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Mummichog (*Fundulus heteroclitus*) are less sensitive to 17α-ethinylestradiol (EE_2) than other common model teleosts: A comparative review of reproductive effects

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ABSTRACT

The environmental estrogen 17α-ethinylestradiol (EE_2) will depress or completely inhibit egg production in many common model teleosts at low concentrations (≤0.5 ng/L; Runnalls et al., 2015). This inhibition is not seen in the estuarine killifish, or mummichog (*Fundulus heteroclitus*), even when exposed to 100 ng/L EE_2. This relative insensitivity to EE_2 exposure indicates species-specific mechanisms for compensating for exogenous estrogenic exposure. This review compares various reproductive responses elicited by EE_2 in mummichog to other common model teleosts, such as zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*), identifying key endpoints where mummichog differ from other studied fish. For example, EE_2 accumulates primarily in the liver/gall bladder of mummichog, which is different than zebrafish and fathead minnow in which accumulation is predominantly in the carcass. Despite causing species-specific differences in fecundity, EE_2 has been shown to consistently induce hepatic vitellogenin in males and cause feminization/sex reversal during gonadal differentiation in larval mummichog, similar to other species. In addition, while gonadal steroidogenesis and plasma steroid levels respond to exogenous EE_2, it is generally at higher concentrations than observed in other species. In mummichog, production of 17β-estradiol (E_2) by full grown ovarian follicles remains high; unlike other teleost models where E_2 synthesis decreases as 17α,20β-dihydroxy-4-prenen-3-on levels increase to induce oocyte maturation. New evidence in mummichog indicates some dissimilarity in gonadal steroidogenic gene expression responses compared to gene expression responses in zebrafish and fathead minnow exposed to EE_2. The role of ovarian physiology continues to warrant investigation regarding the tolerance of mummichog to exogenous EE_2 exposure. Here we present a comprehensive review, highlighting key biological differences in response to EE_2 exposure between mummichog and other commonly used model teleosts.

1. Introduction

One of the most common estrogenic endocrine disrupting compounds (EDCs) in the aquatic environment is 17α-ethinylestradiol (EE_2), a potent estrogen used in the birth control pill that persists in sewage effluents (reviewed in Schröder et al., 2016). EE_2 is typically found in low concentrations (< 0.1 ng/L; Johnson et al., 2013) in aquatic receiving environments, but has been measured at > 30 ng/L (Ternes et al., 1999; Aris et al., 2014). Most fish species exposed to EE_2 exhibit negative impacts on gonadal growth, e.g., gonadosomatic index (Pawlowski et al., 2004), sex steroid production (Salierno and Kane, 2009; Runnalls et al., 2015; Armstrong et al., 2016), sperm quality (Oropesa et al., 2015), egg production (Lin and Janz, 2006; Zha et al., 2008; Armstrong et al., 2016), as well as skewed sex ratios and increased incidence of intersex (Kidd et al., 2007; Luzio et al., 2016a,b).

The majority of studies to date have focussed on the impact of EE_2

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on freshwater species, while only a few estuarine or marine species have been used to investigate the impacts of EE_2 on reproduction (Bosker et al., 2017). One key exception is the estuarine killifish, more commonly known as the mummichog (*Fundulus heteroclitus*), which has emerged as an important and useful marine teleost model for ecotoxicological studies (Burnett et al., 2007; Lister et al., 2011). Mummichog exhibit great physiological plasticity (Whitehead, 2010) and have been used in studies to examine physiological responses induced by natural environmental changes (e.g., changes in temperature, oxygen, salinity; Fangue et al., 2008; Scott et al., 2008; Rees et al., 2009). Due to the dynamic nature of estuaries, mummichog are exposed to a range of salinities and their isosmotic point is estimated around 9 ppt (Marshall et al., 1999; Wood and Grosell, 2009). As salinity fluctuates within estuaries, mummichog have different modes of osmoregulation. These different modes of osmoregulation can alter the potency of contaminants, which has been shown for metals (Hall and Anderson 1995; Wood et al. 2004; Blanchard and Grosell, 2005; Blewett et al., 2016) and polycyclic aromatic hydrocarbons (PAHs; Ramachandran et al., 2006; Shukla et al. 2007). To date, there is only a limited understanding on how salinity affects the actions of EDCs, including EE_2, on fish (Bosker et al., 2017). Saltwater teleosts osmotically lose water across the gills and actively drink to maintain osmotic balance, whereas in freshwater/low salinity conditions, fish are hyperosmotic to their environment and the gills are the main location of osmoregulation (Marshall and Grosell, 2006). Due to their euryhaline tolerances, mummichog are a model species providing the ability to test impacts of EDCs across a range of salinities.

Studies using the northern subspecies of mummichog (*F. h. macrolepidotus*) have resulted in standardized reproductive endocrine bioassays (MacLatchy et al., 2003) and adult fish reproductive tests (Peters et al., 2007; Bosker et al., 2010), which have been used to assess the impacts of complex effluents (e.g., pulp mill; Bosker et al., 2009), environmental contaminants (including EE_2; Bosker et al., 2016), or other model compounds (e.g., androgens; Glinka et al., 2015; Rutherford et al., 2015). A suite of relevant endpoints, spanning various levels of biological organization have been used to assess the effects of EE_2 on reproductive function in mummichog. This range of endpoints has been used to explore potential molecular or biochemical events that underlie tissue (e.g., changes in gonadal development) to whole organism-level (e.g., fecundity) impacts (MacLatchy et al., 2003; Sharpe et al., 2004; Greytak and Callard, 2007; Finley et al., 2009; Bosker et al., 2009; Melvin et al., 2009; Hogan et al., 2010).

However, while some similarities in reproductive responses exist with respect to other teleosts, such as vitellogenin induction in males (Bosker et al, 2016) and feminization of developing fish (Chehade, 2012), EE_2 effects on mummichog reproduction occur at much greater concentrations when compared to other fish species (Table 1). This is most apparent in studies which have shown that mummichog continue to spawn at concentrations of 100 ng/L (Table 2; Bosker et al., 2016). In other fish species, such as the Chinese rare minnow (*Gobiocypris rarus*; Zha et al., 2008), zebrafish (*Danio rerio*; Lin and Janz, 2006), or fathead minnow (*Pimephales promelas*; Armstrong et al., 2016), egg production is significantly reduced by exposure to $\leq$ 10 ng/L EE_2 (Table 2).

These results have highlighted that a range of responses of mummichog to EE_2 are species-specific, and given that mummichog are key model species for estuarine toxicology, the purpose of this review is to summarize the literature and compare the response of mummichog to other common teleost models. We will discuss the effects of EE_2 exposure on i) spawning and gonadal maturation, ii) reproductive steroids and vitellogenin levels, iii) expression of genes within the steroidogenic pathway, iv) sexual differentiation and gonadal development, and v) EE_2 uptake and metabolism. The role of salinity in the reproductive responses of mummichog to EE_2 is also included. Knowledge gaps and research needs are identified in regard to better understanding responses of mummichog exposed to EE_2.

2. Effects of EE_2 on reproductive endpoints

2.1. Spawning and gonadal maturation

In the wild, there are different environmental spawning cues for the northern (*Fundulus heteroclitus macrolepidotus)* and southern (*Fundulus heteroclitus heteroclitus*) subspecies of mummichog (McMullin et al., 2009). In the northern subspecies, increasing water temperature initiates gonadal recrudescence (indicated by increasing GSI), with warmest water temperatures coinciding with highest GSI measurements and a single peak of spawning activity (McMullin et al., 2009). Photoperiod is also correlated to spawning in the northern subspecies, with peak spawning activity occurring during long periods of daylight (McMullin et al., 2009). Conversely, the southern subspecies of mummichog spawn multiple times over the breeding season (Wallace and Selman, 1981), which is generally longer (Kneib, 1986). There is no indication of a lunar periodicity in the northern subspecies, unlike the southern subspecies where a semi-lunar pattern has been reported (Taylor et al., 1979; McMullin et al., 2009). Studies have found that the two subspecies of mummichog present with ovaries that are asynchronous as described in fish from the Northeastern U.S (Wallace and Selman, 1981) and as group-synchronous as found in more southern populations (Taylor and DiMichele, 1980; Hsiao et al., 1994). Mummichog found in Northeastern Canada show high variability in condition, liver size and gonad size throughout the reproductive season and within a population (McMullin et al., 2009).

Adult fish short-term reproductive tests are commonly used to assess the impacts of EDCs, including EE_2 on reproductive output (Bosker et al., 2017). One of the key endpoints assessed in these tests is fecundity (egg production/female/day), as this can be linked to potential population-level effects (Miller and Ankley, 2004; Gurney, 2006). Egg production is considered a sensitive endpoint; two review papers on short-term reproductive tests found it to be among the most sensitive endpoints across a variety of fish species (Dang et al., 2011; Bosker et al., 2017). Mummichog are an important model in estuarine toxicology because they can be manipulated to undergo gonadal recrudescence under laboratory conditions by controlling water temperature and photoperiod, allowing researchers to test this sensitive reproductive stage regardless of the season (Peters et al., 2007; Lister et al., 2011; Bosker et al., 2013).

Adult mummichog exposed to EE_2 show limited adverse effects on reproduction. An initial study that exposed mummichog statically to 22.1 ng/L for seven days showed a significant reduction in egg production (Peters et al., 2007); yet, subsequent studies found no impact of EE_2 after 28 days of exposure on egg production, fertilization, and hatching success despite very high levels up to 3000 ng EE_2/L (actual concentration not measured; data unpublished; Bosker et al., 2016). In addition, there was no effect on egg production in mummichog exposed to 100 ng EE_2/L (actual measured concentration 84.1 $\pm$ 6% of nominal) for 28 days, despite a significant upregulation of hepatic vitellogenin (*vtg1*) gene expression in males that is indicative of exposure to the synthetic estrogen (Bosker et al., 2016). It is unknown whether the different exposure regimes used in the studies contributed to the different reproductive responses observed. In Peters et al. (2007), the sexes were separated for the first 21 days of exposure and combined for the final seven days to assess fecundity. Mummichog do exhibit pairing behaviour between a male and female prior to spawning (Abraham, 1985) but it has not been investigated whether the exposure duration of seven days in Peters et al. (2007) was sufficient for pairing behaviour to develop or if pairing is obligatory. Most exposure studies combined sexes for the entire exposure period and incorporated study designs with greater replication that improved statistical power (Bosker et al., 2009).

Contrary to mummichog, reproduction of several other fish species is consistently affected by EE_2 exposure, even at low concentrations (Table 2). For example, egg production was stimulated in fathead

Table 1
Comparison of commonly quantified endpoints in fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*) during exposure to 17α-ethinylestradiol (EE_2). Endpoints include sex ratio, gonadosomatic indices (GSI), concentrations of circulating plasma testosterone (T), estradiol (E_2), or 11-ketotestosterone (11KT), gene expression analysis of estrogen receptor 1 (*er*), vitellogenin (*Vtg1*), steroidogenic acute regulatory protein (*star*), cytochrome P450 19a1 (*cyp19a1*) and cytochrome P450 17a1 (*cyp17a1*). Lowest observed effect concentration (LOEC) for each endpoint listed, regardless of sex. If endpoint was not changed (NC), then the highest tested concentration is presented. If an effect was only observed at lowest tested concentration, LOEC may be less than or equal to (≤) this concentration. Arrows represent direction of response as increase (↑) or decrease (↓) compared with control treatment.

Endpoint	Species	LOEC (ng/L)	Source Study	Endpoint	Species	LOEC (ng/L)	Source Study
Sex Ratio	Fathead Minnow	100% F, 4	Länge et al., 2001	*er*	Fathead Minnow	↑ 10	Filby et al., 2007
		60% F, 10	Aerle et al., 2002			↑ ≤ 15	Feswick et al., 2017
		> 85% F, 0.32	Parrott and Blunt 2005			↑ 50	Garcia-Reyero et al., 2018
	Zebrafish	86% F, 1	Örn et al., 2003		Zebrafish	↑ ≤ 5	Reyhanian et al., 2014
		80% F, 4	Luzio et al., 2016ab			↑ ≤ 0.4	Santos et al., 2017
		100% F, 5	Örn et al., 2016			↑ ≤ 5.62	Wang et al., 2019
	Mummichog	> 80% F, 100	Peters et al., 2010		Mummichog	↑ ≤ 67.9	Hogan et al. 2010
		> 80% F, 10	Chehade, 2012				
GSI	Fathead Minnow	↓10 (both sexes)	Filby et al., 2007	*Vtg1*	Fathead Minnow	↑ 5	Miracle et al., 2006
		↓≤ 10 (males)	Salierno and Kane, 2009			↑ ≤10	Salierno and Kane 2009
		↓25 (males)	Runnalls et al., 2015			↑ 1.54	Armstrong et al., 2016
	Zebrafish	↓0.029 nM (both sexes)	Van den Belt et al., 2004		Zebrafish	↑ ≤ 45	Michiels et al., 2018
		↓100 (females)	Hoffmann et al., 2006			↑ ≤ 90	Bertotto et al., 2019
		NC 3.92	Armstrong et al., 2016			↑ ≤ 5.62	Wang et al., 2019
		NC 5.62	Wang et al., 2019		Mummichog	↑ ≤ 10	MacLatchy et al., 2003
	Mummichog	↓247.6 (males)	Hogan et al. 2010			↑ ≤ 67.9	Hogan et al. 2010
		NC 250	Doyle et al., 2013			↑ ≤ 50	Chandra et al., 2012
		↑≤100 (males)	Bosker et al., 2016			↑ ≤100	Bosker et al., 2016
Plasma T	Fathead Minnow	↓5	Garcia-Reyero et al., 2009	*star*	Fathead Minnow	↓ 10	Filby et al., 2007
		↓≤10	Salierno and Kane, 2009			↓ 5	Leet et al., 2015
		↓≤10	Filby et al., 2012			↓ ≤ 25	Feswick et al., 2016
	Zebrafish	↓≤5	Nash et al., 2004		Zebrafish	↓ ≤ 100	Urbatzka et al., 2012
		↓ 40	Hoffmann et al., 2006			NC 5	Oikari, 2013
		↓ ≤ 10	Hua et al., 2016			↓ 416	Liang et al., 2017
		↓ ≤ 5	Zhuang et al., 2017		Mummichog	NC 50	Doyle et al., 2013
	Mummichog	↓ 500	MacLatchy et al., 2003			NC 100	Bosker et al., 2016
		↓ 100	Peters et al., 2007				
		↓ 250	Doyle et al., 2013	*cyp19a1*	Fathead Minnow	↓ 88	Hala et al., 2015
Plasma E2	Fathead Minnow	↓ ≤ 10	Salierno and Kane, 2009			↓ 5	Leet et al., 2015
		↓≤ 5.4	Martyniuk et al., 2010			↓ ≤ 25	Feswick et al., 2016
		↓ 1.54	Armstrong et al., 2016		Zebrafish	↓ ≤ 100	Urbatzka et al., 2012
	Zebrafish	↓ ≤10	Hua et al., 2016			↓ ≤ 25	Cosme et al., 2015
		↓ ≤ 5	Zhuang et al., 2017			↑ ≤ 41	Liang et al., 2017
		↓ ≤ 5.62	Wang et al., 2019		Mummichog	NC 50	Doyle et al., 2013
	Mummichog	↓ 500	MacLatchy et al., 2003			NC 100	Bosker et al., 2016
		NC 250	Doyle et al., 2013			NC 74	Kanagasabesan, 2018
		↓ 250	Gillio Meina et al., 2013	*cyp17a1*	Fathead Minnow	↓ 10	Filby et al., 2007
Plasma 11KT	Fathead Minnow	↓ ≤ 10	Salierno and Kane, 2009			↓ 5	Leet et al., 2015
		↓ 88	Hala et al., 2015			↓ ≤ 25	Feswick et al., 2016
		↓ 25	Runnalls et al., 2015		Zebrafish	↓ ≤ 10	Hua et al., 2016
	Zebrafish	↓ 10	Coe et al., 2008			↓ 1.6	Porseryd et al., 2018
		↓ ≤ 10	Hua et al., 2016			NC 5.62	Wang et al., 2019
		↓ ≤ 5	Zhuang et al., 2017		Mummichog		
	Mummichog	↓ 250	Gillio Meina et al., 2013				

minnow exposed to 0.1 ng EE_2/L (Pawlowski et al., 2004). The spawning frequency and the numbers of eggs spawned were decreased in the Chinese rare minnow after a 21-day exposure to 4 ng/L EE_2 (Zha et al., 2008). Zebrafish egg production was depressed after exposure to 1.2 ng EE_2/L for 80 days (Volkova et al., 2015).

A non-monotonic response in egg production has been reported for numerous fish species exposed to EE_2, including zebrafish (Parrott and Blunt, 2005), fathead minnow (Pawlowski et al., 2004) and Japanese medaka (*Oryzias latipes*; Tilton et al., 2005). In these studies, low concentrations of EE_2 (≤1ng/L) increased egg production in exposed females, while a higher concentration (≥3.5 ng/L) led to a complete cessation of egg production. This may indicate that EE_2 can act as both a stimulant and suppressant of egg production in certain species of fish (Overturf et al., 2015). The ability for a synthetic estrogen like EE_2 to stimulate follicular growth at low concentrations is not surprising given the well understood role of estrogens in vitellogenesis and ovarian growth. Studies have not explored low (e.g., < 10 ng/L) EE_2 concentration responses in mummichog sufficiently to compare their response with those of other species. Rather, greater focus has been placed on determining possible mechanisms underlying the lack of reproductive response, particularly their ability to continue to spawn under higher EE_2 exposure concentrations.

Currently, there is little understanding of potential differences in mummichog reproductive physiology that may underlie the ability of the fish to continue to spawn in the presence of high concentrations of exogenous EE_2. A possible explanation may involve the differences that exist in the biosynthesis of ovarian steroids over the reproductive cycle of mummichog compared with other common model teleosts. The granulosa cells in mummichog ovaries are the major site of steroid synthesis, producing 17α,20β-dihydroxy-4-prenen-3-one (17α,20β-P), testosterone (T), and 17β-estradiol (E_2); the thecal cells lack aromatase (CYP19A1) activity and secrete mainly T (Petrino et al., 1989a,b). In contrast, the presence of both granulosa and thecal cells are necessary for the gonadotropin-stimulated synthesis of these steroids in other common model teleosts (the “two cell model”; Nagahama and Yamashita, 2008). In this model, thecal cells synthesize the steroid precursors, which are then converted to17α, 20β-P and E_2 in the granulosa cells. A tenant of this model is that prior to ovulation of the

Table 2
Overview of studies investigating the impact of 17α-ethinylestradiol (EE_2) on egg production of commonly used small bodied fish species (fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*), sheepshead minnow (*Cyprindon variegatus*), Japanese medaka (*Oryzias latipes*), Chinese rare minnow (*Gobiocypris rarus*) and mummichog (*Fundulus heteroclitus*)). Study species and parameters are presented under the appropriate heading and all cited sources were waterborne exposures. Lowest observed effect concentration (LOEC) at which egg production ceased is reported in the LOEC egg production column. If this was the lowest tested concentration, LOEC may be less than or equal to this value (≤).

Species	Duration	Tested Concentrations	LOEC	Source Study
	(days)	(ng/L)	Egg Production	
Fathead Minnow	21	0.47, 1.54, 3.92	↓ ≤ 0.47 ng/L	Armstrong et al., 2016
	21	0.5, 2.5, 12.5, 25	↓ ≤ 0.5 ng/L	Runnalls et al., 2015
	21	0.1, 1, 3, 10, 100	↓ 10 ng/L	Pawlowski et al., 2004
	21	0.1, 1, 3, 10, 100	↓ 10 ng/L	Jobling et al., 2004
	150	0.32, 0.96, 3.5, 9.6, 23	↓ 3.5 ng/L	Parrott and Blunt 2005
Zebrafish	7	25	↓ ≤ 25 ng/L	Cosme et al., 2015
	21	10	↓ ≤ 10 ng/L	Hua et al., 2016
	21	5, 15	↓ 15 ng/L	Zhuang et al., 2017
	80	1.2, 1.6	↓ ≤ 1.2 ng/L	Volkova et al., 2015
Sheepshead Minnow	59	0.2, 2, 20, 200, 400, 800, 1600, 3200	↓ 20 ng/L	Zillioux et al., 2001
Japanese Medaka	14	0.2, 5, 500, 2000	↓ 500 ng/L	Tilton et al., 2005
	60	1, 10, 100	↓ 10 ng/L	Scholz and Gutzeit, 2000
	180	0.2, 2, 10	↓ 10 ng/L	Balch et al., 2004
Chinese Rare Minnow	21	4	↓ ≤ 4 ng/L	Zha et al., 2008
Mummichog	28	0.1, 1, 10, 22.1	↓ 22.1 ng/L	Peters et al., 2007
	28	100	No Effect	Bosker et al., 2016
	28	3000	No Effect	Bosker et al., data unpublished

oocyte, a dramatic shift in steroidogenesis occurs in which the production levels of E_2 give way to increased levels of 17α, 20β-P, which is necessary for the acquisition of maturational competence (reviewed in Senthilkumaran, et al., 2004; Nagahama and Yamashita, 2008).

Despite the fact that E_2 is inhibitory to oocyte maturation in other teleosts, Lin et al. (1987) proposed that in mummichog, high levels of E_2 co-exist with the surge in progestogens at a time when ovarian follicles are undergoing maturation. *In vitro* results in Lin et al. (1987) showed that all stages of developing follicles secreted high basal and gonadotropin-stimulated levels of E_2 (Lin et al., 1987). A study by Petrino et al. (1990) concluded that E_2 does not inhibit oocyte maturation, and Cerda et al. (1996) demonstrated that maturing follicles secrete high levels of E_2 in mummichog. More recent experiments by Kanagasabesan (2018) found considerable secretion of basal and hCG-stimulated E_2 by vitellogenic and mature follicles; significantly greater secretion of E_2 occurred within the mature follicles. The same experiments showed very low levels of 17α, 20β-P in the vitellogenic follicles but a dramatic increase by mature follicles. Combined, these studies, despite the differences in methodologies, support the idea that ovarian E_2 levels in mummichog tend to remain high throughout follicular development. In an *in vivo* experiment in which holding conditions were manipulated to encourage recrudescence and gonadal growth (by increasing water temperatures of stocks of mixed sex fish), levels of plasma E_2 increased significantly and steadily in female mummichog from early recrudescence through to maturation and ovulation (Kanagasabesan, 2018). In the same fish, plasma 17α,20β -P levels were low until a significant increase occurred in females in the late stage of maturation and peaked at ovulation (Kanagasabesan, 2018). Full grown follicles of mummichog undergo maturation in response to gonadotropins and 17α,20β -P (Lin et al., 1987) but the responsiveness of full grown follicles to these hormones is influenced by the follicular cycle (Cerda et al., 1996) and these events may occur in the presence of high endogenous E_2 levels. More comprehensive studies are needed to understand the apparent lack of inhibition of estrogens on maturational competence and ovulation of oocytes of mummichog. Given that E_2 levels have also not been correlated with the early phases of ovarian development (i.e., yolk vesicle formation) in mummichog (Shimizu, 1997, 2003, 2014), exogenous exposure to estrogens may not elicit the ovarian disruption in this species that it does in other fish.

In mummichog, the impact of EE_2 on gonadosomatic index (GSI; a measurement of gonad size relative to body weight) of male and female fish is variable and may be affected by environmental conditions (Gillio Meina et al., 2013). Several studies have found no effect of EE_2 on female GSI across a range (5–3000 ng EE_2/L) encompassing environmentally relevant and higher concentrations (Hogan et al., 2010; Doyle et al., 2013; Gillio Meina et al., 2013; Bosker et al., 2016; unpublished data). However, a study examining the reproductive impacts of differing salinity and temperature regimes in the presence or absence of EE_2 found that temperature-driven increases in gonadal growth were negatively affected in male fish exposed to 250 ng EE_2/L (Gillio Meina et al., 2013). Salinity and temperature increased gonad size in male mummichog, with males held in salt water (32 ppt) or at the elevated temperature of 26 °C having larger testes than males held in brackish (16 ppt) or fresh (0 ppt) water, or at cooler temperatures (8 °C or 10 °C; Gillio Meina et al., 2013). Exposure to 250 ng/L EE_2 at 26 °C negated the increase in male gonadal weight but the same EE_2 concentration at cooler water temperatures had no effect suggesting that the more active stage of testis growth was more sensitive to perturbation (Gillio Meina et al., 2013). Hogan et al. (2010) also found that male GSI was decreased in individuals exposed to 250 ng/L EE_2 for 14 days; conversely, males exposed to 100 ng/L EE_2 for 28 days had a small but significant increase in GSI (Bosker et al., 2016).

Variable effects of EE_2 on GSI in other fish species have also been reported. For example, male GSI in the livebearer *Jenynsia multidentata* was decreased when exposed to 75 or 100 ng/L EE_2 for 28 days; however, 100 ng/L did not impact female GSI over the same time period (Roggio et al., 2014). GSI was decreased in male fathead minnow exposed to 10 ng/L EE_2 (Salierno and Kane, 2009), but not at lower exposure levels of 4 ng/L EE_2 (Armstrong et al., 2016). There was no change in GSI of male or female roach (*Rutulis rutulis*) exposed to 0.3 ng/L EE_2 for 720 days (Tyler, 2009), nor any changes in GSI in Chinese rare minnow exposed to 4 ng/L EE_2 (Zha et al., 2008); yet, female rare minnow GSI was decreased after 28 days of exposure to 5 and 25 ng/L EE_2 (Zha et al., 2007). The extent of the impact of EE_2 exposure on zebrafish GSI in Van den Belt et al. (2002) was shown to be dependent on the duration of exposure and varied by sex where 10 ng EE_2/L caused a decreased in GSI of female and male fish after six and 24 days post exposure, respectively.

2.2. Reproductive steroids and vitellogenin levels

The measurement of reproductive steroid hormones is an important

physiological endpoint that is used to estimate the potential for aquatic contaminants to disrupt normal reproductive function in exposed fish. In part, this is due to the well characterized and critical roles that steroids (i.e., T in both males and females, E_2 in females and 11-keto-testosterone (11KT) in males) have in regulating gonadal growth and development, maturation, and reproductive behaviours that are key to successful spawning (Yaron and Sivan, 2006; Tokarz et al., 2015). Steroid production by gonadal tissues can also be examined using *in vitro* techniques to provide information on the biosynthetic capacity of these tissues (McMaster et al., 2001; Judson et al., 2014). Caution must be exercised when comparing hormone data across multiple laboratories, as calculation errors, methodology and assay types all influence the final reported concentrations of plasma hormones (Feswick et al., 2014). An interlaboratory comparison of E_2, T and 11KT levels in white sucker (*Catostomus commersoni*) reported up to a 14-fold difference in quantified hormone concentrations (ng/ml) among the eight participating laboratories (Feswick et al., 2014). While the final reported concentrations may vary across labs, detection of significant differences between control and treated samples has repeatedly been demonstrated (McMaster et al., 2001; Feswick et al., 2014).

Seasonal changes in reproductive hormone levels and gonadal growth have been characterized in mummichog over their reproductive cycle (e.g., Bradford and Taylor, 1987; Hsiao et al., 1996; Shimizu, 1997; McMullin et al., 2009). Plasma E_2 and T are closely correlated with gonadal recrudescence and maturation over the spawning season, increasing in concentration from early spring (beginning of gonadal recrudescence) and peaking in late summer (immediately before gonadal regression), indicating a key relationship between steroid mediation and gonadal development in later stages (Shimizu, 2003). After the cessation of breeding and subsequent gonadal regression, adult mummichog undergo early phases of gonadal recrudescence, such as development of basal spermatogenesis in males or cortical alveolus development in females, in early autumn (Bradford and Taylor, 1987; Shimizu, 2003, 2014). This is then followed by an over-winter 'refractory period', where gonadal recrudescence does not progress to further stages (Shimizu, 2003). During these two timeframes of gonadal recrudescence there is no correlation between plasma sex steroid levels and gonadal development in adult mummichog (Shimizu, 2003), indicating a potential area of insensitivity in mummichog exposed to exogenous estrogens.

Studies have examined the *in vitro* production of gonadal steroids after incubating tissues with EE_2 or after exposing the adult fish to EE_2 and then excising the gonadal tissues and examining the levels of steroids released to incubation media (Hogan et al., 2010; Peters et al., 2010; Gillio Meina et al., 2013; Kanagasabesan, 2018). *In vitro* experiments on excised gonadal tissue are a common approach to investigate the site of action of an EDC within the gonad. Sex steroids are all products of enzymatic conversion of cholesterol through a series of intermediates before release from the gonads (Arukwe, 2008). Therefore, gonadal tissues treated with stimulants or steroid precursors with or without toxicants followed by steroid measurements in the media provide information on which enzymatic step is affected by the toxicant. A series of experiments conducted by Hogan et al. (2010) demonstrated that *in vitro* production of T was reduced in testis incubations from males exposed to 70 or 250 ng/L EE_2 for 14 days. Basal and hCG-stimulated levels of T were reduced compared to controls indicating EE_2 affected the steroidogenic pathway at and downstream of cholesterol mobilization to P450 side-chain cleavage (P450scc) and/or P450scc conversion of cholesterol to pregnenolone. In the same experiment, T production by incubations of premature ovarian follicles was not significantly affected by the EE_2 treatments. Another *in vitro* mummichog study did not find an effect of EE_2 on basal or hCG-stimulated T or 17α,20β -P production by different stages of ovarian follicles (i.e., cortical alveoli, vitellogenic, early and late maturation) at concentrations of 14.8 or 74 ng EE_2/L (Kanagasabesan, 2018).

The effects of EE_2 on plasma (circulating) reproductive steroids in mummichog have been studied in MacLatchy et al. (2003), Peters et al. (2007), Doyle et al. (2013), and Gillio Meina et al. (2013). The results of these studies suggest that the impact of EE_2 on mummichog steroidogenesis may depend on both the concentration of EE_2 tested, as well as the stage of gonadal maturation of the exposed fish, because not all of the responses observed have been congruent. For example, adult mummichog exposed to high EE_2 concentrations (> 100 ng/L) for 7 to 28 days had depressed circulating steroid levels (E_2 in females and T or 11KT in males) and *in vitro* gonadal steroid synthesis in fish that were in spawning condition (MacLatchy et al., 2003; Peters et al., 2007). However, another experiment using adult mummichog found significantly depressed plasma levels of T, but not E_2 in fish exposed for 14 days to 50 or 250 ng EE_2/L (Doyle et al., 2013). Recrudescent female mummichog exposed to lower EE_2 concentrations (< 10 ng/L) had higher circulating and *in vitro* ovarian tissue production of E_2 but the opposite effect was found in females exposed to > 250 ng/L (MacLatchy et al., 2003). These studies highlight the importance of accounting for gonadal maturity of the fish and exposure concentration when interpreting results.

In both adult and life-cycle reproductive tests using mummichog, EE_2 elicits effects on reproductive steroid levels at concentrations greater than have been shown for other model species (e.g., fathead minnow, Japanese medaka, zebrafish), which tend to respond to more environmentally-relevant EE_2 concentrations (e.g.,0.1 to 10 ng/L) (Balch et al., 2004; Nash et al., 2004; Parrott and Blunt, 2005). According to Bosker et al. (2017) and with few exceptions (Peters et al., 2007), plasma T is a relatively insensitive endpoint for estrogenic exposure (Bosker et al., 2017). This is seen in studies using numerous fish species where plasma T was not impacted by EE_2 exposure (Garcia-Reyero et al., 2014; Hua et al., 2016). As reviewed in Bosker et al. (2017), lowest observed adverse effect concentrations for estrogenic compounds are approximately 70-fold lower in freshwater species compared with saltwater species. Comparisons of plasma sex hormone responses to other estuarine species are hampered by the current lack of studies analyzing these endpoints in species other than mummichog. In the Bosker et al. (2017) review, only mummichog studies conducted in saline environments analyzed plasma sex steroids as endpoints, with studies in sheepshead minnow (*Cyprinodon variegatus*; Folmar et al., 2000), sand goby (*Pomatoschistuc minutus*; Saaristo et al., 2009) and Indian medaka (*Oryzias melastigma*; Lee et al., 2014) not quantifying these endpoints. Salinity was not a factor that impacted steroid hormone responses in mummichog exposed to 50 or 250 ng/L EE_2 for 14 days (Gillio Meina et al., 2013); however, there is currently limited investigation into the role of salinity on natural hormone production and future studies shouldn't disregard salinity as a potential factor in EE_2 impact on reproductive steroid production.

The measurement of increased vitellogenin, an egg yolk precursor protein is used as an indication of estrogenic exposure in fish (Jones et al., 2000) and may involve quantification of hepatic mRNA expression or protein levels. In mummichog, the hepatic induction of vitellogenin by estrogens and its uptake from the plasma into the oocytes has been described (Selman and Wallace, 1983). Male mummichog exposed to 50 or 250 ng/L of EE_2 for 14 days had significant *vtg1* mRNA induction, while varying salinity had no additional impacts on expression (Chandra et al., 2012). Interestingly, male mummichog exposed to 250 ng/L EE_2 at 26 °C had a larger induction of *vtg1* compared with males exposed to the same concentration but held at the cooler temperature of 10 °C (Chandra et al., 2012), similar to differential responses observed in male GSI depending on water temperature. In male mummichog, the induction of vitellogenin by EE_2 appears to be a consistent response occurring at EE_2 concentrations of 10 ng/L and greater (MacLatchy et al., 2003; Hogan et al., 2010; Bosker et al., 2016). In other species, lower EE_2 exposure concentrations have been found to increase vitellogen production. For comparison, hepatic vitellogenin was induced in male fathead minnow exposed to 1.54 ng/L EE_2 for 21 days (Armstrong et al., 2016), in male zebrafish exposed to

2 ng/L EE_2 in a 40-day exposure (Örn et al., 2016), and in male Atlantic salmon (*Salmo salar*) exposed to 11.86 ng EE_2 for 21 days (Breves et al., 2018) via waterborne exposures.

2.3. Impacts of EE_2 on steroidogenic gene expression

Profiling gene expression after exposure to EDCs has the potential to identify the mechanistic basis for higher-level effects, such as depressed plasma sex hormones (Filby et al., 2007), formation of intersex condition (Örn et al., 2016), tissue remodeling in liver (Hultman et al., 2015), or even behavioural changes (Bhandari et al., 2015). In most fish, different estrogens (including EE_2), induce similar gene expression profiles (Moens et al., 2006), which can be detected within 24–48 h post-fertilization (Callard et al., 2001). These profiles are distinct from gene expression profiles elicited from chemicals with other modes of action (Moggs et al., 2004). By investigating impacts of EE_2 on mRNA expression of key steroidogenic enzymes, possible mechanisms of action can be elucidated and may correlate to depressed plasma hormone levels commonly seen in EE_2 exposed fish (Hogan et al., 2010; Doyle et al., 2013; Zhou, 2015; Hua et al., 2016).

The aromatization of androgens to estrogens are facilitated by the cytochrome P450 genes *cyp19a1* and *cyp19b1* (Mouriec et al., 2009). These two isoforms are expressed primarily in the gonad (*cyp19a1*) and brain (*cyp19b1*) of teleosts (Chang et al., 2005) and play key roles in regulating the ratio of androgens to estrogens (Trant et al., 2001). Alteration of aromatase expression can disrupt the rate of estrogen production, impacting local and peripheral levels of estrogens within teleosts (Cheshenko et al., 2008), and *cyp19a1* is commonly used as a biomarker for estrogenic exposure (Urbatzka et al., 2012). Expression patterns of *cyp19a1* are altered after exposure to EE_2 in zebrafish (Urbatzka et al., 2012; Cosme et al., 2015; Liang et al., 2017), roach (Nikoleris et al., 2016), yellow perch (Lynn et al., 2008), rare minnow (Wang et al., 2010) and fathead minnow (Filby et al., 2007; Leet et al., 2015; Feswick et al., 2016). A downregulation of *cyp19a1* correlates to depressed synthesis of endogenous estradiol (Villeneuve, 2016), which is a commonly observed endpoint in EE_2 exposure in fish (Gillio Meina et al., 2013; Wang et al., 2019). This depression of E_2 synthesis may retard follicle growth in developing fish (Cosme et al., 2015). The response of *cyp19a1* to EE_2 exposure is variable and impacted by exposure dose and duration, life stage of fish, and species (Johns et al., 2009; Trubiroha et al., 2012; Nikoleris et al., 2016), but the correlation between transcript levels of this gene and circulating plasma E_2 levels are well established in the literature (Perkins et al., 2015; Muth-Köhne et al., 2016).

In mummichog there is limited impact of EE_2 on *cyp19a1* expression. Doyle et al. (2013) found no changes in expression of *cyp19a1* in individuals exposed to 50 or 250 ng/L EE_2 for 14 days. Similarly, mummichog exposed to 100 ng/L EE_2 for 28 days had no change in *cyp19a1* expression (Bosker et al., 2016). A study investigating the role of temperature and salinity on the effects of EE_2 on mummichog reproduction found no change in *cyp19a1* expression in fish exposed to 50 ng/L EE_2 for 14 days (Gillio Meina et al., 2013). Results from an *in vitro* study exposing separated ovarian follicles, grouped by stage, to a range (14–74 ng/L) of EE_2 also showed no effect on transcript levels of *cyp19a1* at any follicular stage (Kanagasabesan, 2018). In follicles stimulated with hCG and exposed to EE_2 there was no response in *cyp19a1*, while hCG treated follicles without EE_2 induced *cyp19a1* expression (Kanagasabesan, 2018).

The lack of response in *cyp19a1* expression in mummichog is not well understood. The strong correlation between *cyp19a1* levels and E_2 production established in other model teleosts is not apparent in mummichog, though plasma E_2 depression may still be observed in EE_2 exposed mummichog (Greytak et al., 2005; Peters et al., 2007; Doyle et al., 2013; Gillio Meina et al., 2013). As *cyp19a1* is highly conserved between species (Cheshenko et al. 2008), this anomalous response in mummichog is currently unexplained. Several transcriptional elements, including an estrogen response element in *cyp19b1*, the steroidogenic factor 1/adrenal 4 binding protein responsive element and dioxin-responsive elements have been identified in *cyp19* genes (Chang et al., 2005; Kanda et al., 2006; Wong et al., 2006). These promoters are conserved among teleost species and may interact with EDCs and impact transcription of *cyp19* genes (Cheshenko et al., 2008). Whether these transcription factors are present on mummichog *cyp19* genes is currently unknown. Investigation into the presence or absence of these specific regions may account for the unresponsiveness of mummichog *cyp19a1* expression during EE_2 exposure.

Other genes within the steroidogenic pathway of model teleosts that are responsive to EE_2 exposure include cytochrome P450 17a1 (*cyp17;* produces androstenedione), 17β-hydroxysteroid dehydrogenase (*17βhsd;* catalyzes conversion of androstenedione to testosterone) and luteinizing hormone (*lh;* Hua et al., 2016). These genes are downregulated in zebrafish (Hua et al., 2016) and fathead minnow (Filby et al., 2007) exposed to EE_2. Both *cyp17* and *17βhsd* are responsible for androgen synthesis (Filby et al., 2007) and the resulting downregulation may account for depressed circulating levels of plasma sex hormones in exposed fish. There is currently no work in mummichog investigating the impacts of EE_2 exposure on either *cyp17* or *17βhsd*. A current limitation is the lack of studies analyzing a suite of steroidogenic genes, instead mainly relying on gonadal *cyp19* and hepatic *vtg* as biomarkers for estrogenic exposure.

Another family of transcription factors, the estrogen receptor (ER) family, may account for species-specific responses seen during estrogenic exposure (Ankley et al., 2016). The role of ERs in estrogenic signalling is well studied in the literature (Filby and Tyler, 2005; Tarrant et al., 2006). Briefly, estrogen receptors are ligand inducible transcription factors belonging to a superfamily of nuclear receptors (Nelson and Habibi, 2013) capable of activating estrogen response elements in promoter regions of genes (Nilsson and Gustafsson, 2010). Three isoforms of ER's have been identified in mummichog (ERα, ERβ and ERγ) (Greytak and Callard, 2007), similar to other common model fish species (Filby and Tyler, 2005; Nagler et al., 2007; Mu et al., 2013). Mummichog ERα shares over 80% homology with Japanese medaka ERα and is sensitive to estrogenic perturbation when exposed to E_2 (Urushitani et al., 2003). EE_2 can induce hepatic ERα mRNA expression in mummichog (Hogan et al., 2010), and this gene has been classified as estrogen-responsive in fathead minnow (Filby et al., 2007). ERα plays a role in ovarian growth and oocyte development (Filby et al., 2007) and disruption of this gene has been linked to detrimental biological effects in roach populations exposed during development (Nikoleris et al., 2016). Tissue-specific responses in *erα* expression has been found in fathead minnow exposed to EE_2, with hepatic upregulation but ovarian downregulation in exposed fish (Filby et al., 2007). Tissue-specific expression of estrogenic effects can be impacted by interaction of ERα and other ER isoforms (Ankley et al., 2016), which may account for tissue-specific responses seen during estrogenic exposure. The impact of EE_2 exposure on *erα* in mummichog has not currently been investigated in the gonad. Hepatic *erα* expression was increased in male mummichog exposed to 70 ng EE_2 for 14 days (Hogan et al., 2010), but egg production was not an endpoint in that study. The response (if any) of gonadal *erα* to EE_2 exposure may correlate with oocyte development. Well-designed studies examining oocyte development under different *erα* expression patterns will provide the baseline for EE_2 exposure impacts on this gene. The potential interaction between EE_2 and *erα* may provide novel insight into the mummichog's ability to continuously produce eggs during EE_2 exposure.

2.4. Feminization during development by EE_2 exposure

There are two pathways for gonadal differentiation in gonochoristic (i.e., unisex) fish (Strüssmann and Nakamura, 2002). One pathway, as shown in mummichog, the gonads differentiate directly from primordial germ cells into either ovarian or testis tissue (Shimizu et al.,

2002; Urushitani et al., 2002). The second pathway, as exemplified by zebrafish, has an ovarian-like gonad developed in all individuals, followed by sex reversal in select individuals leading to testis formation (Örn et al., 2003; Santos et al., 2017). In general, the process of sexual differentiation in fish is governed by multiple factors, both intrinsic and environmental, and is subjected to perturbation by exogenous sex steroids if administered at the time of sex determination (Devlin and Nagahama, 2002; Strüssmann and Nakamura, 2002). Exposure of gonochoristic fish species to estrogenic EDCs during development can result in sex reversal and skewed sex ratios, which may impact population recruitment in fish (Baroiller and D'Cotta, 2016). Disruptions to gonadal development in fish exposed to estrogenic EDCs, including EE_2, have been widely reported (Bhandari et al., 2015). The feminization process observed in EE_2 exposed juvenile fish is observed both morphologically (differentiation of the ovary in exposed individuals) and molecularly (*cyp19a1*, 11βhsd and other target genes; Pérez et al., 2012, Depiereux et al., 2015).

Female-skewed sex ratios have been found in zebrafish and fathead minnow exposed to environmentally-relevant concentrations of EE_2 (5 ng/L or less; Fenske et al., 2005; Leet et al., 2015; Luzio et al., 2016a,b; Örn et al., 2016; Parrott and Wood, 2002). Exposure of larval fish to estrogenic compounds during the sensitive stage of sexual development can result in all female, or female-skewed, sex ratios, while exposure to the same concentrations of EE_2 at later, adult life stages result in development of female secondary sexual characteristics, but rarely full sex reversal (Santos et al., 2017). Simultaneous exposure of zebrafish to 5 ng/L EE_2 and 50 ng/L 17β-trenbolone, a potent androgen that causes male-skewed sex ratios, found female-biased sex ratios (Örn et al., 2016), demonstrating the potency of EE_2. EE_2 can accelerate ovarian differentiation in fish species, as seen in 46% of larval pejerrey (*Odontesthes bonariensis*) fed 0.1 μg/g EE_2 having differentiated ovaries after six weeks exposure, compared to only 26% of control fish having similarly differentiated ovarian tissue (Pérez et al., 2012).

The impact of EE_2 on gonadal differentiation in mummichog is similar to other model teleosts. Under laboratory conditions suitable for promoting ovarian maturation and spawning (20 °C and 16 light: 8 dark cycle), morphological sex differentiation in mummichog occurs around two weeks post hatch (wph), with full maturity attained by 36 wph (Shimizu et al., 2008). EE_2 accelerates ovarian differentiation in developing mummichog, as seen in larval mummichog exposed to increasing concentrations of EE_2 (10, 50, 250 ng/L) having faster ovarian development than control fish (Chehade, 2012). As early as 1 wph, 60% of larvae had differentiated ovaries when exposed to 50 ng/L EE_2 (Chehade, 2012), and at 10 wph all treatments had female-skewed sex ratios (Chehade, 2012). Similarly, Peters et al. (2010) observed skewed sex ratios in favour of females (> 80% female phenotype) in mummichog exposed to 100 ng/L EE_2 for 52 wph, with some individuals expressing female secondary sex characteristics (such as dull colorization) despite being gonadally male. There was no evidence of inter-sex upon histological examination (Peters et al., 2010). As EE_2 mimics E_2, its presence prior to gonadal differentiation promotes oocyte development by binding to ERs and initiating a positive-feedback loop that increases the number of ERs on germ cells, which disrupts normal levels of gene expression involved in sexual differentiation (Leet et al., 2011).

While specific genes are impacted by EE_2 exposure, the detailed mechanisms underlying teleost sex reversal is not well characterized (Santos et al., 2017). There are several mechanisms which may explain sex reversal in developing fish, including the change in CYP19 enzyme efficiencies (Villeneuve, 2016), cortisol pathway impacts on gonad differentiation (Nozu and Nakamura, 2015) or impacts on pituitary gonadotropins which regulate gametogenesis (Zhao et al., 2015; Cao et al., 2016). The ability of EE_2 to impact expression of luteinizing hormone (*lh)* in zebrafish was found after exposure to 10 ng/L for 21 days (Hua et al., 2016). This decrease in *lh* expression was linked to decreased E_2 synthesis from the gonadal tissue (Hua et al., 2016).

While the specific mechanism(s) of sex reversal in juvenile teleosts may not be currently known, EE_2 has a demonstrable effect on genes associated with sex determination in fish. Sex-determining genes such as double-sex and mab3 related transcription factor 1 (*dmrt1*), sex determining region Y-box 9b (*sox9*), antimullerian hormone (*amh*) and forkhead box protein L2 (*foxl2*) are sensitive to estrogenic exposure in developing fish (Filby et al., 2007; Bhandari et al., 2015; Leet et al., 2015; Muth-Köhne et al., 2016). The *foxl2* gene regulates transcription rates of *cyp19a1* and controls circulating E2 levels. Increased *foxl2* expression was found in testes of fathead minnow exposed to 5 ng/L EE_2 for 96 h (Feswick et al., 2016), possibly causing ovarian development in genetic males due to increased levels of circulating E_2 which prevents female pathway repression (Santi et al., 2019). Conversely, male-associated genes such as *dmrt1, amh* and *sox9* exhibit masculinizing effects on gonadal tissue through proliferation of androgen synthesis (Poonlaphdecha et al., 2013; Diaz and Piferrer, 2015; Lee et al., 2017). In the presence of estrogenic EDCs these male-associated genes are typically downregulated, as evidenced in a study where all three genes were downregulated in male zebrafish exposed to 25 ng/L EE_2 in a waterborne, 14-day exposure (Reyhanian et al., 2014). A similar study, on a much shorter timescale (96 h) showed the rapidity with which EE_2 can downregulate these molecular networks associated with male sex differentiation in fathead minnow (Feswick et al., 2016). This decrease in transcript abundance, and subsequent loss of androgen synthesis capacity, may limit the ability of testes to develop in individuals exposed to EE_2. The current roles, if any, that the aforementioned genes play in mummichog sexual reversal during EE_2 exposure have not yet been investigated.

2.5. EE_2 uptake and metabolism

Investigations have characterized the absorption, distribution, metabolism and excretion (ADME) of EE_2 in tissues of exposed mummichog to provide insight into the sensitivity (or lack of) of the fish to EE_2. The amount of toxicant that is able to access and disrupt critical biological processes will ultimately determine toxicological impact and understanding the ADME of the toxicant could help to explain species specific differences in responses.

The utility of the gill as an uptake route for toxicants such as EE_2 is conferred by its large membrane surface area, small diffusive distances, and high rates of blood perfusion. These are factors that make gill ideal for fundamental transport processes such as gas exchange, ion regulation, and nitrogenous waste excretion. Lipophilic contaminants such as EE_2 (octanol water partition coefficient of 4.12; Yamamoto et al., 2003) are able to cross the lamellar sieve, diffuse across the lipid-rich gill membrane and readily enter the blood of teleost fish (Blewett et al., 2013a,b; Blewett et al., 2014). The diffusion of EE_2 across the branchial epithelium occurs transcellularly in a process similar to that for oxygen (Murphy and Murphy, 1971; Hunn and Allen, 1974; Satchell, 1984; Brauner et al., 1994; Yang and Randall, 1995; Yang et al., 2000). Mummichog exposed to EE_2 show rapid distribution from the gill to other tissues, where it accumulates to higher concentrations in gut, liver, gallbladder, and carcass (Blewett et al., 2013a,b; Blewett et al., 2014). The gall bladder and liver of mummichog account for > 50% of the total EE_2 accumulation after two hours in the reference condition (Blewett et al., 2013a,b). When these organs were separated, the gall bladder accounted for the larger concentration of EE_2, likely indicating very rapid processing of EE_2 into the bile. Biliary secretion likely provides an explanation for the appearance of EE_2 in the gut. Metabolism of EE_2 eventually results in the accumulation of metabolites (and/or potentially EE_2 itself) in the gall bladder where they are incorporated into the bile (Förlin et al., 1995; Blom et al., 2000).

From the perspective of tissue distribution, a study of six fish species (goldfish (*Carassius auratus*), Japanese medaka, rainbow trout (*Oncorhynchus mykiss*), zebrafish, fathead minnow and mummichog) showed few differences in total EE_2 accumulation among species (Blewett et al., 2014). However, tissue-specific distribution was

significantly different. Japanese medaka and mummichog showed relatively high accumulation in the liver and gallbladder, perhaps indicative of more efficient processing of the toxicant. This could explain the relative lack of sensitivity of mummichog to EE_2. In the other species, the carcass (consisting of adipose tissues, bone, kidney, and remaining tissues which were not excised) accumulated higher relative concentrations of EE_2, suggesting a greater capacity of EE_2 to associate with sensitive tissues, and thus to exert toxicological impact (Blewett et al., 2014). Few studies have examined the uptake and distribution of EE_2 in other fish but the work of Gibson et al. (2005) also demonstrated EE_2 accumulation from wastewater effluent exposures in rainbow trout and roach (*Rutilus rutilus*) bile and accumulation of EE_2 in the gonads of the sexually mature roach.

Lipid distribution also differs among fish species, which may influence the accumulation of EE_2 as it is strongly lipophilic and readily associates with fat-rich tissues. Previous research has shown that EE_2 concentrations in shorthead redhorse suckers (*Moxostoma macrolepidotum*) were correlated with total body lipid content (Al-Ansari et al., 2010). This also highlights the importance of biological factors such as the reproductive cycle, which influences fat deposition (Jørgensen et al., 1997; Ekman et al., 2009; Rasheed, 2011) and may affect EE_2 toxicity.

Excretion rates of organic toxicant metabolites also vary among species (Tyler et al., 2005), again illustrating the importance of an understanding of ADME in interpreting the effects of EE_2 on mummichog relative to other fish. As not all of these areas have been probed in mummichog in comparison to other species, the extent of their contribution to EE_2 sensitivity is not currently known.

It is also worth noting that organismal and environmental factors that alter the gill microenvironment play a significant role in determining the fate and effect of EE_2. For example, temperature influences metabolic rate and the demand for oxygen (Hazel and Prosser, 1974) and if ventilation rate increases, increased water flow over the gill via increased buccal pumping, elevated heart rate, and enhanced lamellar perfusion rates by arterial dilation occurs (Davis, 1972; Evans et al., 2005). While these phenomena aid oxygen uptake/delivery, they also increase the amount of EE_2 contacting the uptake surface and increase EE_2 absorption. This was demonstrated in mummichog in Blewett et al. (2013a,b) when EE_2 uptake increased two-fold with a temperature increase from 4 to 26 °C, while a 20-fold increase in EE_2 uptake was measured in exercising versus resting fish. Salinity is another key factor, and one of particular importance for mummichog, which inhabit waters that may vary significantly in salt content. While salinity can alter metabolic rate (Urbina and Glover, 2015), it also fundamentally changes the gill morphology of euryhaline fish, including the mummichog (Copeland, 1950; Evans et al., 2005; Laurent et al., 2006). A three-fold increase in EE_2 uptake rate was found in mummichog exposed in 16 ppt water compared to fish exposed in either 100% salt water (32 ppt) or fresh water (Blewett et al., 2013a). These significant differences were attributed to the development of apical pores in intermediate salinities, which act to decrease diffusion distance across the gill epithelium (Laurent and Dunel, 1980; Laurent, 1984), and thus facilitate EE_2 uptake. In fresh water, cuboidal cells (a cell type that are unique to killifish), and at higher salinities, chloride cells, result in diffusion distances that are greater than those in brackish waters (Wood and Marshall, 1994; Patrick et al., 1997; Patrick and Wood, 1999; Wood and Laurent, 2003), and lead to a comparatively lower EE_2 absorption rate.

An important factor shaping EE_2 toxicity is metabolism. Lipophilic xenobiotics are metabolized by hepatic enzymes. The biotransformation of xenobiotics such as EE_2 involves the functionalization of the xenobiotic (e.g. Phase I) and its eventual conjugation to increase water solubility (Phase II), and thus facilitate excretion. Although the specific pathways by which EE_2 is metabolized in mummichog are unknown, studies in rainbow trout have shown that EE_2 exposure leads to changes in expression of Phase I enzymes such as cytochrome P450 1A, and Phase II pathways such as those mediated by UDP-glucuronosyltransferase and glutathione S-transferase (Hultman et al., 2015). These are all entities which have been shown to be impacted by environmental factors such as salinity and temperature, and thus it is likely that metabolism of EE_2 in mummichog, like absorption, will depend significantly on the physicochemical environment. Examination of EE_2 metabolites in bile of male bream (*Abramis brama*) has identified glucuronic acid and sulfate conjugates (Houtman et al., 2004), lending support to the importance of Phase II metabolism in fish. These compounds may be transformed back into the parent compound once expelled into the intestine, a process aided by bacterial modifications (Bodzek and Dudziak, 2006; Fenlon et al., 2010). However, the specific routes of excretion of EE_2 and its metabolites (i.e. via urine or bile) and the efficiency of excretory pathways, have not been specifically examined in mummichog.

3. Considerations for EE_2 exposure conditions to impact fish responses

Experimental duration (Segner, 2011), temperature (Finley et al., 2009), fish life-stage (Seki et al., 2005) and salinity (Gillio Meina et al., 2013) all impact endpoint responses examined in fish in ecotoxicology studies and researchers must be cognizant of these before making direct comparisons among species. Duration of exposure is a key factor in elucidating impacts on reproductive endpoints during exposure to EE_2 (Segner, 2011). Endpoints differentially impacted by variable exposure durations include development of gonadal intersex (Alan et al., 2008), fecundity (Cripe et al., 2010), gene expression (Cavallin et al., 2016), and fertility (Morthorst et al., 2010). The reversibility of impacts on certain endpoints may also depend on the exposure duration and whether a period of depuration is experienced post-exposure (Villeneuve et al., 2009). Typically, a long-term exposure will elicit responses at lower concentrations than a short-term exposure to higher concentrations of the same chemical (Augspurger et al., 2008). As the mechanism(s) of action of a specific toxicant is dependent on a host of factors, including life-stage, duration of exposure and concentration tested (Dang, 2014), a longer exposure may be required to establish a more stable response in exposed fish (Schroeder et al., 2017). Acute testing (96 h for most toxicity tests done with fish) regimes may not account for compensatory mechanisms elicited during chronic exposures (Cavallin et al., 2016). The exact exposure duration for testing cessation of egg production after EE_2 exposure will vary based on individual species spawning habits (Henriksen et al., 2016) and gonadal development times (Jackson et al., 2019), with most studies running for two to three weeks (Table 2). As EE_2 is a potent estrogen receptor ligand (Legler et al., 2002), the resulting activation of this receptor would need to be of sufficient length to activate specific sequences of events resulting in adverse effects at the level of egg production (Groh et al., 2015).

A short-term reproductive endocrine adult mummichog bioassay has been developed and modified for testing EDC effects on plasma sex steroids, egg production and fertilization success, among other endpoints (MacLatchy et al., 2003; Bosker et al., 2009). This testing regime typically exposes mummichog for 14–28 days and quantifies endpoints at treatment cessation. Other model teleosts, such as zebrafish and fathead minnow, are typically exposed to EE_2 for 14–21 days (Doyle et al., 2013; Reyhanian et al., 2014;Armstrong et al., 2015 ; Leet et al., 2015; DeCourten et al., 2019), the same duration as most mummichog studies (Peters et al., 2007; Hogan et al., 2010; Chandra et al., 2012; Bosker et al., 2016). As reviewed in Petrino et al. (1989), mummichog have asynchronous ovaries, with follicles of all stages present. This allows continuous ovulation into the ovarian cavity throughout the breeding season (Lister et al., 2011), resulting in daily spawning activities (Shimizu, 2003). Zebrafish also possess asynchronous ovaries, with each follicle requiring 10 days for a complete cycle of maturation from Stage I (primary growth) to Stage V (mature egg; Wang and Ge,

2004; Clelland and Peng 2009). Thus, a 21 day exposure duration is a sufficient length to test the ability of EE_2 to impact ovulation rate, as daily spawning events would cease within one to two days due to the prevention of follicle maturation from EE_2.

As discussed above (section 2.2), the main factor inducing gonadal recrudescence in northern mummichog is water temperature (Finley et al., 2009; McMullin et al., 2009). Temperature may impact the rate at which gonadal recrudescence occurs (Santos et al., 2017) and can rapidly alter the reproductive stage of adult fish (Baroiller et al., 2009), which could alter sensitivity of certain endpoints within a study. Both GSI (Gillio Meina et al., 2013) and *vtg1* induction (Chandra et al., 2012) have shown temperature-sensitive responses in mummichog exposed to EE_2. Temperature also alters gonadal development and sex differentiation in zebrafish exposed to EE_2 (Luzio et al., 2016a,b). Zebrafish raised at three distinct temperatures (23, 28 or 33 °C) were exposed to 4 ng/L EE_2 for 60 days post-hatch, with EE_2 increasing gonadal maturation regardless of temperature. However, male fish reared at 33 °C had delayed gonad development, with some individuals still not having fully differentiated gonads at the end of the exposure (Luzio et al., 2016a,b). While a majority of studies examined are conducted at or above room temperature (20–25 °C), the underlying biochemical and physiological processes occurring in a specific species at a specific temperature must be completely understood before comparison to other model teleosts.

Life-stage at exposure is a key parameter in investigating endocrine disrupting compounds and their impact on reproductive endpoints (Kidd et al., 2007). As reviewed in Söffker and Tyler (2012), age of fish during exposure to EE_2 was the strongest variable affecting quantified endpoints. Adults and juveniles respond differently to identical concentrations of EE_2 (Seki et al., 2005), demonstrating that life-stage directly influences reproductive endpoints. In addition to age, stage of gonadal recrudescence is a factor in susceptibility to EE_2 exposure (Liney et al., 2005). Exposure of adult mummichog to EE_2 during the refractory period of gonadal recrudescence may indicate insensitivity to EE_2 exposure due to the relative inactiveness of gonadal growth at this time, even though mummichog have been shown to experience impacts during other stages of gonadal recrudescence (MacLatchy et al., 2003; Hogan et al., 2010; Gillio Meina et al., 2013). Comparisons of responses among model teleosts should account for gonadal stage, not just age, to better identify potential states of insensitivity within the normal cycle of gonadal recrudescence.

The effects of salinity on reproductive endpoints in mummichog exposed to EE_2 have been discussed previously in this review. Euryhaline mummichog offer a unique advantage compared to most other stenohaline model teleosts when testing the impact of salinity in concert with endocrine-active compounds (Burnett et al., 2007). While salinity itself may only play a minor role in mitigating effects of EE_2 on mummichog (such as through the increased buffering ability of seawater; Jury et al., 2013), future research should focus on the physiological changes mummichog employ to tolerate a broad range of salinities, which may have a role in ameliorating exposure to exogenous estrogens.

As a final note, it cannot be ignored that among-laboratory EE_2 exposure concentrations are subject to variability and may affect conclusions based on reported EE_2 levels. Those studies reporting measured (rather than nominal) amounts of EE_2 are more robust in relation to actual exposure concentrations. Similarly, those studies using flow-through waterborne methods and constant EE_2 delivery (Bosker et al., 2016), in comparison to static waterborne exposures with occasional water changes and EE2 delivery (Peters et al., 2007), result in more homogenous and reliable EE_2 concentrations throughout the exposure period. Methods of measuring waterborne EE_2 exposure concentrations include, e.g., radioimmunoassay (Parrott and Blunt, 2005), enzyme-linked immunosorbent assay (Martyniuk et al., 2010), liquid chromatography-mass spectrometry (MS)/MS (Peters et al., 2007; Bosker et al., 2016), and gas chromatography/MS (Armstrong et al., 2016). Detection limits vary for different methodologies and laboratories and need to be taken into consideration when interpreting exposure levels; most studies report a 1–10 ng/L detection limit (Parrott and Blunt, 2005; Bosker et al., 2016).

4. Summary

The continued ability of mummichog to produce eggs while exposed to high concentrations of EE_2 has not been observed in other model fish species. The specific reason(s) mummichog are able to successfully spawn during exposure to exogenous estrogens are not currently known, but may be due to naturally-high levels of local E_2 during ovarian development and maturation. Generally, egg production (fecundity) and reproductive hormone levels of mummichog are not significantly impacted by low exposure concentrations of EE_2 (< 10 ng/L), unlike responses observed by other model fish species (Bosker et al., 2017). While variations in salinity require physiological adaptations, which could alter fish susceptibility to EE_2, results to date in the mummichog do not implicate salinity as a factor involved in their tolerance to exogenous estrogens despite the influence of salinity on EE_2 uptake. Differences in absorption, distribution, metabolism and excretion of EE_2 may account for discrepancies between mummichog and other common teleost models; however; more research is needed in mummichog before their contribution to EE_2 sensitivity can be known. Investigations into the potential differences in estrogen receptor distribution, or apparent lack of steroidogenic gene expression perturbation in response to EE_2 exposure, may reveal species-specific anomalies that account for mummichog insensitivity to EE_2. Once these investigations are better understood, the mummichog will offer a unique model to undertake resistance studies to exogenous estrogens.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113378.

References

Abraham, B.J., 1985. Species profiles: life histories and environmental requirements of coastal fishes and invertebrates – mummichog and striped killifish. U.S. Fish Wildlife Services Biol. Rep. 82, 11–40.

Aerle, R.V.A.N., Pounds, N., Hutchinson, T.O.M.H., Maddix, S.U.E., Tyler, C.R., 2002. Window of Sensitivity for the Estrogenic Effects of Ethinylestradiol in Early Life-Stages of Fathead Minnow , Pimephales promelas 44.

Al-Ansari, A.M., Saleem, A., Kimpe, L.E., Sherry, J.P., McMaster, M.E., Trudeau, V.L., Blais, J.M., 2010. Bioaccumulation of the pharmaceutical 17α-ethinylestradiol in shorthead redhorse suckers (Moxostoma macrolepidotum) from the St. Clair River. Canada. Environ. Pollut. 158, 2566–2571. https://doi.org/10.1016/j.envpol.2010.05.020.

Alan, M.V., Barber, L.B., Gray, J.L., Lopez, E.M., Woodling, J.D., Norris, D.O., 2008. Reproductive disruption in fish downstream from an estrogenic wastewater effluent. Environ. Sci. Technol. 42, 3407–3414. https://doi.org/10.1021/es0720661.

Ankley, G.T., LaLone, C.A., Gray, L.E., Villeneuve, D.L., Hornung, M.W., 2016. Evaluation of the scientific underpinnings for identifying estrogenic chemicals in nonmammalian taxa using mammalian test systems. Environ. Toxicol. Chem. 35, 2806–2816. https://doi.org/10.1002/etc.3456.

Aris, A.Z., Shamsuddin, A.S., Praveena, S.M., 2014. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: A review. Environ. Int. doi: 10.1016/j.envint.2014.04.011.

Armstrong, B.M., Lazorchak, J.M., Jensen, K.M., Haring, H.J., Smith, M.E., Flick, R.W., Bencic, D.C., Biales, A.D., 2016. Reproductive effects in fathead minnows (Pimphales promelas) following a 21 d exposure to 17α-ethinylestradiol. Chemosphere 144, 366–373. https://doi.org/10.1016/j.chemosphere.2015.08.078.

Armstrong, B.M., Lazorchak, J.M., Murphy, C.A., Haring, H.J., Jensen, K.M., Smith, M.E., 2015. and ammonia on fathead minnow (Pimephales promelas) reproduction. Chemosphere 120, 108–114. https://doi.org/10.1016/j.chemosphere.2014.06.049.

Arukwe, A., 2008. Steroidogenic acute regulatory (StAR) protein and cholesterol side-chain cleavage (P450scc)-regulated steroidogenesis as an organ-specific molecular and cellular target for endocrine disrupting chemicals in fish. Cell Biol. Toxicol. 24, 527–540. https://doi.org/10.1007/s10565-008-9069-7.

Augspurger, T., Neves, R.J., Bringolf, R.B., Hammer, E., Dwyer, F.J., Wang, N., Buchwalter, D.B., Cope, W.G., Newton, T.J., Ingersoll, C.G., Barnhart, M.C., 2008. Differential exposure, duration, and sensitivity of unionoidean bivalve life stages to environmental contaminants. J. North Am. Benthol. Soc. 27, 451–462. https://doi.org/10.1899/07-094.1.

Balch, G.C., Mackenzie, C. a, Metcalfe, C.D., 2004. Alterations to gonadal development and reproductive success in Japanese medaka (Oryzias latipes) exposed to 17alpha-ethinylestradiol. Environ. Toxicol. Chem. 23, 782–791. doi: 10.1897/02-539.

Baroiller, J.F., D'Cotta, H., Saillant, E., 2009. Environmental effects on fish sex determination and differentiation. Sex Dev. 3, 118–135.

Baroiller, J.F., D'Cotta, H., 2016. The reversible sex of gonochoristic fish: insights and consequences. Sex. Dev. 10 (5), 242–266.

Bertotto, L.B., Dasgupta, S., Vliet, S., Dudley, S., Gan, J., Volz, D.C., Schlenk, D., 2019. Science of the Total Environment Evaluation of the estrogen receptor alpha as a possible target of bifenthrin effects in the estrogenic and dopaminergic signaling pathways in zebra fish embryos. Sci. Total Environ. 651, 2424–2431. https://doi.org/10.1016/j.scitotenv.2018.10.079.

Bhandari, R.K., Deem, S.L., Holliday, D.K., Jandegian, C.M., Kassotis, C.D., Nagel, S.C., Tillitt, D.E., vom Saal, F.S., Rosenfeld, C.S., 2015. Effects of the environmental estrogenic contaminants bisphenol A and 17α-ethinyl estradiol on sexual development and adult behaviors in aquatic wildlife species. Gen. Comp. Endocrinol. doi: 10.1016/j.ygcen.2014.09.014.

Blanchard, J., Grosell, M., 2005. Effects of salinity on copper accumulation in the common killifish (Fundulus heteroclitus). Environ. Toxicol. Chem. 24, 1403–1413. https://doi.org/10.1897/04-373r.1.

Blewett, T.A., Robertson, L.M., MacLatchy, D.L., Wood, C.M., 2013a. Impact of environmental oxygen, exercise, salinity, and metabolic rate on the uptake and tissue-specific distribution of 17-α-ethynylestradiol in the euryhaline teleost Fundulus heteroclitus. Aquat. Toxicol. 138–139, 43–51. https://doi.org/10.1016/j.aquatox.2013.04.006.

Blewett, T., MacLatchy, D.L., Wood, C.M., 2013b. The effects of temperature and salinity on 17-α-ethynylestradiol uptake and its relationship to oxygen consumption in the model euryhaline teleost (Fundulus heteroclitus). Aquat. Toxicol. 127, 61–71. https://doi.org/10.1016/j.aquatox.2012.04.009.

Blewett, T.A., Chow, T.L., Maclatchy, D.L., Wood, C.M., 2014. A species comparison of 17-α-ethynylestradiol uptake and tissue-specific distribution in six teleost fish. Comp. Biochem. Physiol. – C Toxicol. Pharmacol. 161, 33–40. https://doi.org/10.1016/j.cbpc.2014.01.004.

Blewett, T.A., Ransberry, V.E., McClelland, G.B., Wood, C.M., 2016. Investigating the mechanisms of Ni uptake and sub-lethal toxicity in the Atlantic killifish Fundulus heteroclitus in relation to salinity. Environ. Pollut. 211, 370–381. https://doi.org/10.1016/j.envpol.2016.01.002.

Blom, S., Andersson, T.B., Förlin, L., 2000. Effects of food deprivation and handling stress on head kidney 17α- hydroxyprogesterone 21-hydroxylase activity, plasma cortisol and the activities of liver detoxification enzymes in rainbow trout. Aquat. Toxicol. 48, 265–274. https://doi.org/10.1016/S0166-445X(99)00031-4.

Bodzek, M., Dudziak, M., 2006. Elimination of steroidal sex hormones by conventional water treatment processes. Desalination 198, 24–32.

Bosker, T., Hewitt, L.M., Munkittrick, K.R., Melvin, S.D., MacLatchy, D.L., 2009. The effects of final treated effluent and in-mill waste streams from a Canadian thermo-mechanical pulp and paper mill on mummichog (Fundulus heteroclitus) reproduction. Water Qual. Res. J. Canada 44, 333–344.

Bosker, T., Hewitt, L.M., Munkittrick, K.R., MacLatchy, D.L., 2010. Validation of a refined short-term adult fish reproductive test with improved power for mummichog (Fundulus heteroclitus) to test complex effluents. Ecotoxicol. Environ. Saf. 73, 1596–1601. https://doi.org/10.1016/j.ecoenv.2010.07.037.

Bosker, T., Munkittrick, K., Nacci, D., MacLatchy, D.L., 2013. Laboratory spawning patterns of mummichogs, Fundulus heteroclitus (Cyprinodontiformes: Fundulidae). Copeia 213, 527–538.

Bosker, T., Munkittrick, K.R., Lister, A., Maclatchy, D.L., 2016. Mummichog (Fundulus heteroclitus) continue to successfully produce eggs after exposure to high levels of 17α-ethinylestradiol. Environ. Toxicol. Chem. 35, 1107–1112. https://doi.org/10.1002/etc.3239.

Bosker, T., Santoro, G., Melvin, S.D., 2017. Salinity and sensitivity to endocrine disrupting chemicals: A comparison of reproductive endpoints in small-bodied fish exposed under different salinities. Chemosphere 183, 186–196. https://doi.org/10.1016/j.chemosphere.2017.05.063.

Bradford, C., Taylor, M.H., 1987. Semilunar changes in estradiol and cortisol coincident with gonadal maturation and spawning in the killifish Fundulus heteroclitus. Gen. Comp. Endocrinol. 66, 71–78. https://doi.org/10.1016/0016-6480(87)90351-0.

Brauner, C.J., Newman, J.F., Thurston, R.V., Randall, D.J., 1994. The effect of exposure to 1,2,4,5-tetrachlorobenzene and the relationship between toxicant and oxygen-uptake in rainbow trout (Oncorhyncus mykiss) during exercise. Environ. Toxicol. Chem. 13, 1813–1820.

Breves, J.P., Duffy, T.A., Einarsdottir, I.E., Björnsson, B.T., McCormick, S.D., 2018. In vivo effects of 17A-ethinylestradiol, 17B-estradiol and 4-nonylphenol on insulin-like growth-factor binding proteins (igfbps) in Atlantic salmon. Aquat. Toxicol. 203, 28–39. https://doi.org/10.1016/j.aquatox.2018.07.018.

Burnett, K.G., Bain, L.J., Baldwin, W.S., Callard, G. V., Cohen, S., Di Giulio, R.T., Evans, D. H., Gómez-Chiarri, M., Hahn, M.E., Hoover, C.A., Karchner, S.I., Katoh, F., MacLatchy, D.L., Marshall, W.S., Meyer, J.N., Nacci, D.E., Oleksiak, M.F., Rees, B.B., Singer, T.D., Stegeman, J.J., Towle, D.W., Van Veld, P.A., Vogelbein, W.K., Whitehead, A., Winn, R.N., Crawford, D.L., 2007. Fundulus as the premier teleost model in environmental biology: Opportunities for new insights using genomics. Comp. Biochem. Physiol. - Part D Genomics Proteomics. doi:10.1016/j.cbd.2007.09.001.

Callard, G.V., Tchoudakova, a V., Kishida, M., Wood, E., 2001. Differential tissue distribution, developmental programming, estrogen regulation and promoter characteristics of cyp19 genes in teleost fish. J. Steroid Biochem. Mol. Biol. 79, 305–314.

Cao, F., Zhu, L., Li, H., Yu, S., Wang, C., Qiu, L., 2016. Reproductive toxicity of azoxystrobin to adult zebrafish (Danio rerio). Environ. Pollut. 219, 1109–1121.

Cavallin, J.E., Jensen, K.M., Kahl, M.D., Villeneuve, D.L., Lee, K.E., Schroeder, A.L., Mayasich, J., Eid, E.P., Nelson, K.R., Milsk, R.Y., Blackwell, B.R., Berninger, J.P., Lalone, C.A., Blanksma, C., Jicha, T., Elonen, C., Johnson, R., Ankley, G.T., 2016. Pathway-based approaches for assessment of real-time exposure to an estrogenic wastewater treatment plant effluent on fathead minnow reproduction. Environ. Toxicol. Chem. 35, 702–716. https://doi.org/10.1002/etc.3228.

Cerda, J., Calman, B.G., LaFleur, G.J., Limesand, S., 1996. Pattern of vitellogenesis and follicle maturational competence during the ovarian follicular cycle of *Fundulus heteroclitus*. Gen. Comp. Endocrinol. 103, 24–35.

Chandra, K., Bosker, T., Hogan, N., Lister, A., MacLatchy, D., Currie, S., 2012. Sustained high temperature increases the vitellogenin response to 17a-ethynylestradiol in mummichog (Fundulus heteroclitus). Aquat. Toxicol. 118–119, 130–140. https://doi.org/10.1016/j.aquatox.2012.04.004.

Chang, X., Kobayashi, T., Senthilkumaran, B., Kobayashi-Kajura, H., Sudhakumari, C.C., Nagahama, Y., 2005. Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (Oreochromis niloticus). Gen. Comp. Endocrinol. 141, 101–115.

Chehade, I. The effects of 17a-ethynylestradiol on gonadal development and differentiation in the estuarine killifish, Fundulus heteroclitus, 2012. Theses and Dissertations (Comprehensive). 836. http://scholars.wlu.ca/etd/836.

Cheshenko, K., Pakdel, F., Segner, H., Kah, O., Eggen, R.I.L., 2008. Interference of endocrine disrupting chemicals with aromatase CYP19 expression or activity, and consequences for reproduction of teleost fish. Gen. Comp. Endocrinol. 155 (1), 31–62.

Clelland, E., Peng, C., 2009. Endocrine/paracrine control of zebrafish ovarian development. Mol. Cell. Endocrinol. 312, 42–52. https://doi.org/10.1016/j.mce.2009.04.009.

Coe, T.S., Hamilton, P.B., Hodgson, D., Paull, G.C., Stevens, J.R., Sumner, K., Tyler, C.R., 2008. An environmental estrogen alters reproductive hierarchies, disrupting sexual selection in group-spawning fish. Environ. Sci. Technol. 42, 5020–5025. https://doi.org/10.1021/es800277q.

Copeland, D.C., 1950. Adaptive behaviour of the chloride cell in the gill of Fundulus heteroclitus. J. Morphol. 82, 369–379.

Cosme, M.M., Lister, A.L., Van Der Kraak, G., 2015. Inhibition of spawning in zebrafish (Danio rerio): adverse outcome pathways of quinacrine and ethinylestradiol. Gen. Comp. Endocrinol. 219, 89–101. https://doi.org/10.1016/j.ygcen.2015.01.013.

Cripe, G.M., Hemmer, B.L., Raimondo, S., Goodman, L.R., Kulaw, D.H., 2010. Exposure of three generations of the estuarine sheepshead minnow (Cyprinodon variegatus) to the androgen, 17β-trenbolone: effects on survival, development, and reproduction. Environ. Toxicol. Chem. 29, 2079–2087. https://doi.org/10.1002/etc.261.

Dang, Z.C., Li, K., Yin, H.W., Hakkert, B., Vermeire, T., 2011. Endpoint sensitivity in fish endocrine disruption assays: Regulatory implications. Toxicol. Lett. 202, 36–46. https://doi.org/10.1016/j.toxlet.2011.01.016.

Dang, Z., 2014. Fish biomarkers for regulatory identification of endocrine disrupting chemicals. Environ. Pollut. 185, 266–270. https://doi.org/10.1016/j.envpol.2013.11.006.

Davis, J.C., 1972. An infared photographic technique useful for studying vacularization of fish gills. J. Fish. Res. Bd. Can. 29, 109–111.

DeCourten, B.M., Connon, R.E., Brander, S.M., 2019. Direct and indirect parental exposure to endocrine disruptors and elevated temperature influences gene expression across generations in a euryhaline model fish. e6156. PeerJ 7. https://doi.org/10.7717/peerj.6156.

Depiereux, S., Le Gac, F., De Meulder, B., Pierre, M., Helaers, R., et al., 2015. Meta-analysis of microarray data of rainbow trout fry gonad differentia- tion modulated by ethynylestradiol. PLoS One 10, e0135799.

Devlin, R.H., Nagahama, Y., 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture. https://doi.org/10.1016/S0044-8486(02)00057-1.

Diaz, N., Piferrer, F., 2015. Lasting effects of early exposure to temperature on the gonadal trascriptome at the time of sex differentiation in the European sea bass, a fish with mixed genetic and environmental sex determination. BMC Genom. 16 (1), 679.

Doyle, M.A., Bosker, T., Martyniuk, C.J., Maclatchy, D.L., Munkittrick, K.R., 2013. The effects of 17a-ethinylestradiol (EE2) on molecular signaling cascades in mummichog (Fundulus heteroclitus). Aquat. Toxicol. 134–135, 34–46. https://doi.org/10.1016/j.aquatox.2013.03.001.

Ekman, D.R., Teng, Q., Villeneuve, D.L., Kahl, M.D., Jensen, K.M., Durhan, E.J., Ankley, G.T., Collette, T.W., 2009. Profiling lipid metabolites yields unique information on sexand time-dependent response of fathead minnow (Pimephales promelas) exposed to 17α- ethynylestradiol. Metabolomics 5, 22–32.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177. https://doi.org/10.1152/physrev.00050.2003.

Fangue, N. a, Mandic, M., Richards, J.G., Schulte, P.M., 2008. Swimming performance

and energetics as a function of temperature in killifish Fundulus heteroclitus. Physiol. Biochem. Zool. 81, 389–401. doi:10.1086/589109.
Fenlon, K.A., Johnson, C.A., Tyler, C.R., Hill, M.E., 2010. Gas-liquid chromotography–tandem mass spectrometry methodology for the quantitation of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents and from wild populations. J. Chromatography A 1217, 112–118.
Fenske, M., Maack, G., Schäfers, C., Segner, H., 2005. An environmentally relevant concentration of estrogen induces arrest of male gonad development in zebrafish, Danio rerio. Environ. Toxicol. Chem. 24, 1088. https://doi.org/10.1897/04-096R1.1.
Feswick, A., Ankley, G.T., Denslow, N., Ellestad, L.E., Fuzzen, M., Jensen, K.M., Kroll, K., Lister, A., Maclatchy, D.L., Mcmaster, M.E., Orlando, E.F., Servos, M.R., Tetreault, G.R., Van Den Heuvel, M.R., Munkittrick, K.R., 2014. An inter-laboratory study on the variability in measured concentrations of 17β-estradiol, testosterone, and 11-ketotestosterone in white sucker: Implications and recommendations. Environ. Toxicol. Chem. 33, 847–857. https://doi.org/10.1002/etc.2503.
Feswick, A., Loughery, J.R., Isaacs, M.A., Munkittrick, K.R., Martyniuk, C.J., 2016. Molecular initiating events of the intersex phenotype : Low-dose exposure to 17β-ethinylestradiol rapidly regulates molecular networks associated with gonad differentiation in the adult fathead minnow testis. Aquat. Toxicol. 181, 46–56. https://doi.org/10.1016/j.aquatox.2016.10.021.
Feswick, A., Munkittrick, K.R., Martyniuk, C.J., 2017. Estrogen-responsive gene networks in the teleost liver: What are the key molecular indicators? Environ. Toxicol. Pharmacol. 56, 366–374. https://doi.org/10.1016/j.etap.2017.10.012.
Filby, A.L., Tyler, C.R., 2005. Molecular characterization of estrogen receptors 1, 2a, and 2b and their tissue and ontogenic expression profiles in fathead minnow (Pimephales promelas)1. Biol. Reprod. 73, 648–662. https://doi.org/10.1095/biolreprod.105.039701.
Filby, A.L., Thorpe, K.L., Maack, G., Tyler, C.R., 2007. Gene expression profiles revealing the mechanisms of anti-androgen- and estrogen-induced feminization in fish. Aquat. Toxicol. 81, 219–231. https://doi.org/10.1016/j.aquatox.2006.12.003.
Filby, A.L., Paull, G.C., Searle, F., Ortiz-Zarragoitia, M., Tyler, C.R., 2012. Environmental estrogen-induced alterations of male aggression and dominance hierarchies in fish: A mechanistic analysis. Environ. Sci. Technol. 46, 3472–3479. https://doi.org/10.1021/es204023d.
Finley, M.A., Courtenay, S.C., Teather, K.L., van den Heuvel, M.R., 2009. Assessment of northern mummichog (Fundulus heteroclitus macrolepidotus) as an estuarine pollution monitoring species. Water Qual. Res. J. Canada 44, 323–332.
Folmar, L.C., Hemmer, M., Hemmer, R., Bowman, C., Kroll, K., Denslow, N.D., 2000. Comparative estrogenicity of estradiol, ethynyl estradiol and diethylstilbestrol in an in vivo, male sheepshead minnow (Cyprinodon variegatus), vitellogenin bioassay. Aquat. Toxicol. 49, 77e88.
Förlin, L., Lemaire, P., Livingstone, D.R., 1995. Comparative studies of hepatic xenobiotic metabolizing and antioxidant enzymes in different fish species. Mar. Environ. Res. 39, 201–204. https://doi.org/10.1016/0141-1136(94)00030-S.
Garcia-Reyero, N., Villeneuve, D.L., Kroll, K.J., Liu, L., Orlando, E.F., Watanabe, K.H., Sepulveda, M.S., Ankley, G.T., Denslow, N.D., 2009. Expression signatures for a model androgen and antiandrogen in the fathead minnow (Pimephales promelas) ovary. Environ. Sci. Technol. 43, 2614–2619. https://doi.org/10.1021/es8024484.
Garcia-Reyero, N., Ekman, D.R., Habib, T., Villeneuve, D.L., Collette, T.W., Bencic, D.C., Ankley, G.T., Perkins, E.J., 2014. Integrated approach to explore the mechanisms of aromatase inhibition and recovery in fathead minnows (Pimephales promelas). Gen. Comp. Endocrinol. 203, 193–202. https://doi.org/10.1016/j.ygcen.2014.03.022.
Garcia-Reyero, N., Jayasinghe, B.S., Kroll, K.J., Sabo-Attwood, T., Denslow, N.D., 2018. Estrogen signaling through both membrane and nuclear receptors in the liver of fathead minnow. Gen. Comp. Endocrinol. 257, 50–66. https://doi.org/10.1016/j.ygcen.2017.07.019.
Gibson, R., Smith, M., Spary, C., Tyler, C., Hill, E., 2005. Mixtures of estrogenic contaminants in bile of fish exposed to wastewater treatment works effluents. Environ. Sci. Technol. 39 (8), 2461-2471. doi: 10.1210/en.2008-0384.
Gillio Meina, E., Lister, A., Bosker, T., Servos, M., Munkittrick, K., MacLatchy, D., 2013. Effects of 17a-ethinylestradiol (EE2) on reproductive endocrine status in mummichog (Fundulus heteroclitus) under differing salinity and temperature conditions. Aquat. Toxicol. 134–135, 92–103. https://doi.org/10.1016/j.aquatox.2013.03.014.
Glinka, C.O., Frasca, S., Provatas, A.A., Lama, T., DeGuise, S., Bosker, T., 2015. The effects of model androgen 5α-dihydrotestosterone on mummichog (Fundulus heteroclitus) reproduction under different salinities. Aquat. Toxicol. 165, 266–276. https://doi.org/10.1016/j.aquatox.2015.05.019.
Greytak, S.R., Champlin, D., Callard, G.V., 2005. Isolation and characterization of two cytochrome P450 aromatase forms in killifish (Fundulus heteroclitus): differential expression in fish from polluted and unpolluted environments. Aquat. Toxicol. 71, 371–389.
Greytak, S.R., Callard, G.V., 2007. Cloning of three estrogen receptors (ER) from killifish (Fundulus heteroclitus): Differences in populations from polluted and reference environments. Gen. Comp. Endocrinol. 150, 174–188. https://doi.org/10.1016/j.ygcen.2006.07.017.
Groh, K.J., Carvalho, R.N., Chipman, J.K., Denslow, N.D., Halder, M., Murphy, C.A., Roelofs, D., Rolaki, A., Schirmer, K., Watanabe, K.H., 2015. Development and application of the adverse outcome pathway framework for understanding and predicting chronic toxicity: I. Challenges and research needs in ecotoxicology. Chemosphere 120, 764–777. https://doi.org/10.1016/j.chemosphere.2014.09.068.
Gurney, W.S.C., 2006. Modeling the demographic effects of endocrine disruptors. Environ. Health Perspect. 114, 122–126. https://doi.org/10.1289/ehp.8064.
Hala, D., Petersen, L.H., Martinović, D., Huggett, D.B., 2015. In Silico analysis of perturbed steroidogenesis and gonad growth in fathead minnows (P. promelas) exposed to 17α-ethynylestradiol. Syst. Biol. Reprod. Med. 61, 122–138. https://doi.org/10.3109/19396368.2015.1035817.
Hall, L.W., Anderson, R.D., 1995. The influence of salinity on the toxicity of various classes of chemicals to aquatic biota. crit. Rev. Toxicol. 25, 281–346. https://doi.org/10.3109/10408449509021613.
Hazel, R.J., Prosser, C.L., 1974. Molecular mechanisms of temperature compensation in poikilotherms. Physiol. Rev. 54, 620–688.
Henriksen, P., Beedholm, K., Baatrup, E., 2016. Differences in reproductive behavior between spawning and non-spawning Zebrafish pairs and the effects of 17α-ethinylestradiol (EE2). Toxics 4, 22. https://doi.org/10.3390/toxics4030022.
Hoffmann, J.L., Torontali, S.P., Thomason, R.G., Lee, D.M., Brill, J.L., Price, B.B., Carr, G.J., Versteeg, D.J., 2006. Hepatic gene expression profiling using Genechips in zebrafish exposed to 17 alpha-ethynylestradiol. Aquat. Toxicol. 79, 233–246. https://doi.org/10.1016/j.aquatox.2006.06.009.
Hogan, N.S., Currie, S., LeBlanc, S., Hewitt, L.M., MacLatchy, D.L., 2010. Modulation of steroidogenesis and estrogen signalling in the estuarine killifish (Fundulus heteroclitus) exposed to ethinylestradiol. Aquat. Toxicol. 98, 148–156. https://doi.org/10.1016/j.aquatox.2010.02.002.
Houtman, C.J., Brouwer, A., Lamoree, M.H., 2004. Identification of Estrogenic Compounds in Fish Bile Using Bioassay-Directed Fractionation 38, 6415–6423.
Hsiao, S.M., Greeley Jr., M.S., Wallace, R.A., 1994. Reproductive cycling in female *Fundulus heteroclitus*. Biol. Bull. 186, 271–284.
Hsiao, S.M., Limesand, S.W., Wallace, R.A., 1996. Semilunar follicular cycle of an intertidal fish: the Fundulus model. Biol. Reprod. 54, 809–818.
Hua, J., Han, J., Wang, X., Guo, Y., Zhou, B., 2016. The binary mixtures of megestrol acetate and 17α-ethynylestradiol adversely affect zebrafish reproduction. Environ. Pollut. 213, 776–784. https://doi.org/10.1016/j.envpol.2016.03.031.
Hultman, M.T., Song, Y., Tollefsen, K.E., 2015. 17α-Ethinylestradiol (EE2) effect on global gene expression in primary rainbow trout (Oncorhynchus mykiss) hepatocytes. Aquat. Toxicol. 169, 90–104. https://doi.org/10.1016/j.aquatox.2015.10.004.
Hunn, J.B., Allen, J.L., 1974. Movement of drugs across the gills of fishes. Ann. Rev. Pharmacol. 14, 1–27.
Jackson, L., Felgenhauer, B., Klerks, P., 2019. Feminization, altered gonadal development, and liver damage in least killifish exposed to sublethal concentrations of 17α-ethinylestradiol. Ecotoxicol. Environ. Saf. 170, 331–337.
Jobling, S., Casey, D., Rogers-Gray, T., Oehlmann, J., Schulte-Oehlmann, U., Pawlowski, S., Baunbeck, T., Turner, A.P., Tyler, C.R., 2004. Comparative responses of molluscs and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. 66, 207–222. https://doi.org/10.1016/j.aquatox.2004.01.002.
Johns, S.M., Denslow, N.D., Kane, M.D., Watanabe, K.H., Orlando, E.F., Sepúlveda, M.S., 2009. Effects of estrogens and antiestrogens on gene expression of fathead minnow (Pimephales promelas) early lifestages. Environ. Toxicol. 28, 873–880. https://doi.org/10.1002/tox.20545.
Johnson, A.C., Dumont, E., Williams, R.J., Oldenkamp, R., Cisowska, I., Sumpter, J.P., 2013. Do concentrations of ethinylestradiol, estradiol, and diclofenac in European rivers exceed proposed EU environmental quality standards? Environ. Sci. Technol. 47, 12297–12304. https://doi.org/10.1021/es4030035.
Jones, P.D., De Coen, W.M., Tremblay, L., Giesy, J.P., 2000. Vitellogenin as a biomarker for environmental estrogens. Water Sci. Technol. 42, 1–14.
Jørgensen, E.H., Johansen, S.J.S., Jobling, M., 1997. Seasonal patterns of growth, lipid deposition and lipid depletion in anadromous Arctic charr. J. Fish Biol. 51, 312–326.
Judson, R., Houck, K., Martin, M., Knudsen, T., Thomas, R.S., Sipes, N., Shah, I., Wambaugh, J., Crofton, K., 2014. *In Vitro* and modelling approaches to risk assessment from the U.S. environmental protection agency toxcast programme. Basic Clin. Pharmacol. Toxicol. 115, 69–76. https://doi.org/10.1111/bcpt.12239.
Jury, C.P., Thomas, F.I.M., Atkinson, M.J., Toonen, R.J., 2013. Buffer capacity, ecosystem feedbacks, and seawater chemistry under global change. Water (Switzerland) 5, 1303–1325. https://doi.org/10.3390/w5031303.
Kanagasabesan, T. Molecular Characterization of the Mummichog (Fundulus heteroclitus) Ovarian Steroidogenic Pathway and Implications for Exogenous Estrogen Effects during Follicular Development. 2018. Theses and Dissertations (Comprehensive). Submitted Dec 1, 2017. Wilfrid Laurier University. pp. 130.
Kanda, H., Okubo, T., Omori, N., Niihara, H., Matsumoto, N., Yamada, K., Yoshimoto, S., Ito, M., Yamashita, S., Shiba, T., Takamatsu, N., 2006. Transcriptional regulation of the rainbow trout CYP19a gene by FTZ-F1 homologue. J. Steroid Biochem. Mol. Biol. 99, 85–92.
Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104, 8897–8901. https://doi.org/10.1073/pnas.0609568104.
Kneib, R.T., 1986. Size specific patterns in the reproductive cycle of the killifish, *Fundulus heteroclitus* (Pisces: Fundulidae) from Sapelo Island, Georgia. Copeia 1986, 342–351.
Laurent, P., Dunel, S., 1980. Morphology of gill epithelia in fish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 238, R147–R159.
Länge, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., Sumpter, J.P., 2001. Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 20, 1216–1227. https://doi.org/10.1002/etc.5620200610.
Laurent, P., 1984. Gill internal morphology. In: In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology Vol. 10A. Academic Press, Orlando, pp. 73–183.
Laurent, P., Chevalier, C., Wood, C.M., 2006. Appearance of cuboidal cells in relation to salinity In gills of Fundulus heteroclitus, a species exhibiting branchial Na+ but not Cl- uptake in freshwater. Cell Tiss. Res. 325, 481–492.
Lee, P.Y., Lin, C.Y., Chen, T.H., 2014. Environmentally relevant exposure of 17alphaethinylestradiol impairs spawning and reproductive behavior in the brackish medaka Oryzias melastigma. Mar. Pollut. Bull. 85, 338e343.
Lee, S.L.J., Horsfield, J.A., Black, M.A., Rutherford, K., Fisher, A., Gemmell, N.J., 2017. Histological and transcriptomic effects of 17α-methyltestosterone on zebrafish gonad development. BMC Genom. 18, 1–19. https://doi.org/10.1186/s12864-017-3915-z.

Leet, J.K., Gall, H.E., Sepúlveda, M.S., 2011. A review of studies on androgen and estrogen exposure in fish early life stages: Effects on gene and hormonal control of sexual differentiation. J. Appl. Toxicol. 31, 379–398. https://doi.org/10.1002/jat.1682.

Leet, J.K., Sassman, S., Amberg, J.J., Olmstead, A.W., Lee, L.S., Ankley, G.T., Sepulveda, M.S., 2015. Environmental hormones and their impacts on sex differentiation in fathead minnows. Aquat. Toxicol. 158, 98–107. https://doi.org/10.1016/j.aquatox.2014.10.022.

Legler, J., Zeinstra, L.M,, Schuitemaker, F., Lanser, P.H., Bogerd, J., Brouwer, A., Vethaak, A.D,, De Voogt, P., Murk, A.J., Van der Burg, B., 2002. Comparison of in vivo and in vitro reporter gene assays for short-term screening of estrogenicactivity. Environ. Sci. Technol. 36, 4410–4415.

Liang, Y.Q., Huang, G.Y., Zhao, J.L., Shi, W.J., Hu, L.X., Tian, F., Liu, S.S., Jiang, Y.X., Ying, G.G., 2017. Transcriptional alterations induced by binary mixtures of ethinylestradiol and norgestrel during the early development of zebrafish (Danio rerio). Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 195, 60–67. https://doi.org/10.1016/j.cbpc.2017.02.005.

Lin, Y.W.P., Lamarca, M.J., Wallace, R.A., 1987. Fundulus heteroclitus gonadotropin(s). I. Homologous bioassay using oocyte maturation and steroid production by isolated ovarian follicles. Gen. Comp. Endocrinol. 67, 126–141. https://doi.org/10.1016/0016-6480(87)90212-7.

Lin, L.L., Janz, D.M., 2006. Effects of binary mixtures of xenoestrogens on gonadal development and reproduction in zebrafish. Aquat. Toxicol. 80, 382–395. https://doi.org/10.1016/j.aquatox.2006.10.004.

Liney, K.E., Jobling, S., Shears, J.A., Simpson, P., Tyler, C.R., 2005. Assessing the sensitivity of different life stages for sexual disruption in roach (*Rutilus rutilus*) exposed to effluents from wastewater treatment works. Environ Health Perspect 113, 1299–1307.

Lister, A.L., Van Der Kraak, G.J., Rutherford, R., MacLatchy, D., 2011. Fundulus heteroclitus: ovarian reproductive physiology and the impact of environmental contaminants. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 154, 278–287. https://doi.org/10.1016/j.cbpc.2011.07.004.

Luzio, A., Monteiro, S.M., Rocha, E., Fontaínhas-Fernandes, A.A., Coimbra, A.M., 2016a. Development and recovery of histopathological alterations in the gonads of zebrafish (Danio rerio) after single and combined exposure to endocrine disruptors (17α-ethinylestradiol and fadrozole). Aquat. Toxicol. 175, 90–105. https://doi.org/10.1016/j.aquatox.2016.03.014.

Luzio, A., Santos, D., Fontaínhas-fernandes, A.A., Monteiro, S.M., Coimbra, A.M., 2016b. Effects of 17alpha-ethinylestradiol at different water temperatures on zebrafish sex differentiation and gonad development. Aquat. Toxicol. 174, 22–35. https://doi.org/10.1016/j.aquatox.2016.02.003.

Lynn, S.G., Birge, W.J., Shepherd, B.S., 2008. Molecular characterization and sex-specific tissue expression of estrogen receptor α (esr1), estrogen receptor βa (esr2a) and ovarian aromatase (cyp19a1a) in yellow perch (Perca flavescens). Comp. Biochem. Physiol. B. 149, 126–147.

MacLatchy, D.L., Courtenay, S.C., Rice, C.D., Van der Kraak, G.J., 2003. Development of a short-term reproductive endocrine bioassay using steroid hormone and vitellogenin end points in the estuarine mummichog (Fundulus heteroclitus). Environ. Toxicol. Chem. 22, 996–1008. https://doi.org/10.1897/1551-5028(2003)022<0996:DOASRE>2.0.CO;2.

Marshall, W., Emberley, T., Singer, T., Bryson, S., Mccormick, S., 1999. Time course of salinity adaptation in a strongly euryhaline estuarine teleost, Fundulus heteroclitus: a multivariable approach. J. Exp. Biol. 202 (Pt 11), 1535–1544.

Marshall, W.S., Grosell, M., 2006. Ion transport, osmoregulation, and acid-base balance. In: Evans, D.H., Caliborne, J.B. (Eds.), The Physiology of Fishes, 3rd Edition. CRC Press, Boca Raton, FL, pp. 343–386.

Martyniuk, C.J., Kroll, K.J., Doperalski, N.J., Barber, D.S., Denslow, N.D., 2010. Environmentally relevant exposure to 17 α-ethinylestradiol affects the telencephalic proteome of male fathead minnows. Aquat. Toxicol. 98, 344–353. https://doi.org/10.1016/j.aquatox.2010.03.007.

McMaster, M.E., Jardine, J.J., Ankley, G.T., Benson, W.H., Greeley, M.S., Gross, T.S., Guillette, L.J., MacLatchy, D.L., Orlando, E.F., Van Der Kraak, G.J., Munkittrick, K.R., 2001. An interlaboratory study on the use of steroid hormones in examining endocrine disruption. Environ. Toxicol. Chem. 20, 2081–2087.

McMullin, V.A., Munkittrick, K.R., Methven, D.A., 2009. Latitudinal variability in lunar spawning rhythms: absence of a lunar pattern in the northern mummichog Fundulus heteroclitus macrolepidotus. J. Fish Biol. 75, 885–900. https://doi.org/10.1111/j.1095-8649.2009.02338.x.

Melvin, S.D., Munkittrick, K.R., Bosker, T., MacLatchy, D.L., 2009. Detectable effect size and bioassay power of mummichog (Fundulus heteroclitus) and fathead minnow (pimephales promelas) adult reproductive tests. Environ. Toxicol. Chem. 28, 2416–2425. https://doi.org/10.1897/08-601.1.

Michiels, E.D.G., Vergauwen, L., Lai, F.Y., Town, R.M., Covaci, A., van Nuijs, A.L.N., Van Cruchten, S.J., Knapen, D., 2018. Advancing the zebrafish embryo test for endocrine disruptor screening using micro-injection: ethinyl estradiol as a case study. Environ. Toxicol. Chem. 9999, 1–15. https://doi.org/10.1002/etc.4343.

Miller, D.H., Ankley, G.T., 2004. Modeling impacts on populations: Fathead minnow (Pimephales promelas) exposure to the endocrine disruptor 17b-trenbolone as a case study. Ecotoxicol. Environ. Saf. 59, 1–9. https://doi.org/10.1016/j.ecoenv.2004.05.005.

Miracle, A., Ankley, G., Lattier, D., 2006. Expression of two vitellogenin genes (vg1 and vg3) in fathead minnow (Pimephales promelas) liver in response to exposure to steroidal estrogens and androgens. Ecotoxicol. Environ. Saf. 63, 337–342. https://doi.org/10.1016/j.ecoenv.2005.12.002.

Moens, L.N., van der Ven, K., Van Remortel, P., Del-Favero, J., De Coen, W.M., 2006. Expression profiling of endocrine disrupting compounds using a customized *Cyprinus carpio* cDNA microarray. Toxicol. Sci. 93, 298–310.

Moggs, J.G., Ashby, J., Tinwell, H., Lim, F.L., Moore, D.J., Kimber, I., Orphanides, G., 2004. The need to decide if all estrogens are intrinsically similar. Environ. Health Perspect. 112, 1137–1142.

Morthorst, J.E., Holbech, H., Bjerregaard, P., 2010. Trenbolone causes irreversible masculinization of zebrafish at environmentally relevant concentrations. Aquat. Toxicol. 98, 336–343. https://doi.org/10.1016/j.aquatox.2010.03.008.

Mouriec, K., Lareyre, J.J., Tong, S.K., Le Page, Y., Vaillant, C., Pellegrini, E., Pakdel, F., Chung, B.C., Kah, O., Anglade, I., 2009. Early regulation of brain aromatase (cyp19a1b) by estrogen receptors during zebrafish development. Dev. Dyn. 238, 2641–2651.

Mu, W.J., Wen, H.S., Shi, D., Yang, Y.P., 2013. Molecular cloning and expression analysis of estrogen receptor betas (ER??1 and ER??2) during gonad development in the Korean rockfish, Sebastes schlegeli. Gene 523, 39–49. https://doi.org/10.1016/j.gene.2013.03.109.

Murphy, P.G., Murphy, J.V., 1971. Correlations between respiration and direct uptake of DDT in the mosquito fish Gambusia affinis. Bull. Environ. Contam. Toxicol. 6, 581–588.

Muth-Köhne, E., Westphal-Settele, K., Brückner, J., Konradi, S., Schiller, V., Schäfers, C., Teigeler, M., Fenske, M., 2016. Linking the response of endocrine regulated genes to adverse effects on sex differentiation improves comprehension of aromatase inhibition in a Fish Sexual Development Test. Aquat. Toxicol. 176, 116–127. https://doi.org/10.1016/j.aquatox.2016.04.018.

Nagahama, Y., Yamashita, M., 2008. Regulation of oocyte maturation in fish. Dev Growth Differ 50 Suppl 1, S195-219. https://doi.org/DGD1019 [pii]\r10.1111/j.1440-169X.2008.01019.x.

Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad, C., 2007. The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ERα2 and both ERβ isoforms. Gene 392, 164–173. https://doi.org/10.1016/j.gene.2006.12.030.

Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P., Tyler, C.R., 2004. Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. Environ. Health Perspect. 112, 1725–1733. https://doi.org/10.1289/ehp.7209.

Nelson, E.R., Habibi, H.R., 2013. Estrogen receptor function and regulation in fish and other vertebrates. Gen. Comp. Endocrinol. 192, 15–24. https://doi.org/10.1016/j.ygcen.2013.03.032.

Nikoleris, L., Hultin, C.L., Hallgren, P., Hansson, M.C., 2016. 17α-Ethinylestradiol (EE2) treatment of wild roach (Rutilus rutilus) during early life development disrupts expression of genes directly involved in the feedback cycle of estrogen. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 180, 56–64. https://doi.org/10.1016/j.cbpc.2015.12.002.

Nilsson, Stefan, Gustafsson, Jan-Åke, 2010. Estrogen Receptors: Their Actions and Functional Roles in Health and Disease. In: Bunce, Chris M., Campbell, Moray J. (Eds.), Nuclear Receptors. Springer Netherlands, Dordrecht, pp. 91–141. https://doi.org/10.1007/978-90-481-3303-1_5.

Nozu, R., Nakamura, M., 2015. Cortisol administration induces sex change from ovary totestis in the protogynous Wrasse Halichoeres trimaculatus. Sex Dev. 9, 118–124.

Oikari, T.P.S., 2013. Use of early juvenile zebrafish danio rerio for in-vivo assessment of endocrine modulation by xenoestrogens. J. Environ. Anal. Toxicol. 04, 1–13. https://doi.org/10.4172/2161-0525.1000202.

Örn, S., Holbech, H., Madsen, T.H., Norrgren, L., Petersen, G.I., 2003. Gonad development and vitellogenin production in zebrafish (Danio rerio) exposed to ethinylestradiol and methyltestosterone. Aquat. Toxicol. 65, 397–411.

Örn, S., Holbech, H., Norrgren, L., 2016. Sexual disruption in zebrafish (Danio rerio) exposed to mixtures of 17α-ethinylestradiol and 17β-trenbolone. Environ. Toxicol. Pharmacol. 41, 225–231. https://doi.org/10.1016/j.etap.2015.12.010.

Oropesa, A.L., Martín-Hidalgo, D., Fallola, C., Gil, M.C., 2015. Effects of exposure to 17-alpha-ethynylestradiol on sperm quality of tench (Tinca tinca). Ecotoxicol. Environ. Saf. 120, 318–325. https://doi.org/10.1016/j.ecoenv.2015.06.016.

Overturf, M.D., Anderson, J.C., Pandelides, Z., Beyger, L., Holdway, D.A., 2015. Pharmaceuticals and personal care products: a critical review of the impacts on fish reproduction. Crit. Rev. Toxicol. 45, 469–491. https://doi.org/10.3109/10408444.2015.1038499.

Parrott, J.L., Wood, C.S., 2002. Fathead minnow lifecycle tests for detection of endocrine-disrupting substances in effluents. Water Qual. Res. J. Canada 37, 651–667.

Parrott, J.L., Blunt, B.R., 2005. Life-cycle exposure of fathead minnows (Pimephales promelas) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20, 131–141. https://doi.org/10.1002/tox.20087.

Patrick, M.L., Pärt, P., Marshall, W.S., Wood, C.M., 1997. Characterization of ion and acid-base transport in the fresh water adapted mummichog (Fundulus heteroclitus). J. Exp. Zool. 279, 208–219. https://doi.org/10.1002/(SICI)1097-010X(19971015)279:3<208::AID-JEZ2>3.0.CO;2-R.

Patrick, M.L., Wood, C.M., 1999. Ion and acid-base regulation in the freshwater mummichog (Fundulus heteroclitus): a departure from the standard model for freshwater teleosts. Comp. Biochem. Physiol. - A Mol. Integr. Physiol. 122, 445–456. https://doi.org/10.1016/S1095-6433(99)00030-6.

Pawlowski, S., van Aerle, R., Tyler, C.R., Braunbeck, T., 2004. Effects of 17alpha-ethinylestradiol in a fathead minnow (Pimephales promelas) gonadal recrudescence assay. Ecotoxicol. Environ. Saf. 57, 330–345. https://doi.org/10.1016/j.ecoenv.2003.07.019.

Pérez, M.R., Fernandino, J.I., Carriquiriborde, P., Somoza, G.M., 2012. Feminization and altered gonadal gene expression profile by ethinylestradiol exposure to pejerrey, Odontesthes bonariensis, a South American teleost fish. Environ. Toxicol. Chem. 31, 941–946. https://doi.org/10.1002/etc.1789.

Perkins, E.J., Antczak, P., Burgoon, L., Falciani, F., Garcia-Reyero, N., Gutsell, S., Hodges,

G., Kienzler, A., Knapen, D., McBride, M., Willett, C., 2015. Adverse outcome pathways for regulatory applications: Examination of four case studies with different degrees of completeness and scientific confidence. Toxicol. Sci. 148, 14–25. https://doi.org/10.1093/toxsci/kfv181.

Peters, R.E.M., Courtenay, S.C., Cagampan, S., Hewitt, M.L., MacLatchy, D.L., 2007. Effects on reproductive potential and endocrine status in the mummichog (Fundulus heteroclitus) after exposure to 17a-ethynylestradiol in a short-term reproductive bioassay. Aquat. Toxicol. 85, 154–166. https://doi.org/10.1016/j.aquatox.2007.08.010.

Peters, R.E.M., Courtenay, S.C., Hewitt, L.M., MacLatchy, D.L., 2010. Effects of 17a-ethynylestradiol on early-life development, sex differentiation and vitellogenin induction in mummichog (Fundulus heteroclitus). Mar. Environ. Res. 69, 178–186. https://doi.org/10.1016/j.marenvres.2009.10.002.

Petrino, T.R., Lin, Y.W., Wallace, R.A., 1989a. Steroidogenesis in Fundulus heteroclitus. I. Production of 17 alpha-hydroxy,20 beta-dihydroprogesterone, testosterone, and 17 beta-estradiol by prematurational follicles in vitro. Gen. Comp. Endocrinol. 73, 147–156. doi: 0016-6480(89)90154-8 [pii].

Petrino, T.R., Greeley Jr., M.S., Selman, K., Lin, Y.W., Wallace, R.A., 1989b. Steroidogenesis in Fundulus heteroclitus. II. Production of 17 alpha- hydroxy-20 beta-dihydroprogesterone, testosterone, and 17 beta-estradiol by various components of the ovarian follicle. Gen. Comp. Endocrinol. 76, 230–240.

Petrino, T.R., Hoch, K.L., Lin, Y.W., Wallace, R.A., 1990. Steroidogenesis in *Fundulus heteroclitus* III. Evidence for involvement of cAMP and protein synthesis in the gonadotropic modulation of ovarian steroid production and aromatase activity. J. Exp. Zool. 253, 177–185.

Poonlaphdecha, S., Pepey, E., Canonne, M., de Verdal, H., Baroiller, J.F., D'Cotta, H., 2013. Temperature induced-masculinization in the Nile tilapia causes rapipd upregulation of both dmrt1 and amh expression. Gen. Comp. Endocrinol. 193, 234–242.

Porseryd, T., Caspillo, N.R., Volkova, K., Elabbas, L., Källman, T., Dinnétz, P., 2018. General and Comparative Endocrinology Testis transcriptome alterations in zebrafish (Danio rerio) with reduced fertility due to developmental exposure to 17 a -ethinyl estradiol. Gen. Comp. Endocrinol. 262, 44–58. https://doi.org/10.1016/j.ygcen.2018.03.011.

Ramachandran, S.D., Sweezey, M.J., Hodson, P.V., Boudreau, M., Courtenay, S.C., Lee, K., King, T., Dixon, J.A., 2006. Influence of salinity and fish species on PAH uptake from dispersed crude oil. Mar. Pollut. Bull. 52, 1182–1189. https://doi.org/10.1016/j.marpolbul.2006.02.009.

Rasheed, R.O., 2011. Seasonal variation in fat percentage and water content ofmuscle tissues of some fishes endemic to Derbendikham reservoir. J. Bab. Univ. Pure. Appl. Sci. 19, 435–443.

Rees, B.B., Figueroa, Y.G., Wiese, T.E., Beckman, B.S., Schulte, P.M., 2009. A novel hypoxia-response element in the lactate dehydrogenase-B gene of the killifish Fundulus heteroclitus. Comp. Biochem. Physiol. – A Mol. Integr. Physiol. 154, 70–77. https://doi.org/10.1016/j.cbpa.2009.05.001.

Reyhanian, N., Volkova, K., Hallgren, S., Olsson, P., Porsch-hällström, I., 2014. Comparative Biochemistry and Physiology, Part C Short-term treatment of adult male zebra fi sh (Danio Rerio) with 17 α-ethinyl estradiol affects the transcription of genes involved in development and male sex differentiation. Comp. Biochem. Physiol. Part C 164, 35–42. https://doi.org/10.1016/j.cbpc.2014.04.003.

Roggio, M.A., Guyón, N.F., Hued, A.C., Amé, M.V., Valdés, M.E., Giojalas, L.C., Wunderlin, D.A., Bistoni, M.A., 2014. Effects of the synthetic estrogen 17??-ethinylestradiol on aromatase expression, reproductive behavior and sperm quality in the fish Jenynsia multidentata. Bull. Environ. Contam. Toxicol. 92, 579–584. https://doi.org/10.1007/s00128-013-1185-2.

Runnalls, T.J., Beresford, N., Kugathas, S., Margiotta-Casaluci, L., Scholze, M., Scott, A.P., Sumpter, J.P., 2015. From single chemicals to mixtures-Reproductive effects of levonorgestrel and ethinylestradiol on the fathead minnow. Aquat. Toxicol. 169, 152–167. https://doi.org/10.1016/j.aquatox.2015.10.009.

Rutherford, R., Lister, A., Hewitt, L.M., Maclatchy, D., 2015. Effects of model aromatizable (17a-methyltestosterone) and non-aromatizable (5a-dihydrotestosterone) androgens on the adult mummichog (Fundulus heteroclitus) in a short-term reproductive endocrine bioassay. Comp. Biochem. Physiol. Part - C Toxicol. Pharmacol. 170, 8–18. https://doi.org/10.1016/j.cbpc.2015.01.004.

Saaristo, M., Craft, J.A., Lehtonen, K.K., Björk, H., Lindström, K., 2009. Disruption of sexual selection in sand gobies (Pomatoschistus minutus) by 17α-ethinyl estradiol, an endocrine disruptor. Horm. Behav. 55, 530–537. https://doi.org/10.1016/j.yhbeh.2009.01.006.

Salierno, J.D., Kane, A.S., 2009. 17α-ethinylestradiol alters reproductive behaviors, circulating hormones, and sexual morphology in male fathead minnows (Pimephales promelas). Environ. Toxicol. Chem. 28, 953. https://doi.org/10.1897/08-111.1.

Santi, S., Gennotte, V., Muller, M., Melard, C., Toguyeni, A., Mandiki, S.N.M., Rougeot, C., 2019. Sex-ratio, early sex steroid profiles and cyp19a1b, dmrt1 and foxl2 gene expressions upon high temperature treatment of undifferentiated African catfish juveniles (Clarias gariepinus). Aquaculture 499, 140–148. https://doi.org/10.1016/j.aquaculture.2018.09.033.

Santos, D., Luzio, A., Coimbra, A.M., 2017. Zebrafish sex differentiation and gonad development: a review on the impact of environmental factors. Aquat. Toxicol. 191, 141–163. https://doi.org/10.1016/j.aquatox.2017.08.005.

Satchell, G.H., 1984. Respiratory toxicology of fishes In: Weber, LH (Ed), Vol. 2. Aquatic Toxicology. Raven Press, New York, pp. 1–50.

Scholz, S., Gutzeit, H.O., 2000. 17 alpha-ethinylestradiol affects reproduction, sexual differentiation and aromatase gene expression of the medaka (Oryzias latipes). Aquat. Toxicol. 50, 363–373.

Schröder, P., Helmreich, B., Škrbić, B., Carballa, M., Papa, M., Pastore, C., Emre, Z., Oehmen, A., Langenhoff, A., Molinos, M., Dvarioniene, J., Huber, C., Tsagarakis, K.P., Martinez- Lopez, E., Pagano, S.M., Vogelsang, C., Mascolo, G., 2016. Status of hormones and painkillers in wastewater effluents across several European states—considerations for the EU watch list concerning estradiols and diclofenac. Environ. Sci. Pollut. Res. 23, 12835–12866. https://doi.org/10.1007/s11356-016-6503-x.

Schroeder, A.L., Ankley, G.T., Habib, T., Garcia-Reyero, N., Escalon, B.L., Jensen, K.M., Kahl, M.D., Durhan, E.J., Makynen, E.A., Cavallin, J.E., Martinovic-Weigelt, D., Perkins, E.J., Villeneuve, D.L., 2017. Rapid effects of the aromatase inhibitor fadrozole on steroid production and gene expression in the ovary of female fathead minnows (Pimephales promelas). Gen. Comp. Endocrinol. 252, 79–87. https://doi.org/10.1016/j.ygcen.2017.07.022.

Scott, G.R., Baker, D.W., Schulte, P.M., Wood, C.M., 2008. Physiological and molecular mechanisms of osmoregulatory plasticity in killifish after seawater transfer. J. Exp. Biol. 211, 2450–2459. https://doi.org/10.1242/jeb.017947.

Segner, H., 2011. Moving beyond a descriptive aquatic toxicology: the value of biological process and trait information. Aquat. Toxicol. 104, 50–55.

Seki, M., Yokota, H., Maeda, M., Kobayashi, K., 2005. Fish full life-cycle testing for 17β-estradiol on medaka (*Oryzias latipes*). Environ. Toxicol. Chem. 24, 1259–1266.

Selman, K., Wallace, R.A., 1983. Oogenesis in Fundulus heteroclitus. 3. Vitellogenesis. J. Exp. Zool. 226, 441–457.

Senthilkumaran, B., Yoshikuni, M., Nagahama, Y., 2004. A shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. Mol. Cell. Endocrinol. 27, 11–18. https://doi.org/10.1016/j.mce.2003.11.012.

Sharpe, R.L., MacLatchy, D.L., Courtenay, S.C., Van Der Kraak, G.J., 2004. Effects of a model androgen (methyl testosterone) and a model anti-androgen (cyproterone acetate) on reproductive endocrine endpoints in a short-term adult mummichog (Fundulus heteroclitus) bioassay. Aquat. Toxicol. 67, 203–215. https://doi.org/10.1016/j.aquatox.2004.01.009.

Shimizu, A., 1997. Reproductive cycles in a reared strain of the mummichog, a daily spawner. J. Fish Biol. 51, 724–737. https://doi.org/10.1111/j.1095-8649.1997.tb01994.x.

Shimizu, A., Yamashita, M., 2002. Purification of mummichog (Fundulus heteroclitus) gonadotropins and their subunits, using an immunochemical assay with antisera raised against synthetic peptides. Gen. Comp. Endocrinol. 125, 79–91. https://doi.org/10.1006/gcen.2001.7741.

Shimizu, A., 2003. Effect of photoperiod and temperature on gonadal activity and plasma steroid levels in a reared strain of the mummichog (Fundulus heteroclitus) during different phases of its annual reproductive cycle. Gen. Comp. Endocrinol. 131, 310–324. https://doi.org/10.1016/S0016-6480(03)00026-1.

Shimizu, A., Hamaguchi, M., Ito, H., Ohkubo, M., Udagawa, M., Fujii, K., Kobayashi, T., Nakamura, M., 2008. Appearances and chronological changes of mummichog Fundulus heteroclitus FSH cells and LH cells during ontogeny, sexual differentiation, and gonadal development. Gen. Comp. Endocrinol. 156, 312–322. https://doi.org/10.1016/j.ygcen.2008.01.022.

Shimizu, A., 2014. Reproductive physiology of the mummichog- an excellent experimental fish. Aqua-BioScience Monographs 7, 79–116.

Shukla, P., Gopalani, M., Ramteke, D.S., Wate, S.R., 2007. Influence of salinity on PAH uptake from water soluble fraction of crude oil in Tilapia mossambica. Bull. Environ. Contam. Toxicol. 79, 601–605. https://doi.org/10.1007/s00128-007-9272-x.

Söffker, M., Tyler, C.R., 2012. Endocrine disrupting chemicals and sexual behaviors in fish a critical review on effects and possible consequences. Crit. Rev. Toxicol. 42, 653–668. https://doi.org/10.3109/10408444.2012.692114.

Strüssmann, C.A., Nakamura, M., 2002. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. Fish Physiol. Biochem. https://doi.org/10.1023/A: 1023343023556.

Tarrant, A.M., Greytak, S.R., Callard, G.V., Hahn, M.E., 2006. Estrogen receptor-related receptors in the killifish Fundulus heteroclitus: Diversity, expression, and estrogen responsiveness. J. Mol. Endocrinol. 37, 105–120. https://doi.org/10.1677/jme.1.01976.

Taylor, M.H., Leach, G.J., Dimichele, L., Levitan, W.M., Jacob, W.F., 1979. Lunar spawning in the mummichog, *Fundulus heteroclitus* (Pisces: Cyprinodontidae). Copeia 1979, 291–297.

Taylor, M.H., DiMichele, L., 1980. Ovarian changes during the lunar spawning cycle of *Fundulus heteroclitus*. Copeia 1980, 118–125.

Ternes, T.A., Stumpf, M., Mueller, J., Haberer, K., Wilken, R.D., Servos, M., 1999. Behavior and occurrence of estrogens in municipal sewage treatment plants - I. Investigations in Germany. Canada and Brazil. Sci. Total Environ. 225, 81–90. https://doi.org/10.1016/S0048-9697(98)00334-9.

Tilton, S.C., Foran, C.M., Benson, W.H., 2005. Relationship between ethinylestradiol-mediated changes in endocrine function and reproductive impairment in Japanese medaka (Oryzias latipes). Environ. Toxicol. Chem. 24, 352–359.

Tokarz, J., Möller, G., Hrabě De Angelis, M., Adamski, J., 2015. Steroids in teleost fishes: A functional point of view. Steroids 103, 123–144. https://doi.org/10.1016/j.steroids.2015.06.011.

Trant, J.M., Gavasso, S., Ackers, J., Chung, B.C., Place, a R., 2001. Developmental expression of cytochrome P450 aromatase genes (CYP19a and CYP19b) in zebrafish fry (Danio rerio). J. Exp. Zool. 290, 475–483.

Trubiroha, A., Kroupova, H., Wuertz, S., Kloas, W., 2012. Up-regulation of gonadotropin mRNA-expression at the onset of gametogenesis in the roach (Rutilus rutilus): evidence for an important role of brain-type aromatase (cyp19a1b) in the pituitary. Gen. Comp. Endocrinol. 178, 529–538.

Tyler, C.R., Spary, C., Gibson, R., Santos, E.M., Shears, J., Hill, E.M., 2005. Accounting for the differences in oestrogenic responses in rainbow trout (Oncorrhynchus mykiss: Salmonidae) and roach (Rutilus rutilus) exposed to effluents from wastewater treatment works. Environ. Sci. Technol. 39, 2599–2607. https://doi.org/10.1021/es0488939.

Tyler, C.R., 2009. Sexual Reprogramming and Estrogenic Sensitization in Wild Fish

Exposed to Ethinylestradiol 43, pp. 1219–1225.
Urbatzka, R., Rocha, E., Reis, B., Cruzeiro, C., Monteiro, R.A.F., Rocha, M.J., 2012. Effects of ethinylestradiol and of an environmentally relevant mixture of xenoestrogens on steroidogenic gene expression and specific transcription factors in zebrafish. Environ. Pollut. 164, 28–35. https://doi.org/10.1016/j.envpol.2012.01.018.
Urbina, M.A., Glover, C.N., 2015. Effect of salinity on osmoregulation, metabolism and nitrogen excretion in the amphidromous fish, inanga (Galaxias maculatus). J. Exp. Mar. Bio. Ecol. 473, 7–15. https://doi.org/10.1016/j.jembe.2015.07.014.
Urushitani, H., Shimizu, A., Katsu, Y., Iguchi, T., 2002. Early estrogen exposure induces abnormal development of Fundulus heteroclitus. J. Exp. Zool. 293, 693–702. https://doi.org/10.1002/jez.10161.
Urushitani, H., Nakai, M., Inanaga, H., Shimohigashi, Y., Shimizu, A., Katsu, Y., Iguchi, T., 2003. Cloning and characterization of estrogen receptor α in mummichog, Fundulus heteroclitus. Mol. Cell. Endocrinol. 203, 41–50. https://doi.org/10.1016/S0303-7207(03)00118-7.
Van den Belt, K., Wester, P.W., van der Ven, L.T., Verheyen, R., Witters, H., 2002. Effects of ethynylestradiol on the reproductive physiology in zebrafish (Danio rerio): time dependency and reversibility. Environ. Toxicol. Chem. 21, 767–775.
Van den Belt, K., Berckmans, P., Vangenechten, C., Verheyen, R., Witters, H., 2004. Comparative study on the in vitro/in vivo estrogenic potencies of 17β-estradiol, estrone, 17α-ethynylestradiol and nonylphenol. Aquat. Toxicol. 66, 183–195. https://doi.org/10.1016/j.aquatox.2003.09.004.
Villeneuve, D., 2016. Adverse outcome pathway on aromatase inhibition leading to reproductive dysfunction in fish. OECD Series on Adverse Outcome Pathways, No 4 OECD Publishing, Paris, France. https://doi.org/10.1787/5jlsv05mx433-en.
Villeneuve, D.L., Mueller, N.D., Martinović, D., Makynen, E.A., Kahl, M.D., Jensen, K.M., Durhan, E.J., Cavallin, J.E., Bencic, D., Ankley, G.T., 2009. Direct effects, compensation, and recovery in female fathead minnows exposed to a model aromatase inhibitor. Environ. Health Perspect. 117, 624–631. https://doi.org/10.1289/ehp.11891.
Volkova, K., Reyhanian, N., Porseryd, T., Hallgren, S., Dinnétz, P., Porsch-hällström, I., 2015. Hormones and Behavior Developmental exposure of zebra fish (Danio rerio) to 17 α -ethinylestradiol affects non-reproductive behavior and fertility as adults, and increases anxiety in unexposed progeny. Horm. Behav. 73, 30–38. https://doi.org/10.1016/j.yhbeh.2015.05.014.
Wallace, R.A., Selman, K., 1981. The reproductive activity of *Fundulus heteroclitus* females from Woods Hole, Massachusetts, as compared with more southern locations. Copeia 1981, 212–215.
Wang, J., Liu, X., Wang, H., Wu, T., Hu, X., Qin, F., Wang, Z., 2010. Expression of two cytochrome P450 aromatase genes is regulated by endocrine disrupting chemicals in rare minnow Gobiocypris rarus juveniles. Comp. Biochem. Physiol. – C Toxicol. Pharmacol. 152, 313–320. https://doi.org/10.1016/j.cbpc.2010.05.007.
Wang, Q., Li, W., Chen, L., Liu, H., 2019. Long-term exposure of xenoestrogens with environmental relevant concentrations disrupted spermatogenesis of zebra fish through altering sex hormone balance, stimulating germ cell proliferation, meiosis and enhancing apoptosis. Environ. Pollut. 244, 486–494. https://doi.org/10.1016/j.envpol.2018.10.079.
Wang, Y., Ge, W., 2004. Cloning of zebrafish ovarian P450c17 and characterization of its expression in gonadal and extra-gonadal tissues. Gen. Comp. Endocrinol. 135, 241–249.
Whitehead, A., 2010. The evolutionary radiation of diverse osmotolerant physiologies in killifish (Fundulus sp.). Evolution (N. Y) 64, 2070–2085. https://doi.org/10.1111/j.1558-5646.2010.00957.x.
Wong, T.T., Ijiri, S., Zohar, Y., 2006. Molecular biology of ovarian aromatase in sex reversal: complementary DNA and 50-flanking region isolation and differential expression of ovarian aromatase in the gilthead seabream (Sparus aurata). Biol. Reprod. 74, 857–864.
Wood, C.M., Grosell, M., 2009. TEP on the tide in killifish (Fundulus heteroclitus): Effects of progressively changing salinity and prior acclimation to intermediate or cycling salinity. J Comp. Physiol. B Biochem. Syst. Environ. Physiol. 179, 459–467. https://doi.org/10.1007/s00360-008-0323-3.
Wood, C.M., Laurent, P., 2003. Na + versus Cl-transport in the intact killifish after rapid salinity transfer. Biochim. Biophys. Acta - Biomembr. 106–119. https://doi.org/10.1016/j.bbamem.2003.08.014.
Wood, C.M., Marshall, W.S., 1994. Ion balance, acid-base regulation, and chloride cell function in the common killifish, Fundulus heteroclitus-a euryhaline estuarine teleost. Estuaries 17, 34–52. https://doi.org/10.1007/BF02694901.
Wood, C.M., McDonald, M.D., Walker, P., Grosell, M., Barimo, J.F., Playle, R.C., Walsh, P.J., 2004. Bioavailability of silver and its relationship to ionoregulation and silver speciation across a range of salinities in the gulf toadfish (Opsanus beta). Aquat. Toxicol. 70, 137–157. https://doi.org/10.1016/j.aquatox.2004.08.002.
Yamamoto, H., Liljestrand, H.M., Shimizu, Y., Morita, M., 2003. Effects of physical-chemical characteristics on the sorption of selected endocrine disruptors by dissolved organic matter surrogates. Environ. Sci. Technol. 37, 2646–2657. https://doi.org/10.1021/es026405w.
Yang, R., Randall, D., 1995. Relationships between xenobiotic uptake/depuration and oxygen consumption by fishes: A physiological model. EPA, 600.
Yang, R., Brauner, C., Thurston, V.V., Neuman, J., Randall, D.J., 2000. Relationship between toxicant transfer kinetic processes and fish oxygen consumption. Aquatic Toxicol. 48, 95–108.
Yaron, Z., Sivan, B., 2006. Reproduction. In: Evans, D.H., Caliborne, J.B. (Eds.), The Physiology of Fishes, 3rd Ed. CRC Press, Boca Raton, FL, pp. 343–386.
Zha, J., Wang, Z., Wang, N., Ingersoll, C., 2007. Histological alteration and vitellogenin induction in adult rare minnow (Gobiocypris rarus) after exposure to ethynylestradiol and nonylphenol. Chemosphere 66, 488–495.
Zha, J., Sun, L., Spear, P.A., Wang, Z., 2008. Comparison of ethinylestradiol and nonylphenol effects on reproduction of Chinese rare minnows (Gobiocypris rarus). Ecotoxicol. Environ. Saf. 71, 390–399. https://doi.org/10.1016/j.ecoenv.2007.11.017.
Zhao, Y., Xie, L., Yan, Y., 2015. Microcystin-LR impairs zebrafish reproduction by affecting oogenesis and endocrine system. Chemosphere 120, 115–122.
Zhou, B.S., 2015. Adverse outcome pathway: framework, application, and challenges in chemical risk assessment. J. Environ. Sci. 35, 191e193.
Zhuang, T., Jiang, H., Liyu, L., Jianghuan, H., 2017. Reproductive toxicity caused by co-exposure of zebrafish to progestin megestrol acetate and estrogen 17 alpha-ethinylestradiol. Environ. Chem. 36, 1440–1450.
Zillioux, E.J., Johnson, I.C., Kiparissis, Y., Metcalfe, C.D., Wheat, J.V., Ward, S.G., Liu, H., 2001. The sheepshead minnow as an in vivo model for endocrine disruption in marine teleosts: a partial life-cycle test with 17α-ethynylestradiol. Environ. Toxicol. Chem. 20, 1968–1978. https://doi.org/10.1002/etc.5620200915.