Is seven days enough? Comparing a 7-day exposure to the classical 21-day OECD TG 229 fish short-term reproduction assay in fathead minnow

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Research Article

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Abstract

The OECD (Organisation for Economic Co-operation and Development) test guidelines (TG) 229 - fish short-term reproduction assay (FSTRA) is one of the gold standard methods used to identify endocrine disrupting chemicals (EDCs). While informative, the FSTRA's 5 to 6-week duration makes it difficult to use routinely. Prior studies have shown that EDCs' impact on fecundity, vitellogenin (VTG) and steroid levels can be detected after less than one week of exposure suggesting the FSTRA could be shortened. This study compares both 7- and 21-day FSTRAs using fathead minnows (*Pimephales promelas*) for three known EDCs: 17α-ethinylestradiol (EE2; 40 ng/L), 17β-trenbolone (TRB; 50 µg/L), and propiconazole (PRP; 500 µg/L). All three compounds led to arrested fertility after 24 h of exposure, except for the 7-day EE2 treatment which still decreased reproduction. Moreover, independently of time of exposure, EE2 induced VTG production in males, and decreased estrogen levels in females and testosterone levels in males. In contrast, TRB induced VTG production in males, while the levels were not different from controls in females even though testosterone levels increased, and masculinization was observed. Finally, PRP led to a decrease in VTG levels which was only significant during the 21-day exposure, and surprisingly, no effect on steroid levels were observed despite its known effects on steroidogenesis. Apart from minor differences, both times of exposure led to similar outcomes supporting the shortening of the FSTRA to seven days. This proposed 7-day FSTRA could be used to screen EDCs in routine monitoring of environmental samples.

1. Introduction

Endocrine disrupting chemicals (EDCs) are substances which can alter the normal function of hormones and give rise to adverse effects in intact organisms or their progeny (WHO/ICPS 2002). EDCs are known to reduce fecundity, gonadal function, and sexual development as well as to lead to intersex conditions and feminization in fish (reviewed in Marlatt et al. 2022; Matthiessen et al. 2017; WHO/UNEP 2012). Moreover, EDCs can have a more generalized impact at the ecosystem level. One classic example is the addition of 7 ng/L of 17α-ethinylestradiol (EE2) in a Canadian natural lake, which provoked the collapse of the fathead minnow (*Pimephales promelas*) population (Kidd et al. 2007).

To identify EDCs, international efforts were made to standardized bioassays, especially in fish species. One of the earliest developed bioassays was the fish short-term reproduction assay (FSTRA). It was first developed for fathead minnows by the Environmental Protection Agency of the United States (US-EPA 2009; Ankley et al. 2001; Ankley et al. 2003; Ankley et al. 2002; Harries et al. 2000). The FSTRA was also adapted for Japanese medaka (*Oryzias latipes*) (Kang et al. 2003; IJ Kang et al. 2002; IKJ Kang et al. 2002; Seki et al. 2002) and zebrafish (*Danio rerio*) (Van den Belt et al. 2001; van der Ven et al. 2003). It was later standardized as one of the test guidelines (TG) of the Organisation for Economic Co-operation and Development (OECD TG 229; 2012). The FSTRA (Ankley et al. 2001; OECD 2012; US-EPA 2009) recommends evaluating fecundity success for a period of 2 to 3 weeks before starting treatment for an additional 21 days. During this later 21-day exposure period, the number of eggs produced is being quantified daily. At the end of the exposure, several other endpoints are measured, including total fish

weight, gonadal and liver weight, assessment of secondary sexual characteristics, level of plasmatic or hepatic vitellogenin (VTG, a female biomarker), concentrations of circulating steroids, and optionally, gonadal histopathology.

The FSTRA is currently one of the gold standards used to evaluate the impact of EDCs on fish reproduction as it is included in multiple international framework for the identification of EDCs from the United States (EDSTAC 1998), Japan (Japan-MoE 2016), the European Union (Andersson et al. 2018), and in OECD guidance (OECD 2018). While the FSTRA has short-term in its name, its current form remains long (5 to 6 weeks) and labour intensive. For those reasons, the FSTRA is difficult to apply on a routine basis to analyze environmental water samples for endocrine activity. A previous study assessing pulp and paper mill effluents has suggested shortening the duration of the FSTRA to a 4-day pre-exposure and to 5 to 6 days of exposure (Tibor Kovacs et al. 2007). This experimental time reduction would be possible as most of the FSTRA endpoints are known to be sensitive after only a few days of exposure in fathead minnows. For example, VTG gene expression and protein synthesis were induced after a minimum of 24 h when exposed to EE2 (Cavallin et al. 2014; Ekman et al. 2008; Feswick et al. 2017; Martinović et al. 2007; Reddy et al. 2015). Similarly, VTG and 17β-estradiol (E2) levels decreased in female fish after at least 48 h up to 8 days when exposed to fadrozole, an inhibitor of the aromatase (Villeneuve et al. 2009). For female fathead minnows exposed to 17β-trenbolone (TRB), a potent androgen, levels of E2 and VTG in the plasma decreased after 24 h and 4 days, respectively (Ekman et al. 2011). Furthermore, when caged for 4 days in the field near wastewater treatment plants, testosterone levels were altered in fathead minnows (Ankley et al. 2021). In the laboratory, induction of VTG was observed in male fathead minnows when exposed 48 h to wastewater (Wehmas et al. 2011). For pulp and paper mill effluents, gene expression of VTG, estrogen receptor α (ERα) and β (ERβ), and the androgen receptor (AR) was modified in males after an exposure of 6 days (Werner et al. 2010). Moreover, reduction in egg spawning is often observed during the first week of FSTRA (Ankley et al. 2001; Ankley et al. 2003; Ankley et al. 2002; Cavallin et al. 2016; Tibor Kovacs et al. 2007; Salierno and Kane 2009; Skolness et al. 2013; Werner et al. 2010).

While a shorter version of the FSTRA has been proposed, there is no published studies on its feasibility. The objective of this study was to compare the standard OECD TG 229 FSTRA of 21 days to a shorter version of 7 days. To compare the exposures, three known EDCs were selected to encompass the most studied modalities of endocrine disruption for reproduction namely the modalities for estrogen, androgen, and steroidogenesis (EAS). The first chosen EDCs was EE2, the active compound of the contraceptive pill, which is commonly found in municipal wastewater and lead to decreased reproduction and induce feminization in fish population (Jobling et al. 1998; Larsson et al. 1999; Purdom et al. 1994). The second one was TRB, the active metabolite of trenbolone acetate, a potent androgen used for growing beef and found in the environment near beef feedlots (Ankley et al. 2018; Balter 1999; Durhan et al. 2006; Orlando et al. 2004). At last, the propiconazole (PRP), a fungicide that affects the synthesis of steroids (Huang et al. 2022; Skolness et al. 2013; Teng et al. 2020) was chosen.

2. Method

Chemicals and stock solutions

Compounds used include, 17α-ethinylestradiol (EE2; Sigma #E4876-1g, lot WXBc6630V), 17β-trenbolone (TRB; Steraloids, #E3170-000, lot B1588), and propiconazole (PRP; Sigma #45642-250mg, lot BCBW6694). The use of TRB on animals was approved by Health Canada under the permits HC6-53-39-97 and HC6-70-242-8.

The used concentrations of each compound were selected based on the scientific literature to allow observing effects at 21 days of exposure in fathead minnows and were not selected to be environmentally relevant as it was not the objective of the study. Fish were either exposed for 7 or 21 days to 40 ng/L of EE2 (Salierno and Kane 2009), 50 µg/L of TRB (Ankley et al. 2003) and 500 µg/L of PRP (Skolness et al. 2013). Fresh stock solutions were prepared in 100% ethanol every day at 4 g/L for EE2, 5 g/L for TRB, and 50 g/L for PRP. For EE2, few serial dilutions were done afterwards to obtain a 4 mg/L solution. The stock solutions were then diluted in reconstituted water to obtain concentration of 2 µg/L, 2.5 mg/L, and 25 mg/L for EE2, TRB and PRP respectively. Finally, to obtain the desired concentrations, 200 mL of each solution was added to the aquarium for which 60% of the water was removed and then refilled to 10 L with reconstituted water every day. The final concentration of ethanol was of 0.001%.

Fish husbandry

Fathead minnow (*P. promelas*) eggs were obtained by reproducing fish from the Institut national de la recherche scientifique (INRS)'s colony. Fish were then raised at 25 ± 1°C in an environmental room in reconstituted water (for 100 L of water: 20 g $NaHCO_3$, 6 g $MgSO_4$, 6 g $CaSO_4$ and 0.4 g KCl) with a light cycle of 16:8. Procedures for raising fish and conducting the exposures were reviewed and approved by INRS' *Comité institutionnel de protection des animaux* (CIPA; #1903-01 and #1909-01).

Fish short-term reproduction assay (FSTRA)

The protocol was based on standard protocols (OECD 2012; US-EPA 2009) and adapted for semi-static systems. Fish were distributed randomly into 24 glass aquaria of 10 L. Each tank contained two male and four female fathead minnows of 11 to 12 months old. Each tank was aerated to maintain over 60% of dissolved oxygen. Water quality was assessed every day for dissolved oxygen (> 60%), pH (6.5–8.0), temperature (24.0–25.5°C), conductivity (250–300 µS/cm), and ammonia (< 1 µg/L). Throughout the exposure, 60% of the water was changed daily either by reconstituted water for the pre-exposure and controls, or by reconstituted water spiked with compounds at the selected concentration. Fish were fed spirulina flakes (Aquatic Animals Accessories, AAA premium) in the morning and artemias (Hikari, Bio-Pure frozen brine shrimp) in the afternoon.

To evaluate fecundity, freshly laid eggs were collected every day from the tiles. Eggs were incubated for 24 h in reconstituted water and fertilized eggs were counted. Before starting any exposures, the fecundity of every tank was assessed during a pre-test of three weeks. Fish were changed during the first two weeks of pre-exposure if they were not producing fertilized eggs. At the beginning of the exposure, each tank was assigned to a treatment in a semi-random approach starting with the tanks with the highest reproduction. The experiment included a total of 8 treatment with the control (CTRL), 40 ng/L of EE2, 50 µg/L TRB, and 500 µg/L PRP for both times of exposure. Each treatment had three replicates.

Endpoints at exposure completion

Fecundity and mortality were recorded daily for the entirety of the exposure. At the end of each exposure (7- or 21-day), fish were anaesthetized with 500 mg/L of tricaine methanesulfonate (MS-222, Sigma, #A5040-25g) buffered with the same amount of sodium bicarbonate. The weight of each fish was then recorded. Blood was collected from the caudal vein with heparinized capillaries (Fisher, #22-362-566), transferred into a 0.6 mL-tube containing 0.55 U of aprotinin (Millipore Sigma, #61637010MG), and centrifuged at 6,000 g for 1 min to collect the plasma. The plasma was then flash frozen in dry ice and stored at -80°C for ELISA analysis. Fish were euthanized by decapitation, dissected, and organs were weighted (liver and gonads).

Secondary sexual characteristics were also evaluated, and scoring were attributed for all individuals. Presence, size, and/or numbers of ovipositor, fatpad, bands, and tubercles were observed. A male index was used to score the animals based on previous studies (Tibor Kovacs et al. 2007; Martel et al. 2010; Parrott and Blunt 2005). First, the fatpad was graded on a scale from 0 to 3 (0 = absent; 1 = beginning or resorbing fatpad; 2 = small fatpad; and 3 = big fatpad). Then, the banding pattern was also graded from 0 to 3 based on the darkness of the head and the prominence of the vertical band on the body (0 = absent; 1 = darker body; 2 = dark head and body; and 3 = dark body + band). The tubercle index was equal to Eq. 1. Finally, the male index was calculated by summing all three indexes.

Equation 1. $$Tubercle index = \frac{Total number of tubercles + (2X number of large tubercles)}{10}$$

ELISA for vitellogenin and steroids in plasma

VTG analysis was performed using the ELISA kits from Biosense Laboratories (#V01018401). For sexual steroids, Cayman chemical kits were used for 17β-estradiol (E2; #501890) and testosterone (T; #582701). The methods and calculations were done according to the fabricants' instructions. The ELISA plates were read using a spectrometer Filtermax F5 from Molecular Devices. For each of the methods, plasma was diluted in the respective assay buffer with the following ratio: 1:50 to 1: $5x10^{-17}$ for VTG; 1:10 to 1:50 for E2; and 1:20 for T. Following fabricant's indications, the limits of detection (LODs) for the sample preparation used were of 4.68 ng/mL VTG, 60 pg/mL E2, and 100 pg/mL T in plasma. To include non-detected samples in statistical analysis, half of the respective LOD of each assay was used. Two samples for the female VTG analysis had concentrations surpassing the linear range of the standard

curve and could not be assessed again due to the volume of the sample. To include them in the analysis, their concentration was equal to the highest standard multiplied by the dilution factor used.

Chemical analysis

Chemical analysis was performed for each compound at time 0, 24 h, and 21 days by the *Centre d'expertise en analyse environnementale du Québec* (CEAEQ, Quebec City, Canada) using standard protocols. Briefly, for the steroids and cholesterol, samples were prepared by solid-phase extraction with a C-18 column and analyzed by GC-MS (MA.404 – Steroids 1.0, CEAEQ 2023), while for PRP, samples were prepared by liquid-liquid extraction using dichloromethane and analyzed by GC-MS (MA.400 – Pest. 1.0, CEAEQ 2016). The LOD were of 12 ng/L for EE2, 1.4 µg/L for TRB, 0.18 µg/L for PRP, 80 ng/L for cholesterol, 5 ng/L for coprostan-3-ol and 20 ng/L for coprostan-3-one. The recoveries of internal standards for the steroid method varied between 49–120% and between 90–140% for the PRP method.

Statistics

One fish from the 7-day PRP treatment was removed from the statistical analysis as it was misidentified as a female at the beginning of the experiment. The arithmetic means and standard deviation were first calculated. The Shapiro-Wilk's normality test and the Levene's test for the homogeneity were then performed. A log-transformation was only used for the VTG levels. The 7-day and 21-day exposure were analyzed separately by ANOVA with a post-hoc Dunnett's test or when the data was considered nonparametric by Kruskal-Wallis's analysis with a post-hoc Dunn's test. The statistical significance indicated refers to the post-hoc p-value (< 0.05). To assess if there was any significant difference between the same treatment for both times of exposure, an unpaired t-test or a nonparametric Mann-Whitney's test was performed. Statistical analysis and graphs were made using GraphPad PRISM 10.

3. Results

Chemical analyses

Chemical analysis on the water was performed at the start of the exposure to verify the concentration added to the water. As seen in Table 1, the concentration of EE2 was close to the nominal concentration of 40 ng/L. However, the concentration of TRB was around 80 µg/L instead of 50 µg/L, and PRP were closer to half the concentration at ~ 290 µg/L.

Table 1
Nominal and observed chemical concentrations. (n = 2, both values indicated)

Compound	CAS	Concentration unit	Nominal concentration	Observed concentrations		
				0 h	24 h	21 d
17α-ethinylestradiol (EE2)	57-63-6	ng/L	40	37–42	< 12	< 12
17β-trenbolone (TRB)	10161-33-8	µg/L	50	76–81	38–53	31–38
Propiconazole (PRP)	60207-90-1	µg/L	500	250–330	180–230	480–490

To evaluate if the concentrations of compounds were stable throughout the experiment and were not accumulating overtime as only 60% of the water was changed every day, the concentration of each compound was assessed at 24 h and on the last day of the 21-day exposure. EE2 was not detectable after 24 h (Table 1) suggesting high uptake by the fish or rapid degradation. TRB was detectable at concentration around 40 µg/L at both 24 h and 21 days suggesting its concentration was relatively stable for the entirety of the experiment. PRP had a concentration of 205 µg/L after 24 h and reached about 485 µg/L at the end of the 21 days which represents more than the double of the initial concentration suggesting an accumulation of PRP during the experiment. For the controls, none of the studied compounds were detected. As part of the steroid analysis, trace of cholesterol and its metabolites were detected in the controls and in the EE2 treatment (Table 2).

Table 2
Concentrations of cholesterol and metabolites of steroids. (n = 2, both values indicated)

Treatments	Cholesterol (ng/L)	Coprostan-3-ol (ng/L)	Coprostan-3-one (ng/L)
Control	< 400–660	< 5	< 20–47
EE2–0 h	300–320	< 5	< 20
EE2–24 h	2800–8200	25–66	210–510
EE2–21 days	21000–68000	29–41	130–280

Mortality

No mortality was observed in any of the control, EE2 and PRP treatments; however, in the TRB treatment, two females of the 7-day exposure and 2 males of the 21-day exposure died. All of them exhibited abnormal behaviour prior to death by swimming slowly at the surface. Only one fish was necropsied, and the only abnormality detected was the gallbladder which was swollen and contained dark liquid.

Weight, HSI, and GSI

For the weight, no significant difference was observed for male (Table 3) and female (Table 4). For the hepatosomatic index (HSI), there was a significant increase of 1.5-fold for the females exposed for 21-day to EE2 ($p = 0.046$) and TRB ($p = 0.013$) in comparison to their control. For the gonadosomatic index (GSI), there was a significant decrease of over 2-fold for both males ($p = 0.008$) and females ($p = 0.014$) exposed to EE2 for 21-day. Noteworthy, it was the only treatment where there was a significative difference between the 7-day and 21-day exposure.

Table 3
Weight, HSI, GSI, and secondary sexual characteristics of males for the 7-day and 21-day exposure.

Duration of exposure	Treatments	n	Weight (g)	HSI	GSI
7-day	CTRL	6	5.00 ± 0.41	0.014 ± 0.006	0.011 ± 0.003
	EE2	6	5.40 ± 1.45	0.019 ± 0.003	0.009 ± 0.002‡
	TRB	6	5.65 ± 1.47	0.014 ± 0.005	0.008 ± 0.002
	PRP	6	5.64 ± 0.73	0.018 ± 0.005	0.011 ± 0.003
21-day	CTRL	6	4.79 ± 1.38	0.016 ± 0.007	0.011 ± 0.004
	EE2	6	4.54 ± 1.30	0.019 ± 0.009	0.004 ± 0.002*‡
	TRB	4	5.85 ± 1.00	0.020 ± 0.006	0.011 ± 0.003
	PRP	6	6.32 ± 1.05	0.019 ± 0.005	0.010 ± 0.004
*p-value < 0.5					
‡ Nonparametric Mann-Whitney's test with a p-value < 0.01					

Table 4
Weight, HSI, GSI, and secondary sexual characteristics of females for the 7-day and 21-day exposure.

Duration of exposure	Treatments	n	Weight (g)	HSI	GSI
7-day	CTRL	12	2.76 ± 0.44	0.018 ± 0.006	0.096 ± 0.041
	EE2	12	2.27 ± 0.29	0.023 ± 0.009	0.116 ± 0.045†
	TRB	9	2.46 ± 0.71	0.021 ± 0.008	0.112 ± 0.043
	PRP	11	2.38 ± 0.53	0.013 ± 0.006	0.119 ± 0.033
21-day	CTRL	12	2.62 ± 0.55	0.017 ± 0.006	0.125 ± 0.054
	EE2	11	2.66 ± 0.67	0.026 ± 0.008*	0.054 ± 0.051*†
	TRB	12	3.14 ± 1.09	0.027 ± 0.007*	0.088 ± 0.044
	PRP	12	2.39 ± 0.40	0.014 ± 0.004	0.119 ± 0.036
*p-value < 0.5					
† T-test or nonparametric Mann-Whitney's test with a p-value < 0.01					

Secondary sexual characteristics

For the male index, there was no statistical difference for males across treatments (Fig. 1A). However, resorbed nuptial tubercles were observed in males exposed to EE2 for 21 days and the fatpad were generally smaller. For females, the presence of male secondary sexual characteristics was only observed and significant in the 21-day TRB treatment (Fig. 1B, $p < 0.001$). Eight females out of 12 were exhibiting secondary sexual characteristics with all of them showing a darker coloration, five showing a beginning of fatpad, and four exhibiting four to six small tubercles. For the ovipositor, females of all treatment had an ovipositor. For males, ovipositors were only observed at 21 days for five males out of six in the EE2 treatment.

Fecundity

The cumulative number of eggs for each duration of exposure is represented in Fig. 2. The cumulative number of eggs was normalized to day 1 of each of the exposures to simplify the comparison as the reproductive capacity of the fish varied between treatment during the pre-exposure. The control treatment continued to produce eggs throughout both durations of exposure. For most treatments, the fish stopped producing eggs after the first or second day of the exposure (Fig. 2, $p < 0.001$). The only exception was the 7-day EE2 exposure ($p = 0.40$) which still had an important decrease in reproduction to about one third of the 7-day control. Only one of the triplicates was still able to reproduce. This contrast with the 21-day EE2 exposure for which the reproduction stopped completely.

Plasma levels of vitellogenin and sexual steroids

VTG levels in plasma were evaluated by ELISA for males (Fig. 3A) and females (Fig. 3B). For males, there was a significant increase of 10 orders of magnitude in VTG for the 7- ($p = 0.016$) and 21-day EE2 ($p = 0.01$) and 7-day TRB treatment ($p = 0.016$) in comparison to their respective control. For the TRB-treated males exposed for 21 days, the increase in VTG was close to being significant ($p = 0.078$). For females, a similar increase was observed for the same treatments, but was not significant as the control females already have high levels of VTG in comparison to males. For PRP, there was a significant decrease only in females exposed for 21 days in comparison to the control ($p < 0.001$). For both sexes, there was no significant difference between the results from the 7-day and 21-day exposure across treatments.

For E2 levels in plasma, there were no significant changes between treatments for males (Fig. 3C). However, there were significant decrease between the 7- and 21-day exposure for the control ($p = 0.041$) and PRP ($p = 0.015$) for which the levels of E2 decreased by a factor of 4 and 8, respectively. This could be linked to the high variation of the samples of 7-day exposure for both the CTRL and PRP treatment. For females (Fig. 3D), E2 levels were significantly decreased by 3- and 25-fold for the 7- ($p = 0.010$) and 21-day EE2 ($p < 0.001$) exposures, respectively, in comparison to their controls. Moreover, this decrease is likely underestimated as some samples had concentrations under the limit of detection.

For T levels in plasma, there was a significant decrease of 22- to 17-fold for the 7- ($p = 0.004$) and 21-day EE2 ($p = 0.005$) treatments for males in comparison to their respective control (Fig. 3E). For females, the decrease was not considered significant (Fig. 3F). However, for both sexes, the decrease is underestimated as the levels were 2/3 of the time under the LOD. This suggests that independently of time of exposures and sex, EE2 decreased T levels. For females, there was also a significant increase in T levels by respectively 2- and 4-fold for the 7- ($p = 0.022$) and 21-day TRB ($p = 0.035$) exposures in comparison to their control. There was no significant difference observed in T levels for PRP exposure in both sexes.

4. Discussion

The objective of this study was to evaluate if a 7-day FSTRA could replace the standard 21-day FSTRA. This was considered possible as fecundity, VTG quantification, and steroid plasma levels can be affected in the first week of treatment by various chemicals (Ankley et al. 2021; Cavallin et al. 2014; Cavallin et al. 2016; Ekman et al. 2008; Ekman et al. 2011; Feswick et al. 2017; Tibor Kovacs et al. 2007; Martinović et al. 2007; Reddy et al. 2015; Villeneuve et al. 2009; Wehmas et al. 2011; Werner et al. 2010). To verify this hypothesis, a 7-day and a 21-day FSTRAs were compared using three known EDC-related compounds (EE2, TRB, and PRP) with different mechanisms of action for the disruption of the reproductive axis, which include estrogenicity, androgenicity, and the interference with the steroid synthesis.

17α-ethinylestradiol

As a potent synthetic estrogen, EE2 is primarily known to induce VTG, which can occur in both sexes in fish at concentrations ranging from 10–488 ng/L (Doyle et al. 2013; Park et al. 2009; Salierno and Kane

2009; Seki et al. 2002; Van den Belt et al. 2001; Zhang et al. 2008). This increase in VTG was significative in males for both EE2-FSTRAs. Another common effect is the decrease, and in some cases, the full arrest in reproduction at concentrations of 5 -100 ng/L for exposure of 7 days to two months (Park et al. 2009; Pawlowski et al. 2004; Scholz and Gutzeit 2000; Van den Belt et al. 2001). In the 21-day FSTRA, there was a clear stop in reproduction, while a non-significant decrease in spawning was observed for the 7-day FSTRA due to one replicate out of three which was still reproducing. In addition, EE2 can lead to feminization and female bias sex ratio at 1-100 ng/L (Parrott and Blunt 2005; Pawlowski et al. 2004; Salierno and Kane 2009; Scholz and Gutzeit 2000). The 21-day FSTRA led to feminization with the development of ovipositors in males accompanied with the resorption of their nuptial tubercles. This contrast with the 7-day FSTRA in which there was no change. Moreover, the 7-day FSTRA decreased GSI in females, but not in males, while in the 21-day FSTRA, GSI reduced it in both sexes. This observation is commonly seen in both sexes for EE2 treatment and is linked to a decrease in gametogenesis (Parrott and Blunt 2005; Pawlowski et al. 2004; Salierno and Kane 2009; Scholz and Gutzeit 2000; Van den Belt et al. 2001). Finally, a decrease in E2 in females and in T in males was observed in both FSTRAs. It is difficult to compare those results to the scientific literature as effects on sexual steroids have rarely been studied and EE2 impacts vary depending on sex and species (Doyle et al. 2013; Salierno and Kane 2009). Nevertheless, both FSTRAs were able to capture the main effects of EE2 on VTG levels, egg production, and even GSI, and had the same effects on steroid levels. The main difference between both exposure durations was the male feminization, which was only apparent after 21 days of treatment.

Trenbolone

TRB is an anabolic steroid used to grow beef cattle muscle (aka meat). In fish, TRB can negatively impact egg production at 0.5–500 µg/L (Ankley et al. 2003; Hemmer et al. 2008; Park et al. 2009; Zhang et al. 2008) like the arrest in reproduction observed in both FSTRAs. The TRB-FSTRAs also induced VTG synthesis in males and increased T in females. While identical in each TRB-FSTRA, those results are counterintuitive as TRB is a potent androgen and will usually lead to a decrease in VTG levels, and sometimes, in E2 levels in females (Ankley et al. 2003; Brockmeier et al. 2013; Ekman et al. 2011; Seki et al. 2006; Zhang et al. 2008). Despite being unusual, the observed increase in VTG in males was previously described by others (Ankley et al. 2003). Male VTG induction is hypothesized to be linked to its weak ability to activate the estrogen receptor and not to its capacity to be aromatized like T (Ankley et al. 2018; Ankley et al. 2003; Yarrow et al. 2010). The ability of TRB to induce the estrogen receptor was confirmed in the transgenic cyp19a1b-GFP zebrafish embryo (Brion et al. 2012) and in a luciferase assay for the human estrogen receptor from INDIGO Biosciences by the authors (unpublished data). Moreover, Ankley et al. (2003) observed a U-shaped correlation for levels of VTG, E2, and T in females, which decreased from 0.005 to 0.5 µg/L of TRB and increased slightly at 5 and 50 µg/L. This U-shape could explain the lack of effect on the female VTG levels. Hence, while atypical, the observed effects on VTG and circulating steroids have been observed previously like the arrest in reproduction.

In contrast, some differences were observed between the TRB-FSTRAs for other endpoints. TRB is known to induce masculinization in fish at concentrations ranging from 0.05 to 50 µg/L (Ankley et al. 2003;

Brockmeier et al. 2013; Seki et al. 2006; Sone et al. 2005). In the 21-day TRB-FSTRA, females developed nuptial tubercles and a fatpad, while no change was detected during the 7-day TRB-FSTRA similarly as the feminization outcome measured by the EE2-FSTRAs. Comparably, another effect that was only seen in the 21-day TRB-FSTRA was the increase in HSI that has been previously reported (Hemmer et al. 2008; Park et al. 2009). The inability of the 7-day TRB-FSTRA to assess the impact on the secondary sexual characteristics and HSI is not surprising as those are deeper physiologic modification which takes time to develop (Brockmeier et al. 2013; Tibor Kovacs et al. 2007). While it would be interesting to observe those effects (secondary sexual characteristics, HSI) on a smaller experimental duration, the evaluation of the other endpoints, such as egg production and VTG levels, can already inform on the endocrine disruptive capacity of substances.

Propiconazole

PRP has been shown to impact egg production (Skolness et al. 2013; Teng et al. 2020) as seen in both PRP-FSTRAs. PRP will affect reproduction by disrupting steroidogenesis, like other azole fungicides, and more specifically, by inhibiting the aromatase (Doering et al. 2019; Huang et al. 2022). Due to its impact on the aromatase, PRP is expected to decrease E2 synthesis and in turn VTG synthesis at concentrations of 0.1–1000 µg/L (Huang et al. 2022; Skolness et al. 2013; Teng et al. 2020). In the present study, VTG was decreased in all PRP treatments, but was only significative in females of the 21-day PRP-FSTRA, while no effect was observed on E2. Hence, egg production and VTG were both impacted similarly by both PRP-FSTRAs.

Conclusion

In conclusion, a 7-day FSTRA was able to induce expected effects for an estrogenic, androgenic, and steroidogenic mode of action as the traditional 21-day FSTRA for egg production, VTG levels, and steroid levels. As expected, endpoints linked to phenotype, such as modifications in secondary sexual characteristics, occur over a longer period and were only captured by the longer exposures. However, the inability of the shorten FSTRA to assess those longer endpoints do not hinder the evaluation of endocrine disruption. Hence, as suggested by Tibor Kovacs et al. (2007), the standard 21-day FSTRA can be reduced to a shorter version. With further testing on complex mixture, this 7-day FSTRA could facilitate the study of environmental samples (e.g., wastewater) on a more regular basis to assess the impact of EDC mixtures and could be used to inform on risk to better protect aquatic ecosystems.

Declarations

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Competing interests

The authors have no financial or proprietary interests in any material discussed in this article.

Author's contribution

All authors contributed to the study conception, design, and data interpretation. Animal care, material preparation and data collection except for analytical chemistry data were performed by J. Robitaille with the help of M. Lefebvre-Raine. Analysis of chemical concentration was organized by E. Veilleux. The original draft was written by J. Robitaille and revised and approved by all the other authors.

Ethics approval

The animal protocols were approved by the CIPA of INRS' LNBE for the fathead minnow colony (#1903-01) and the FSTRA (#1909-01). The use of TRB on animals was approved by Health Canada under the permit HC6-53-39-97 and HC6-70-242-8.

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Figures

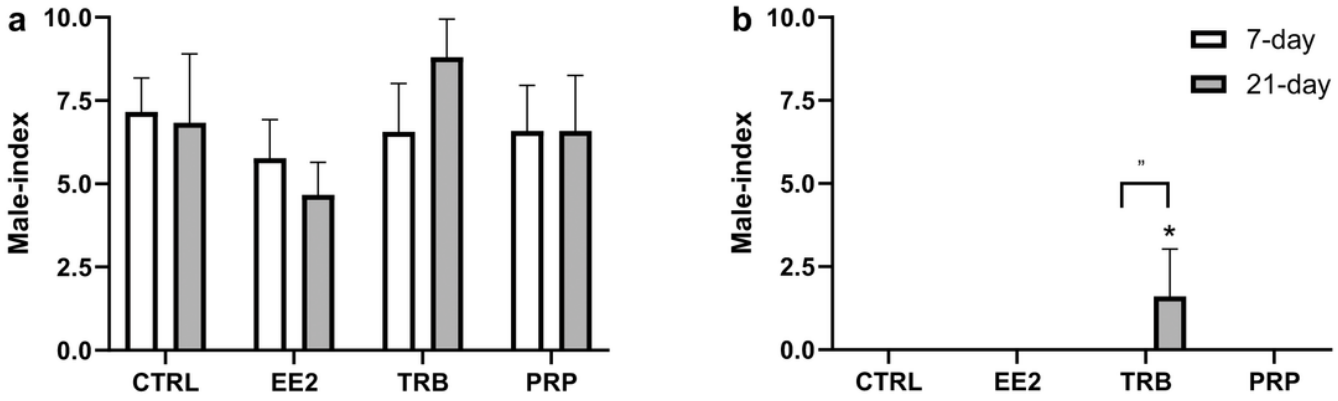

Figure 1

Male index for males (a) and females (b).

The 7-day and 21-day exposures are represented in white and grey, respectively. For males, the n was of 6, except for the 21-day TRB treatment, which had an n of 4. For females, the n was of 12 except for the 7-day TRB and PRP, which had respectively a n of 10 and 9. Stars (*) and deltas (Δ) represent statistical significance (p-value < 0.05). Stars are for a Kruskal-Wallis' test with a post-hoc Dunn's test in comparison to the respective control; deltas (Δ) with a bracket are for a Mann-Whitney's test.

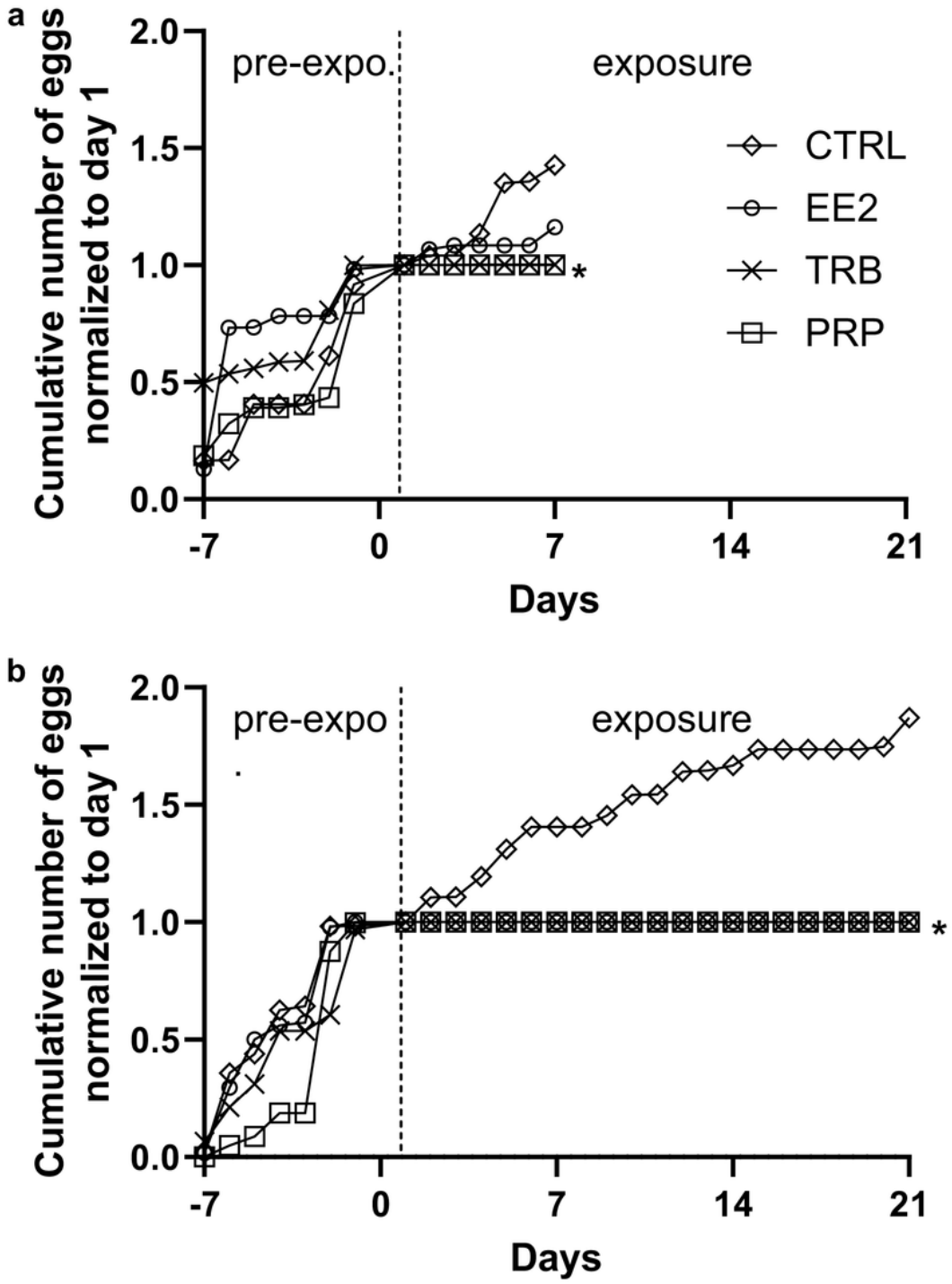

Figure 2

The cumulative number of eggs normalized to the first day of exposure for the 7-day (a) or 21-day (b) exposure.

The n was of 3. The stars (*) represent the statistical significance ($p < 0.05$) with a Kruskal-Wallis' test for the daily egg production.

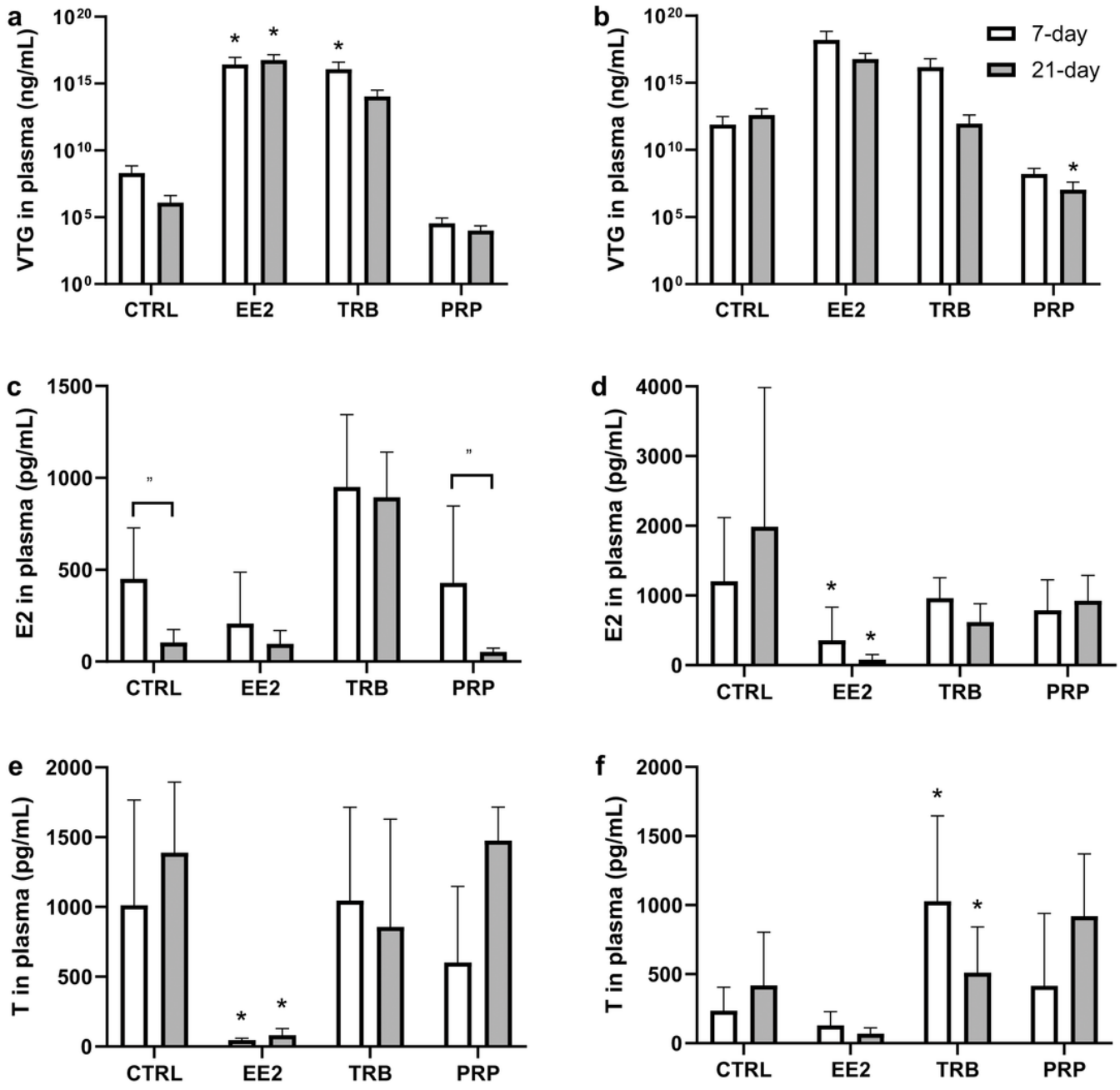

Figure 3

Concentration of vitellogenin (VTG; a and b), 17β-estradiol (E2; c and d) and testosterone (T; e and f) in plasma for males (a, c and e) and females (b, d and f).

The 7-day and 21-day exposure are represented in white and grey respectively. For females, the n was of 12 except for the 7-day EE2, TRB and PRP which had respectively an n of 11, 10 and 9. For males, the n was of 6 except for the 21-day TRB treatment which had an n of 4. The smaller n was either explained by death or the inability to collect blood from some fish. Stars (*) and deltas (Δ) represent statistical significance (p-value < 0.05). Stars are statistical significance in comparison to the respective control

based on the Kruskal-Wallis's test with a post-hoc Dunn's test; the deltas with brackets are statistical significance from Mann-Whitney's test to compare times for the same treatment.