis

spectrophotometer (Thermo Fisher Scientific, MA, USA). After quantification, 500 ng of RNA was reverse-transcribed into cDNA using the PrimeScript RT Master Mix (Perfect Real-Time) (Takara Bio, Inc., Shiga, Japan). The cDNA concentration was determined using EASY Dilution for real-time PCR (Takara Bio, Inc.) to 10 ng/μL. Samples were then stored at −30 °C.

Expression levels of six genes related to reproduction (*kiss1, kiss2, ghnrh1, ghnrh2, fshb,* and *lhb*) were investigated using RT-qPCR. The primers used are listed in Supplementary Table 1. The analysis was performed on a LightCycler 96 system (Roche Diagnostics, Basel, Switzerland) using FastStart SYBR Green Master Mix (Nippon Genetics, Tokyo, Japan). The reaction volume for each PCR was 20 μL, consisting of 0.2 μL primer (20 μM) for each gene, 1 μL cDNA (10 ng/μL), 10 μL PCR Master Mix (2 ×), and 8.6 μL PCR water. Duplicates were performed for each gene and the obtained data were analyzed using LightCycler 96 SW 1.1 software (Roche Diagnostics). The data were then transferred to Microsoft Excel and target gene expression was normalized using the housekeeping gene elongation factor 1a (*ef1a*) (2-⊿⊿Ct method) (Livak and Schmittgen, 2001).

2.5. Statistical analysis

Statistical analyses were performed using EZR, a statistical software that extended the capabilities of R and R Commander. For analyzing significant differences in behavioral abnormalities and reproductive behavior, Fisher's exact probability test with the Holm–Bonferroni method was used ($p < 0.05$). McNamer's test was used to determine significant differences during and 24 h after stopping exposure to phenytoin ($p < 0.05$). The Kruskal–Wallis test was used to determine significant differences in the number of attacks ($p < 0.05$). The Mann–Whitney U test was used to determine significant differences in gene expression ($p < 0.05$).

3. Results

3.1. Phenytoin exposure induced abnormal swimming behavior

No swimming behavioral abnormalities were observed in the control group. However, upon phenytoin exposure, equilibrium abnormalities were found in 86 of 100 individuals and all 100 individuals in the 2.25 and 9 mg/L concentration groups (Fig. 1a), whereas rotational behavior was observed in 35 of 100 and 33 of 100 individuals in the 2.25 and 9 mg/L concentration groups, respectively (Fig. 1b). Vertical swimming was found 7 of 100 individuals in the 9 mg/L concentration group but not in the 2.25 mg/L concentration group (Fig. 1c). Rolling over behavior was observed in 4 of 100 and 15 of 100 individuals in the 2.25 and 9 mg/L concentration groups, respectively (Fig. 1d).

When phenytoin exposure was stopped, the individuals exhibiting these behavioral abnormalities reduced (Fig. 2). Equilibrium abnormalities reduced by 27 % and 20 % in the 2.25 mg/L and 9 mg/L concentration groups, respectively (Fig. 2a). Rotational behavior reduced by 71 % and 64 % in the 2.25 mg/L and 9 mg/L concentration groups, respectively (Fig. 2b). Vertical swimming reduced by 86 % in the 9 mg/L group (Fig. 2c). Rolling over behavior reduced by 50 % and 93 % in the 2.25 mg/L and 9 mg/L concentration groups, respectively (Fig. 2d).

Fig. 3 shows the changes in aggression after phenytoin exposure; no changes in aggression were observed in the 2.25 mg/L concentration group compared to the control group, but aggression increased significantly in the 9 mg/L concentration group (Fig. 3a). However, 24 h after phenytoin exposure was stopped, aggression decreased and did not differ from that of the control (Fig. 3b).

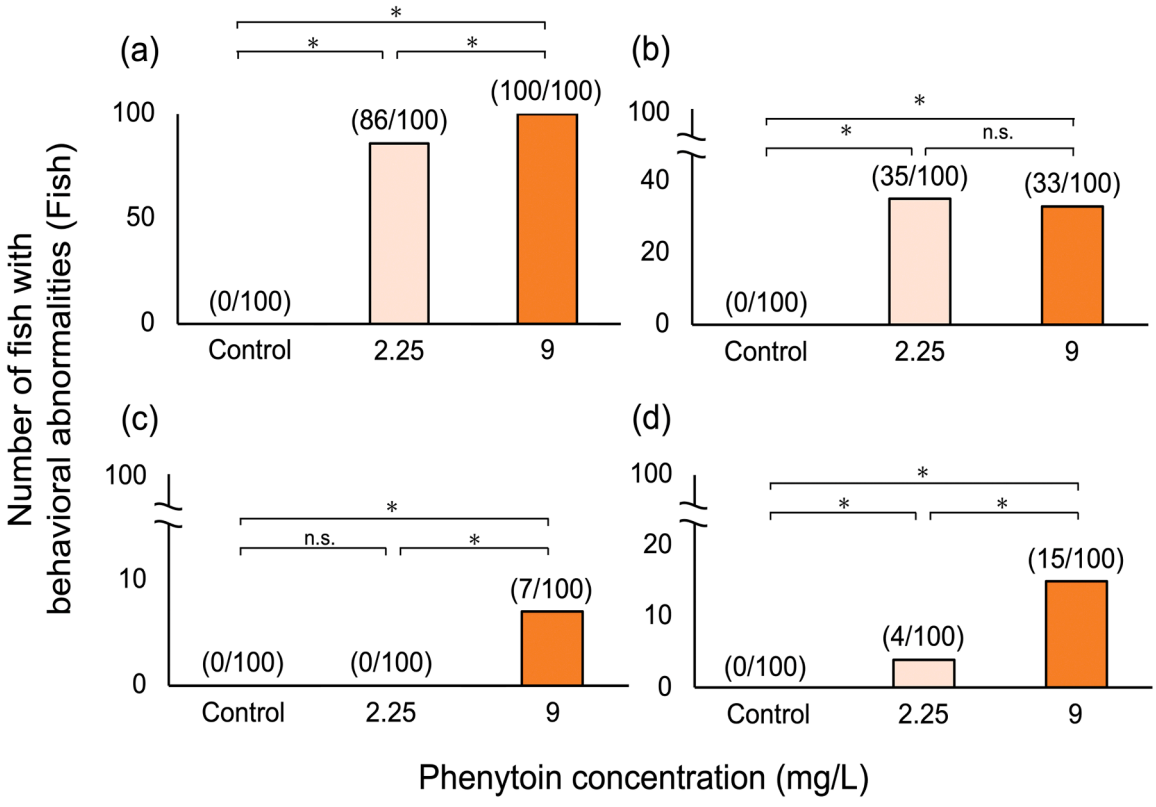

Fig. 1. Effect of phenytoin on swimming behavior in Japanese medaka.
Phenytoin exposure induced (a) equilibrium abnormalities, (b) rotational behavior, (c) vertical swimming, (d) rolling over in Japanese medaka. Numbers above the bars indicate the number of fish with behavioral abnormalities; $n = 100$. Holm-Bonferroni method was used to determine significant differences ($p < 0.05$); n.s = not significant.

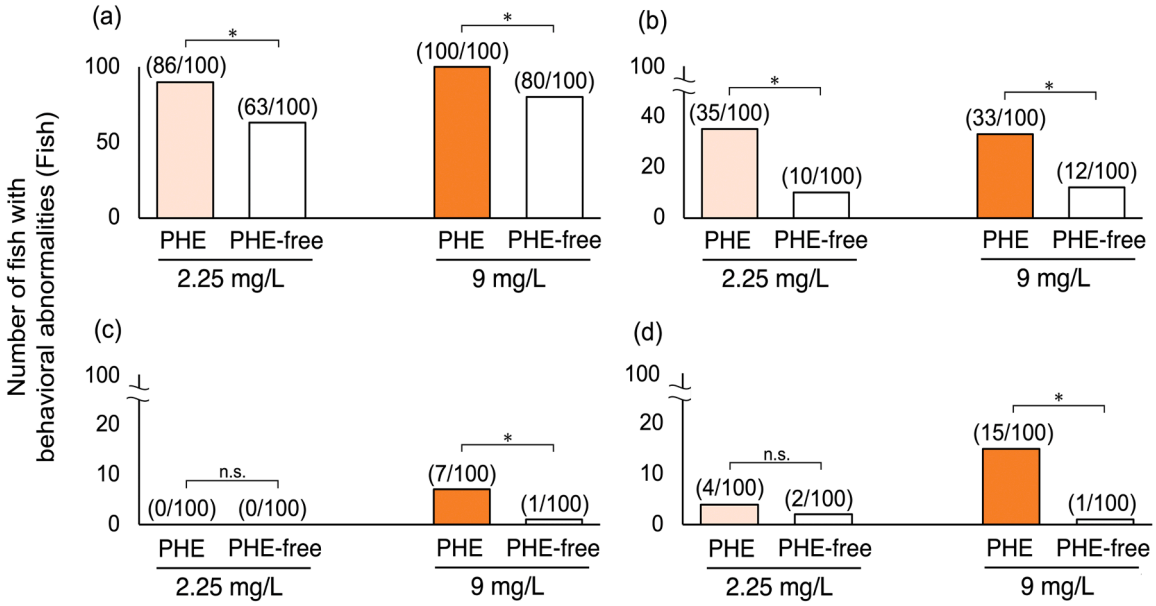

Fig. 2. Effect on swimming behavior 24 h after phenytoin exposure was stopped in Japanese medaka.
(a) Equilibrium abnormalities, (b) rotational behavior, (c) vertical swimming, (d) rolling over. Numbers above the bars indicate the number of fish with behavioral abnormalities; $n = 100$. McNamer's test was used to determine significant differences during and 24 h after phenytoin exposure was stopped ($p < 0.05$); n.s = not significant.

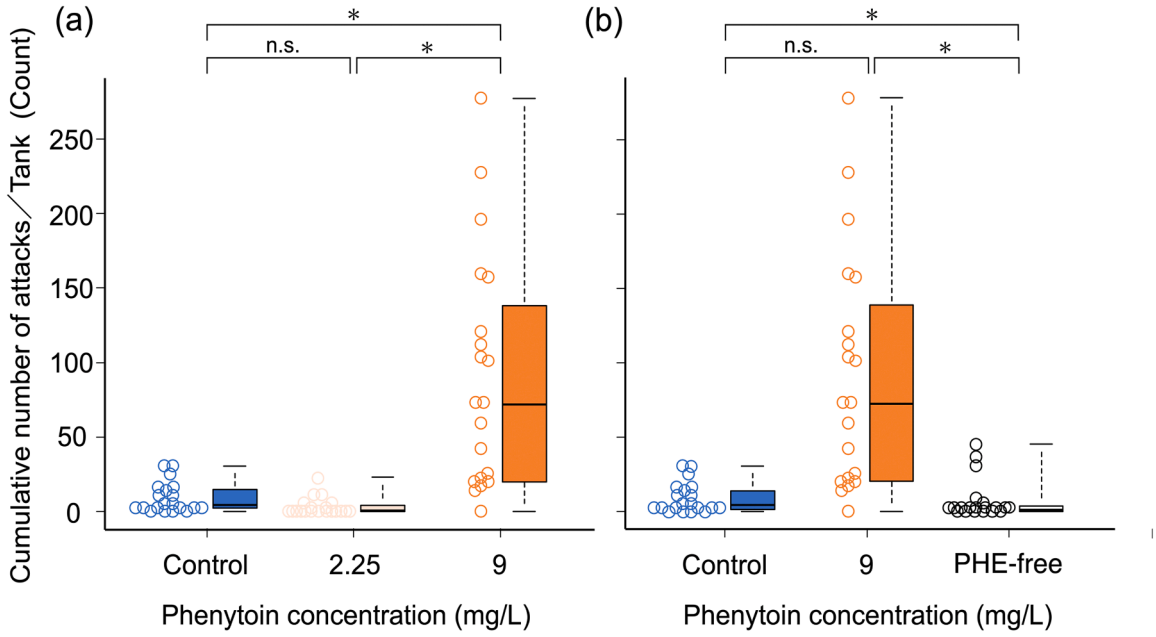

Fig. 3. Effect of phenytoin on aggression in Japanese medaka.
(a) Aggression significantly increased in the 9 mg/L concentration group. (b) 24 h after phenytoin exposure was stopped, aggression significantly decreased and reached the same level as that of the control. Kruskal-Wallis test used to determine significant differences ($p < 0.05$); n.s = not significant; $n = 20$.

3.2. Phenytoin exposure reduced reproduction

In the control and 2.25 mg/L exposure groups, all pairs (10 of 10) spawned (Fig. 4). By contrast, only 5 of 20 pairs spawned eggs in the 9 mg/L exposure group, exhibiting a significant decrease in the spawning pair proportion compared to that in the control group (Fig. 4a). Male-initiated courtship behavior was observed in all pairs in the control, 2.25 mg/L, and 9 mg/L exposure groups (Fig. 4b, Video 2).

3.3. Effect of phenytoin on gene expression

When both males and females were exposed to phenytoin, *gnrh1, gnrh2, fshb*, and *lhb* expression remained unchanged compared with that in the control (Fig. 5). In contrast, *kiss1* expression was significantly suppressed due to phenytoin exposure in both males and females compared with that in the control group (Fig. 5). In addition, *kiss2* expression was significantly suppressed in females compared to that in the control group; males showed no changes in *kiss2* expression (Fig. 5).

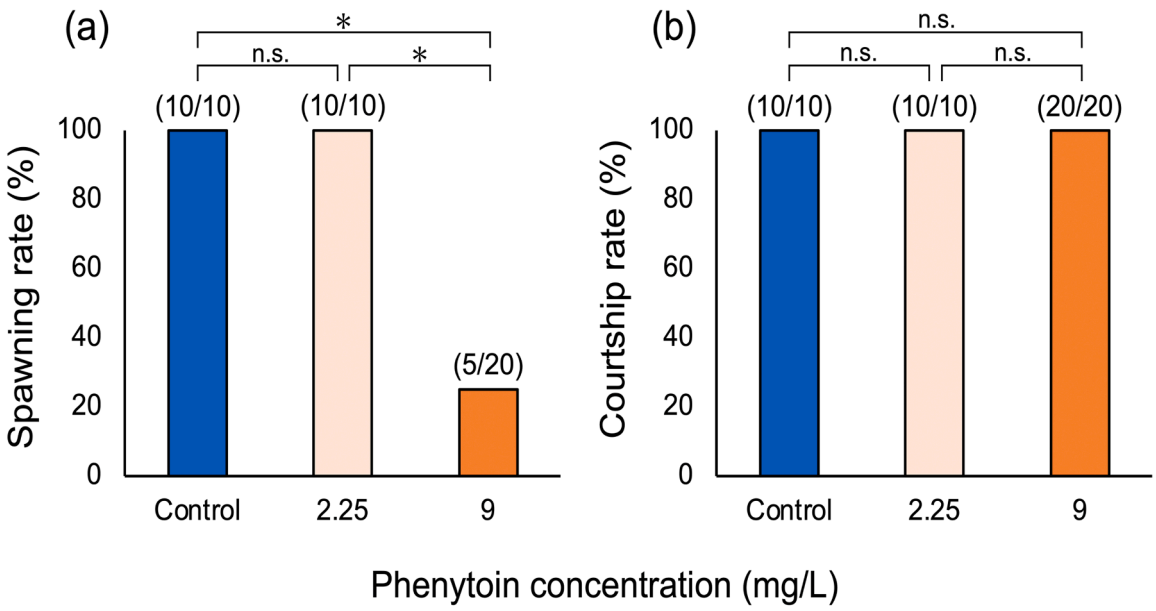

Fig. 4. Effect of phenytoin on (a) Spawning rate and (b) Courtship rate in Japanese medaka.
Numbers above the bars indicate the pairs with spawning or courtship behavior; $n = 10$ or 20. Holm-Bonferroni method was used to determine significant differences compared with the control group ($p < 0.05$); n.s = not significant.

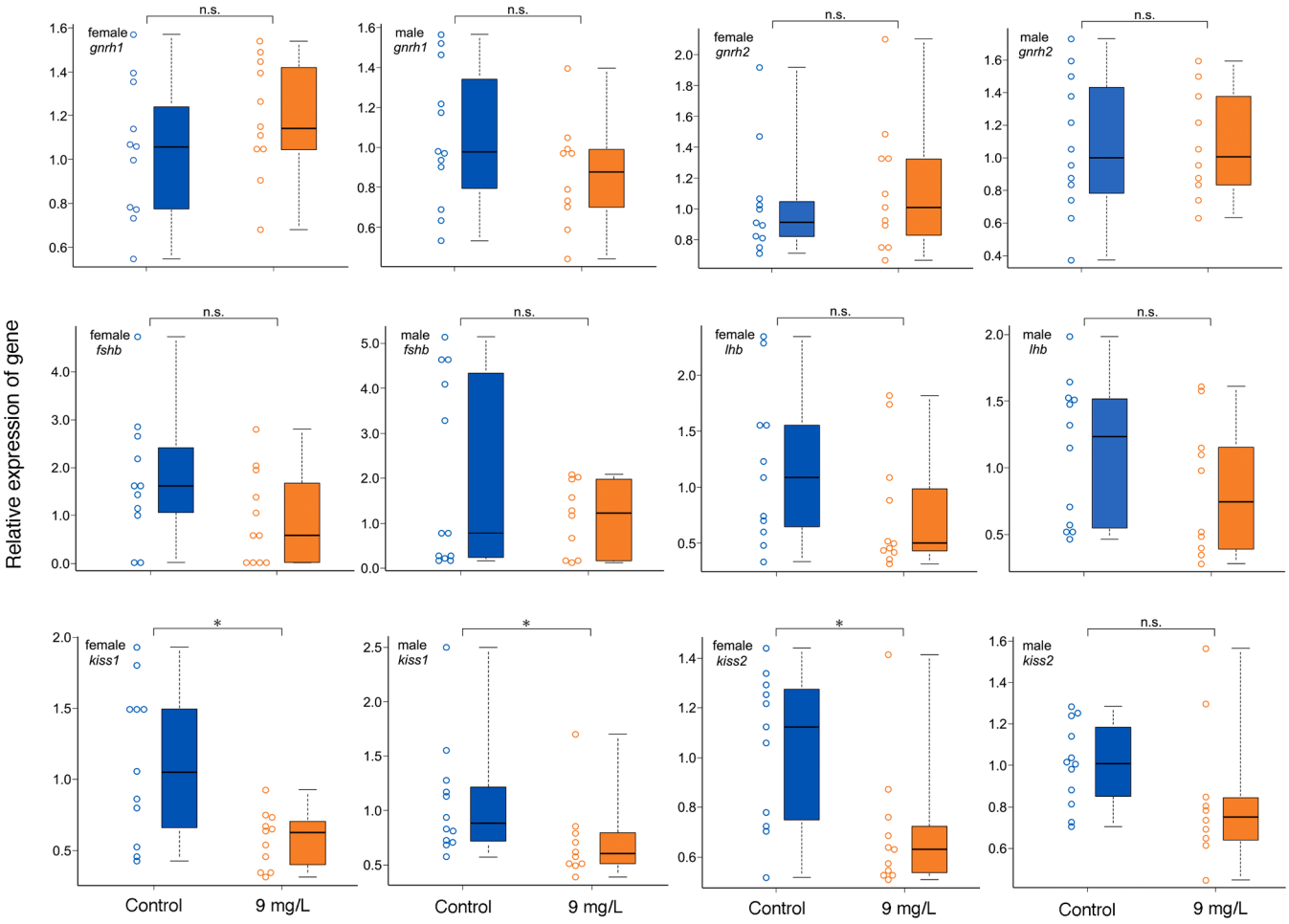

Fig. 5. Effect of phenytoin on *gnrh1, gnrh2, fshb, lhb, kiss1*, and *kiss2* expression.
Each plot indicates the expression level in each individual. Mann–Whitney's U test was used to determine significant differences compared with the control group ($p < 0.05$); n.s = not significant.

4. Discussion

To date, several studies have reported that antiepileptic drug exposure induces abnormalities in the swimming behavior of fish. For example, Pieróg et al. (2021) reported that exposure to valproate, phenytoin, diazepam, and phenobarbital caused anxiety-like behaviors in adult zebrafish. Zhou et al. (2019) reported that exposure to carbamazepine increased the swimming speed of zebrafish larvae. Martinez et al. (2018) reported that phenobarbital exposure increased the spontaneous movement of zebrafish larvae. Qiang et al. (2016) reported that carbamazepine exposure increased the touch-evoked escape response in zebrafish larvae. In contrast, Nassef et al. (2010) reported that carbamazepine exposure decreased the swimming speed in Japanese medaka. Wang et al. (2024) also reported that diazepam exposure decreased the swimming performance of female Japanese medaka. In this study, phenytoin exposure induced abnormalities in swimming behavior in Japanese medaka similar to the findings of these previous studies.

In humans, adverse effects may occur when blood phenytoin levels increase excessively owing to phenytoin overdosage, with initial symptoms including dizziness, ataxia, lethargy, and occurrence of a coma in severe cases (Patocka et al., 2020). When the side effects of phenytoin were compared with behavioral abnormalities identified in this study, the equilibrium abnormalities and rotational behavior were similar to those of ataxia, whereas the vertical swimming and rolling over behaviors were similar to those of lethargy and coma. Phenytoin is a drug that exerts its effects via acting on the central nervous system. As the basic structure of the central nervous system is preserved in vertebrates such as fish, including medaka, and mammals, including humans (Mink al. 1981), phenytoin likely functions in medaka in the same manner as in humans. These results suggest that phenytoin is a novel and potent inhibitor of the central nervous system in medaka. Based on these findings, the occurrence of behavioral abnormalities upon phenytoin exposure may be caused by adverse effects of overdosing on phenytoin.

In humans, the mean elimination half-life of phenytoin at 100 mg is 40 h (Ahn et al., 2008). Although the half-life of phenytoin in Japanese medaka remains unknown, the phenytoin levels in the Japanese medaka body, as in humans, are likely decreasing, because the percentage of behavioral abnormalities was observed to decrease 24 h after phenytoin exposure was stopped.

Phenytoin is generally used as an antiepileptic drug; however, it has also been found to suppress aggressive behaviors. For example, Barratt et al. (1997) reported that phenytoin significantly reduced aggressive behavior by 71 % and aggression intensity by 60 % in humans. Stanford et al. (2009) stated that clinically, they recommended phenytoin (initial dose of 100 mg three times daily) as the first-line treatment for impulsive aggressive outbursts among antiepileptic drugs. No previous studies have reported on the effects of phenytoin on increasing aggression in fish; this is the first report to do so. Unlike in humans, phenytoin exposure increases aggression in Japanese medaka and its cessation results in the absence of aggression. However, the relationship between phenytoin levels and increased aggression remains unclear. Therefore, future studies are required to elucidate the underlying mechanism of this observed behavior.

The reproductive system is regulated by endocrine hormones in the hypothalamus, pituitary glands, and gonads (HPG axis). The hypothalamus secretes gonadotropin-releasing hormone, which regulates gonadotropin (luteinizing hormone and follicle-stimulating hormone) secretion from gonadotropin-producing cells in the pituitary gland. Luteinizing and follicle-stimulating hormones promote ovarian maturation and ovulation. In this study, phenytoin exposure did not alter *gnrh1, gnrh2, fshb*, or *lhb* expression, suggesting that the suppression of reproductive ability due to phenytoin exposure did not involve these hormone axis systems. By contrast, phenytoin exposure suppressed *kiss1* expression in both male and female medaka.

Kisspeptin is a type of neuropeptide, that uses its receptor Gpr54, to regulate gonad function via the HPG axis control mechanism in mammals (de Roux et al., 2003; d'Anglemont de Tassigny et al., 2007). However, Nakajo et al. (2018) reported that kisspeptin was not involved in HPG axis regulation in Japanese medaka. Kisspeptin is highly associated with sexual behavior in these fish. For example, Nakajo et al. (2024) reported that the Kiss1-Gpr54–1 system, and not the Kiss2-Gpr54–2 system, could be involved in upregulating female spawning and downregulating male motivation. In particular, male individuals of Gpr54–1 knockout medaka exhibit increased courtship behavior, whereas female individuals become less motivated and less receptive to males. In this study, phenytoin-exposed males exhibited courtship behavior toward females; however, the females were not interested (Video 3, Supplemental Figure 3). This suggests that phenytoin exposure decreased *kisspeptin 1* expression in both males and females, and that females became less interested in males.

For ecological risk assessment of chemicals on aquatic organisms, the no observed effect concentration (NOEC), lowest observed effect concentration (LOEC), and actual concentrations of chemicals in the aquatic environment should be known. Phenytoin has been detected in surface water (United States of America, 150 ng/L; Laws et al., 2011), urban watersheds (United States of America, 145 ng/L; Bai et al., 2018), hospital effluent (United States of America, 60–100 ng/L; Oliveira et al., 2015), rivers (Japan, 3.1–26 ng/L; (Simazaki et al., 2015), WWTP (Spain, 31–2375 ng/L; Mijangos et al., 2018), and estuaries (Spain, 3–1401 ng/L; Mijangos et al., 2018). The LOECs of phenytoin in Japanese medaka obtained in the present study—for abnormal swimming behavior (2.25 mg/L), and reproduction (9 mg/L)—were approximately $>$ 1000 and 3700 times higher than in WWTP in Spain. Therefore, the ecological risk associated with phenytoin is low in Japan. These results suggest that phenytoin may not be affecting fish swimming behavior and reproduction in the aquatic environment. However, in the actual aquatic ecosystem, fish live under more severe conditions than under laboratory conditions with regard to certain aspects, such as sunshine hours, pH, dissolved oxygen, and feed. Therefore, the ecological impact concentration of phenytoin in the nature environment is possibly lower than the value of LOEC obtained in the present study. Therefore, continuously surveying phenytoin levels in the aquatic environment is required.

5. Conclusion

In this study, phenytoin disrupts swimming behavior and causes aggression in Japanese medaka. Phenytoin exposure also significantly reduced their reproductive ability and decreased *kisspeptin* 1 expression, which controlled reproductive behavior in Japanese medaka. While males displayed courtship behavior, females showed no interest in males. This confirms *kisspeptin* 1 suppression due to phenytoin exposure; consequently, no eggs were laid. This is the first study to show that phenytoin exposure induces behavioral abnormalities and suppresses *kiss1* expression and reproductive performance in Japanese medaka.

Data statement

All data underlying the findings are fully available without restriction.

Supplemental Figure 1. Experimental flowchart showing the effect of phenytoin on behavioral abnormalities in Japanese medaka.

Supplemental Figure 2. Experimental flowchart showing the effect of phenytoin on the reproductive ability of Japanese medaka.

Supplemental Figure 3. Number of females who showed interest in the courtship behavior of males. Numbers above the bars indicate the number of females with interesting in the courtship behavior of males.

CRediT authorship contribution statement

Kensuke Mitsunaga: Writing – original draft, Methodology, Investigation, Data curation. **Sheikh Shohag:** Writing – review & editing. **Chew Jia Ming:** Writing – review & editing. **Chee Kong Yap:** Writing –

review & editing. **Yoshifumi Horie:** Writing – original draft, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2024.107007.

References

Ahn, J.E., Cloyd, J.C., Brundage, R.C., Marino, S.E., Conway, J.M., Ramsay, R.E., White, J.R., Musib, L.C., Rarick, J.O., Birnbaum, A.K., Leppik, I.E., 2008. Phenytoin half-life and clearance during maintenance therapy in adults and elderly patients with epilepsy. Neurology 71 (1), 38–43. https://doi.org/10.1212/01.wnl.0000316392.55784.57.

Bai, X., Lutz, A., Carroll, R., Keteles, K., Dahlin, K., Murphy, M., Nguyen, D., 2018. Occurrence, distribution, and seasonality of emerging contaminants in urban watersheds. Chemosphere 200, 133–142. https://doi.org/10.1016/j.chemosphere.2018.02.106.

Barratt, E.S., Stanford, M.S., Felthous, A.R., Kent, T.A., 1997. The effects of phenytoin on impulsive and premeditated aggression: a controlled study. J. Clin. Psychopharmacol. 17 (5), 341–349. https://doi.org/10.1097/00004714-199710000-00002.

Brandão, F.P., Rodrigues, S., Castro, B.B., Gonçalves, F., Antunes, S.C., Nunes, B., 2013. Short-term effects of neuroactive pharmaceutical drugs on a fish species: biochemical and behavioural effects. Aquat. Toxicol. 144-145, 218–229. https://doi.org/10.1016/j.aquatox.2013.10.005.

Cardoso-Vera, J.D., Gómez-Oliván, L.M., Islas-Flores, H., García-Medina, S., Elizalde-Velázquez, G.A., Orozco-Hernández, J.M., Heredia-García, G., Rosales-Pérez, K.E., Galar-Martínez, M., 2022a. Multi-biomarker approach to evaluate the neurotoxic effects of environmentally relevant concentrations of phenytoin on adult zebrafish *Danio rerio*. Sci. Total. Environ. 834, 155359 https://doi.org/10.1016/j.scitotenv.2022.155359.

Cardoso-Vera, J.D., Gómez-Oliván, L.M., Islas-Flores, H., García-Medina, S., Orozco-Hernández, J.M., Heredia-García, G., Elizalde-Velázquez, G.A., Galar-Martínez, M., SanJuan-Reyes, N., 2022b. Acute exposure to environmentally relevant concentrations of phenytoin damages early development and induces oxidative stress in zebrafish embryos. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 253, 109265 https://doi.org/10.1016/j.cbpc.2021.109265.

ClinCalc, 2020. The Top 300 Drugs of 2020 https://clincalc.com/DrugStats/Top300Drugs.aspx. (Accessed June 24, 2024).

d'Anglemont de Tassigny, X., Fagg, L.A., Dixon, J.P.C., Day, K., Leitch, H.G., Hendrick, A.G., Zahn, D., Franceschini, I., Caraty, A., Carlton, M.B.L., et al., 2007. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc. Natl. Acad. Sci. USA. 104, 10714–10719. https://doi.org/10.1073/pnas.0704114104.

de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc. Natl. Acad. Sci. USA. 100, 10972–10976. https://doi.org/10.1073/pnas.1834399100.

Espinosa-Jovel, C., Toledano, R., Aledo-Serrano, Á., García-Morales, I., Gil-Nagel, A., 2018. Epidemiological profile of epilepsy in low income populations. Seizure 56, 67–72. https://doi.org/10.1016/j.seizure.2018.02.002.

Laws, B.V., Dickenson, E.R., Johnson, T.A., Snyder, S.A., Drewes, J.E., 2011. Attenuation of contaminants of emerging concern during surface-spreading aquifer recharge. Sci. Total. Environ. 409 (6), 1087–1094. https://doi.org/10.1016/j.scitotenv.2010.11.021.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta C(T)) method. Methods 25 (4), 402–408. https://doi.org/10.1006/meth.2001.1262.

Martinez, C.S., Feas, D.A., Siri, M., Igartúa, D.E., Chiaramoni, N.S., Del, V., Alonso, S., Prieto, M.J., 2018. *In vivo* study of teratogenic and anticonvulsant effects of antiepileptics drugs in zebrafish embryo and larvae. Neurotoxicol. Teratol. 66, 17–24. https://doi.org/10.1016/j.ntt.2018.01.008.

Mijangos, L., Ziarrusta, H., Ros, O., Kortazar, L., Fernández, L.A., Olivares, M., Zuloaga, O., Prieto, A., Etxebarria, N., 2018. Occurrence of emerging pollutants in estuaries of the Basque Country: analysis of sources and distribution, and assessment of the environmental risk. Water. Res. 147, 152–163. https://doi.org/10.1016/j.watres.2018.09.033.

Mink, J.W., Blumenschine, R.J., Adams, D.B., 1981. Ratio of central nervous system to body metabolism in vertebrates: its constancy and functional basis. Am. J. Physiol. 241 (3), R203–R212. https://doi.org/10.1152/ajpregu.1981.241.3.R203.

Nakajo, M., Kanda, S., Karigo, T., Takahashi, A., Akazome, Y., Uenoyama, Y., Kobayashi, M., Oka, Y., 2018. Evolutionally conserved function of kisspeptin neuronal system is nonreproductive regulation as revealed by nonmammalian study. Endocrinology 159 (1), 163–183. https://doi.org/10.1210/en.2017-00808.

Nakajo, M., Kanda, S., Oka, Y., 2024. Involvement of the kisspeptin system in regulation of sexual behaviors in medaka. iScience 27 (2), 108971. https://doi.org/10.1016/j.isci.2024.108971.

Nassef, M., Matsumoto, S., Seki, M., Khalil, F., Kang, I.J., Shimasaki, Y., Oshima, Y., Honjo, T., 2010. Acute effects of triclosan, diclofenac and carbamazepine on feeding performance of Japanese medaka fish (*Oryzias latipes*). Chemosphere 80 (9), 1095–1100. https://doi.org/10.1016/j.chemosphere.2010.04.073.

OECD, 2012. Guidelines for the testing of chemicals, test No. 229: Fish short term reproduction assay. OECD Publishing.

Oliveira, T.S., Murphy, M., Mendola, N., Wong, V., Carlson, D., Waring, L., 2015. Characterization of Pharmaceuticals and Personal Care products in hospital effluent and waste water influent/effluent by direct-injection LC-MS-MS. Sci. Total. Environ. 518-519, 459–478. https://doi.org/10.1016/j.scitotenv.2015.02.104.

Ono, Y., Uematsu, T., 1957. Mating ethogram in *Oryzias latipes*. J. Fac. Sci. Hokkaido Univ. Ser. VI Zool. 13, 197–202.

Patocka, J., Wu, Q., Nepovimova, E., Kuca, K., 2020. Phenytoin - An anti-seizure drug: overview of its chemistry, pharmacology and toxicology. Food. Chem. Toxicol. 142, 111393 https://doi.org/10.1016/j.fct.2020.111393.

Pieróg, M., Socała, K., Doboszewska, U., Wyska, E., Guz, L., Szopa, A., Serefko, A., Poleszak, E., Wlaź, P., 2021. Effects of classic antiseizure drugs on seizure activity and anxiety-like behavior in adult zebrafish. Toxicol. Appl. Pharmacol. 415, 115429 https://doi.org/10.1016/j.taap.2021.115429.

Qiang, L., Cheng, J., Yi, J., Rotchell, J.M., Zhu, X., Zhou, J., 2016. Environmental concentration of carbamazepine accelerates fish embryonic development and disturbs larvae behavior. Ecotoxicology 25 (7), 1426–1437. https://doi.org/10.1007/s10646-016-1694-y.

Sawada, A., Dorcas, U., Horie, Y., 2024. Behavioral profile alterations and predation susceptibility of Japanese medaka fish exposed to phenytoin, an antiepileptic drug. Environ. Toxicol. Pharmacol. 108, 104474 https://doi.org/10.1016/j.etap.2024.104474.

Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68 (4), 369–392. https://doi.org/10.1016/j.aquatox.2004.03.016.

Simazaki, D., Kubota, R., Suzuki, T., Akiba, M., Nishimura, T., Kunikane, S., 2015. Occurrence of selected pharmaceuticals at drinking water purification plants in Japan and implications for human health. Water. Res. 76, 187–200. https://doi.org/10.1016/j.watres.2015.02.059.

Stanford, M.S., Anderson, N.E., Lake, S.L., Baldridge, R.M., 2009. Pharmacologic treatment of impulsive aggression with antiepileptic drugs. Curr. Treat. Options Neurol. 11 (5), 383–390. https://doi.org/10.1007/s11940-009-0043-3.

Wang, S., Dong, J., Chen, K., Shi, Y., Qiu, X., 2024. Effects of chronic diazepam exposure on the behaviors and oxidative stress homeostasis in the eyes and brains of female Japanese medaka. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 276, 109812 https://doi.org/10.1016/j.cbpc.2023.109812.

Zhou, S., Chen, Q., Di, Paolo, C., Shao, Y., Hollert, H., Seiler, T.B., 2019. Behavioral profile alterations in zebrafish larvae exposed to environmentally relevant concentrations of eight priority pharmaceuticals. Sci. Total. Environ. 664, 89–98. https://doi.org/10.1016/j.scitotenv.2019.01.300.