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Maternal exposure to mixtures of dienestrol, linuron and flutamide. Part I: Feminization effects on male rat offspring

Elga Schreiber[a], Tània Garcia[a], Neus González[a], Roser Esplugas[a], Raju Prasad Sharma[a,b], Margarita Torrente[a,c], Vikas Kumar[b,d], Toine Bovee[e], Efrosini S. Katsanou[f], Kyriaki Machera[f], José Luis Domingo[a], Mercedes Gómez[a,*]

[a] Laboratory of Toxicology and Environmental Health, School of Medicine, IISPV, Universitat Rovira i Virgili, Reus, Catalonia, Spain
[b] Environmental Analysis and Management Group, Department of Chemical Engineering, Universitat Rovira i Virgili, Catalonia, Spain
[c] Research Center in Behavioral Assessment (CRAMC), Department of Psychology, Universitat Rovira i Virgili, Tarragona, Catalonia, Spain
[d] IISPV, Hospital Universitari Sant Joan de Reus, Universitat Rovira i Virgili, Reus, Spain
[e] Wageningen Food Safety Research, Department of Bioassays and Biosensors, WUR, Wageningen, the Netherlands
[f] Laboratory of Pesticides' Toxicology, Department of Pesticides Control and Phytopharmacy, Benaki Phytopathological Institute, Athens, Greece

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ABSTRACT

Exposure to endocrine-disrupting compounds (EDCs) during pregnancy can result in negative health effects in later generations, including sex changes and feminization. The present study assessed the feminization effects on male offspring rats of three EDCs: Dienestrol (DIES), Linuron (LIN), and Flutamide (FLU). Sexually mature female rats were exposed from gestation day (GD) 6 until postnatal day (PND) 21 to: 0.37, 0.75, 1.5, 3.12 or 6.25 μg/kg/day of DIES, 1.5, 3, 6, 12.5, 25 or 50 mg/kg/day of LIN, 3.5, 6.7, 12.5, 25 or 50 mg/kg/day of FLU, and the following mixtures: FLU + DIES (mg/kg/day + μg/kg/day), 3.5 + 0.37, or 3.5 + 3, 25 + 0.37, or 25 + 3; FLU + LIN (mg/kg/day + mg/kg/day), 3.5 + 12.5, or 25 + 12.5; and DIES + LIN (μg/kg/day + mg/kg/day), 0.37 + 12.5, or 3 + 12.5. Anogenital distance (AGD), nipple retention (NR) and cryptorchidism were evaluated. FLU produced a decrease of AGD, an increase of NR, and an increase of cryptorchidism at the highest dose. None of these three endpoints were significantly affected by LIN or DIES treatments alone. Combinations of FLU + LIN and FLU + DIES increased NR, and decreased AGD, while DIES + LIN did not produce any effects in male pups. Results show that FLU is able to induce feminization in male pups, while binary combinations of LIN and DIES did not modify the effects produced by FLU.

1. Introduction

Many chemicals are essential components of a number of products that are part of our daily lives. However, exposure to some of these chemicals can cause adverse health effects. Though many of these single chemicals are tested regarding their toxicological profile this is not the case for mixtures of chemicals and especially under real-life exposure scenarios (Tsatsakis et al., 2016, 2019; Tsatsakis, 2020; Docea et al., 2018; Kostoff et al., 2018; Hernández et al., 2020). This is why testing of chemical mixture is of great importance. A group of these compounds are known as endocrine-disrupting compounds (Ewence et al., 2015; Monneret, 2017). An endocrine-disrupting compound (EDC) has been defined by the World Health Organization/International Programme on Chemical Safety as "*An exogenous substance or mixture that alters function(s) of the endocrine system, and consequently, causes adverse health effects in an intact organism, or its progeny, or (sub) populations*" (WHO/IPCS, 2002). The presence of EDCs in both the environment and foodstuffs has raised concerns about the potentially harmful effects of exposure to such chemicals (ECHA, 2018a; Roszko et al., 2018).

Human exposure to chemicals, drugs, and stress, can affect the hormonal balance and have serious consequences for the health. EDCs have been linked to cancer and reproductive disturbances in wildlife (Marty et al., 2011; EEA, 2012). A well-known example of a human drug, that showed severe increases in hormone related cancers, in both pregnant mothers and their baby girls, is diethylstilbestrol (DES) (Metzler and Fischer, 1981). Humans are exposed to EDCs via several ways, e.g. medical drugs, oral consumption of food and water, contact with the skin, inhalation, transfer from mother to child via the placenta and after birth via breast milk, and even intravenously (e.g. EDCs

* Corresponding author.
E-mail address: mariamercedes.gomez@urv.cat (M. Gómez).

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coming from an infuse) (Kabir et al., 2015; Roszko et al., 2018). One of the mechanisms by which EDCs can potentially interrupt the endocrine system and alter hormonal functions is by imitating the natural hormones.

It has been shown that exposure to EDCs during pregnancy can result in severe health effects in later generations (WHO, 2012). Regarding possible sex changes or feminization, tremendous efforts have been made in recent years in order to establish whether mixtures of (anti)estrogens or (anti)androgens and its combinations may have additive effects (Christiansen et al., 2009; Yin et al., 2017) or not (Bhatia et al., 2015). In a previous study, in-silico docking on estrogen and androgen receptors (ER and AR), in-vitro ER and AR transcriptional activation bioassays, and the in-vitro H295R steroidogenesis assay, were used to characterize compounds on their potential (anti)estrogenic and (anti)androgenic potencies (Bovee et al., unpublished data). With the specific in-silico receptor docking and in-vitro transcriptional activation bioassays, it is not possible to compare the potency of an estrogen with that of an anti-androgen as well as to test for mixture effects with compounds having a dissimilar mode of action. For these reasons, three compounds were selected for further *in vivo* testing individually and in binary mixtures: dienestrol as an ER-agonist, and flutamide and linuron as AR-antagonists.

This study belongs to Euromix project (supported by European Commission). In this project, there were different type of studies and some of them were tailored by the results of the previous ones. The present endocrine *in vivo* rat study was preceded by a *in silico*, *in vitro* and *in vivo* studies (zebra fish). Most part of these studies have not published yet, despite they had been submitted to journals. For the purpose of EuroMix, substances have been identified for feminization (EuroMix Chemical Inventory). The compounds forming the main mixtures were selected from combined exposure and their potential to cause feminization of a developing organism. Compounds and endpoints were chosen from human studies (Crépet et al., 2018).

Dienestrol (DIES), a catabolic product of diethylstilbestrol (DES), is a stilbene derivative with estrogenic activity comparable to that of estradiol-17β (E2), the main natural endogenous estrogen (Metzler and Fischer, 1981). DES exposure was related with human cancer, particularly breast cancer (Green et al., 2005; Greenberg et al., 1984). As a result, DES was banned as a prescription drug for women with high-risk pregnancies in 1971 in the United States and in 1978 in Europe (Giusti et al., 1995). However, its use continued to be allowed in cattle and sheep, where DES promoted the growth of the animal, until 1979, when trans placental contamination was found. Notwithstanding, recently, high concentrations of (natural) estrogenic steroid hormones (DES included) in water (Chen et al., 2010; Xu et al., 2011), sediments (Pimentel et al., 2016), foods (Zhang et al., 2008), supplements (Toorians et al., 2010) and urine (Zhang et al., 2017) have been detected.

Dienestrol (4,4'-(diethylideneethylene)diphenol)) has three different isomers, Z,Z-DIES, E,E-DIES and E,Z-DIES. Regarding estrogenic activity, the major metabolite (Z,Z-DIES) does not display estrogenicity (Inano et al., 1993), whereas the E,E-DIES stereoisomer is about 200 times more potent on the ERα than the endogenous E2 and even 400 times more potent on the ERβ (Kuiper et al., 1997). According to the latest United Nations data on drugs, DIES is officially withdrawn in Austria, Italy, Kuwait, Saudi Arabia and Venezuela (UN, 2005) and this substance is registered under REACH Regulation and has a self-classification as Carcinogen Cat. 1 and as Toxic to reproduction Cat. 2 (it is suspected that DIES damages fertility or the unborn child) (ECHA, 2018b). Unlike DES, there are no available data regarding the possible feminization of DIES, either separately or in binary combinations.

Linuron (LIN) (3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea) is a widely used herbicide in the production of fruit, wheat, sugar cane, peanuts, cotton, corns, soybeans, carrots and other vegetables (Ding et al., 2017). Humans are exposed to LIN either through contaminated food or drinking water, or by dermal contact (Narayanan et al., 2015). LIN both inhibits cytochrome P450 enzymes that are critical for de novo synthesis of testosterone in the testis and display AR antagonist activity (McIntyre et al., 2002a,b; Wilson et al., 2009). Prenatal exposure to LIN induces dose-dependent alterations in the androgen-mediated reproductive development in male rats. Such a deformity of the male reproductive system is irreversible and persistent, and may last for lifetime (Ding et al., 2017; McIntyre et al., 2000). Gestational exposure to a high dose level of LIN can cause a reduction of testosterone synthesis in male offspring rats (Ding et al., 2017; Hotchkiss et al., 2004), reduces neonatal anogenital distance (AGD) and can lead to smaller testis's along with severe tissue lesion, sperm reduction, acrosome malformation and pathological damage in seminiferous tubules of sexually matured offspring male rats (Ding et al., 2017; Gray et al., 2001; Kang et al., 2004; Wilson et al., 2009). In line with these findings, in utero exposure to LIN has also been shown in male offspring to lead to hypospadias, cryptorchidism, prostate hyperplasia, and the retention of areolae and nipples in a dose-dependent manner (Hass et al., 2007; Hotchkiss et al., 2004; McIntyre et al., 2000; Sultan et al., 2001). In 2010, it was suggested that LIN acts as an environmental endocrine disruptor (Ewence et al., 2015; Rider et al., 2010).

Flutamide (FLU) (2-methyl-N-[4-nitro-3-(trifluoromethyl)-phenyl] propamide) is a well-known pure anti-androgen, reported to block binding of androgens to the AR ligand-binding pocket. As a pharmaceutical, FLU is used in a therapy of advanced androgen-dependent prostate cancer (Hejmej and Bilinska, 2018). Although the effects of FLU on the development and function of the male reproductive system were reported (Anahara et al., 2006; Hejmej and Bilinska, 2018; McIntyre et al., 2001), the mechanisms by which this drug alters the function of seminiferous, epididymal, and prostate epithelia are not fully understood. Several studies in rats revealed FLU off target effects, such as decreased weight of the accessory gland, alteration in sex hormone levels in the male rat, and prolongation of the oestrous cycle in female rats (Shin et al., 2002; Toyoda et al., 2000). Consistent to the OECD Test Guideline 407, repeated-dose (28 days) oral toxicity study, for screening for endocrine disrupting compounds (EDCs), FLU was listed as one of the EDCs (Toyoda et al., 2000).

Taking the above into account, the aim of the current study was to assess the feminization effects (anogenital distance, nipple retention and cryptorchidism) in offspring male rats whose mothers were exposed to DIES, LIN and FLU either individually or in binary mixtures, in order to evaluate whether these compounds act additively.

2. Materials and methods

2.1. Chemicals and doses

Dienestrol (DIES) (purity ≥ 95%, CAS Number: 84-17-3, Sigma-Aldrich, St. Louis, MO, USA), linuron (LIN) (purity ≥ 98%, CAS Number: 330-55-2, Sigma-Aldrich, St. Louis, MO, USA), and flutamide (FLU) (purity ≥ 99%, CAS Number: 13311-84-7, Sigma-Aldrich, St. Louis, MO, USA), were dissolved in absolute ethanol (0.2% of total volume), followed by dilution with commercial sunflower oil as vehicle.

The doses of FLU and LIN were directly taken from the literature (Foster and McIntyre, 2002). Nominal doses were 1.5, 3, 6, 12.5, 25, and 50 mg/kg b.w./day for LIN and 3.5, 6.7, 12.5, 25, and 50 mg/kg b.w./day for FLU (Table 1). No dose references of DIES in experimental rodents were available in the literature. As DIES and DES displayed a similar potency *in vitro* (Bovee et al., 2006), the administered doses were based on diethylstilbestrol (DES) concentrations as previously reported (Metzler and Fischer, 1981), being the nominal doses of DIES 0.37, 0.75, 1.5, 3.12, and 6.25 μg/kg b.w./day (Table 1). Two different control groups were added to the experimental groups: one control without vehicle (control 0), and another control with vehicle only (control-oil).

For mixture experiments (binary mixtures), doses selected were lowest and one-half of the highest doses for each compound: clear effect

Table 1
Given-doses to pregnant rats (n = 5 pregnant females).

Single	
DIES	0.37; 0.75; 1.5; 3.12; and 6.25 μg/kg/day
LIN	1.5; 3; 6; 12.5; 25; and 50 mg/kg/day
FLU	3.5; 6.7; 12.5; 25; and 50 mg/kg/day
Binary mixtures	
FLU + DIES (mg/kg/day + μg/kg/day)	3.5 + 0.37; 3.5 + 3; 25 + 0.37; 25 + 3
FLU + LIN (mg/kg/day + mg/kg/day)	3.5 + 12.5; 25 + 12.5
DIES + LIN (μg/kg/day + mg/kg/day)	0.37 + 12.5; 3 + 12.5

DIES, dienestrol; LIN, linuron; FLU, flutamide.

differentiation dose and clear reference of effect dose. Selected doses for binary mixtures were the following: FLU (mg/kg b.w./day) + DIES (μg/kg b.w./day), 3.5 + 0.37, 3.5 + 3, 25 + 0.37, and 25 + 3; FLU (mg/kg b.w./day) + LIN (mg/kg b.w./day), 3.5 + 12.5, and 25 + 12.5; DIES (μg/kg b.w./day) + LIN (mg/kg b.w./day), 0.37 + 12.5, and 3 + 12.5 (Table 1). As no feminization effects in male pups were observed after single doses of LIN only the highest viable dose of LIN, which not cause mortality in pups, was used in binary mixtures.

2.2. Animals and experimental design

Mature female and male Sprague Dawley rats were obtained from Charles River (Saint-Germain-Nuelles, France). Animals were individually housed in an animal room maintained at 22 ± 2 °C, a relative humidity of 50 ± 10%, and a 12-hr light/dark automatic light cycle (light: 08.00–20.00). All animals were allowed free access to food (Panlab rodent chow, Barcelona, Spain) and tap water. After one week of acclimation, the oestral cycle of females was evaluated during 15 consecutive days. After this period, female rats were mated with males (2:1) overnight. Vaginal smears were collected the next morning to detect the presence of sperm. The day of sperm detection was considered as gestation day 0 (GD0). Positive GD0 females were individually housed in plastic cages and randomly distributed into different groups (5 pregnant females/group). The use of animals and the experimental protocol, were approved by the Animal Care and Use Committee of the Rovira i Virgili University (Tarragona, Catalonia, Spain).

At GD6 pregnant females received the different doses of each chemical or mixture. The doses were administered daily by *gavage* (1 mL/400 g b.w.) from GD6 to postnatal day (PND21), except the day of parturition called PND0/1 (Fig. 1). To avoid possible implantations loss, treatment began at GD6. During the treatment, food and water consumption were recorded twice a week. Female body weight was also recorded twice a week during the gestation period and at GD0, 6, and 21, and during the lactation period at PND0/1, 4, 7, 14, and 21. The sex of the pups was determined at PND0/1. In the FLU groups, sex was evaluated at PND4 because at PND0/1 it was not possible to differentiate between males and females due to feminizating effects caused by FLU. Signs of toxicity were daily monitored in females and pups, e.g. presence of tremors, convulsions, changes in activity levels, posture etc. Death of pups at birth and during the treatment were also included.

Four male pups per litter (20 male pups/5 pregnant females in each group) were chosen and followed during the whole lactation period (Fig. 1). At certain time points (see section 2.3.), the AGD, nipple retention (NR) and cryptorchidism were evaluated as feminization endpoints. In addition, pups body weight was recorded at PND0/1, 4, 7, 14, and 21. At PND21, male pups were intraperitoneally anesthetised with medetomidine/ketamine (0.5 mg/kg b.w. + 75 mg/kg b.w.) being liver and testicles excised immediately, weighted. Females and the rest of the pups were sacrificed by CO_2 asphyxiation.

2.3. Feminization endpoints

The anogenital distance is the distance from the base of the genitalia to the anus, and was measured at PND0/1, 4, 7, 14, and 21 using a Vernier caliper. Results are given as mm (AGD) and as mm/g (relative AGD). The nipple retention (NR) is the presence of nipples in male pups. Male pups were examined at PND14 and presence of nipples or areolas was observed. Results are given as the number of nipples/pup and NR frequency, i.e. percentage of pups with NR/litter. Cryptorchidism is the absence of one or both testicles in the scrotum. At PND21, male pups were anesthetised and the position of both testicles with respect to the scrotum (descended testes) was assessed visually and recorded on a qualitative scale where 0 means that both testicles has descended nicely into the scrotum, 1 means that one testicle has not descended, and 2 that both testicles have not descended into the scrotum. Results are given as percentage of pups/litter with 0, 1 or both (2) testicles not descended. Details of the exact position of the testicles were also recorded.

2.4. Statistics

The litter was the statistical unit. Four pups per litter were used in order to assess the endpoints, being the average then calculated (Festing, 2006).

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS v.25) it included ANOVA, followed by the Dunnett post-hoc test when variances among treated groups were homogeneous. When variances were not homogeneous, the Kruskal-Wallis test, followed by the Dunns post-hoc test, was used. Levene's test was used to asses homogeneity of variances. To determine differences due to the vehicle, a two-tailed *t*-test was conducted between control and control-vehicle groups. If no differences between these groups were found, ANOVAs were done with treated groups and the control-vehicle. If control groups were different, both controls were

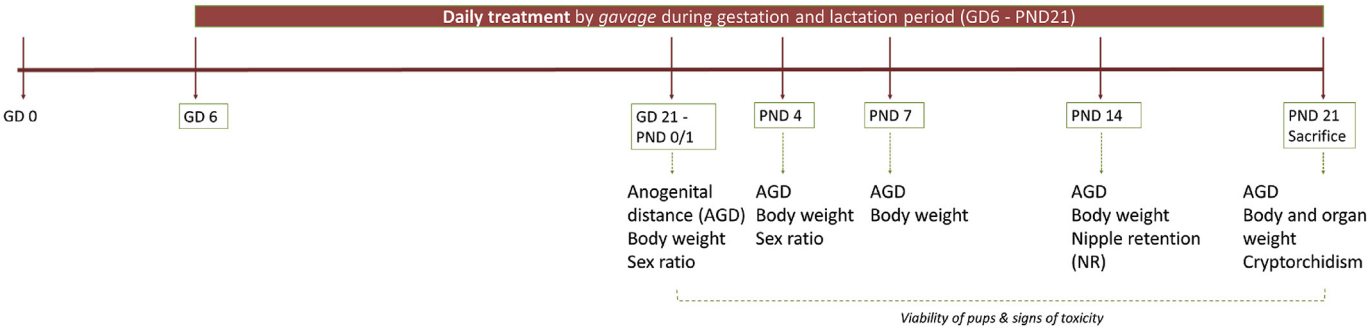

Fig. 1. Scheme of the treatment and experimental design.

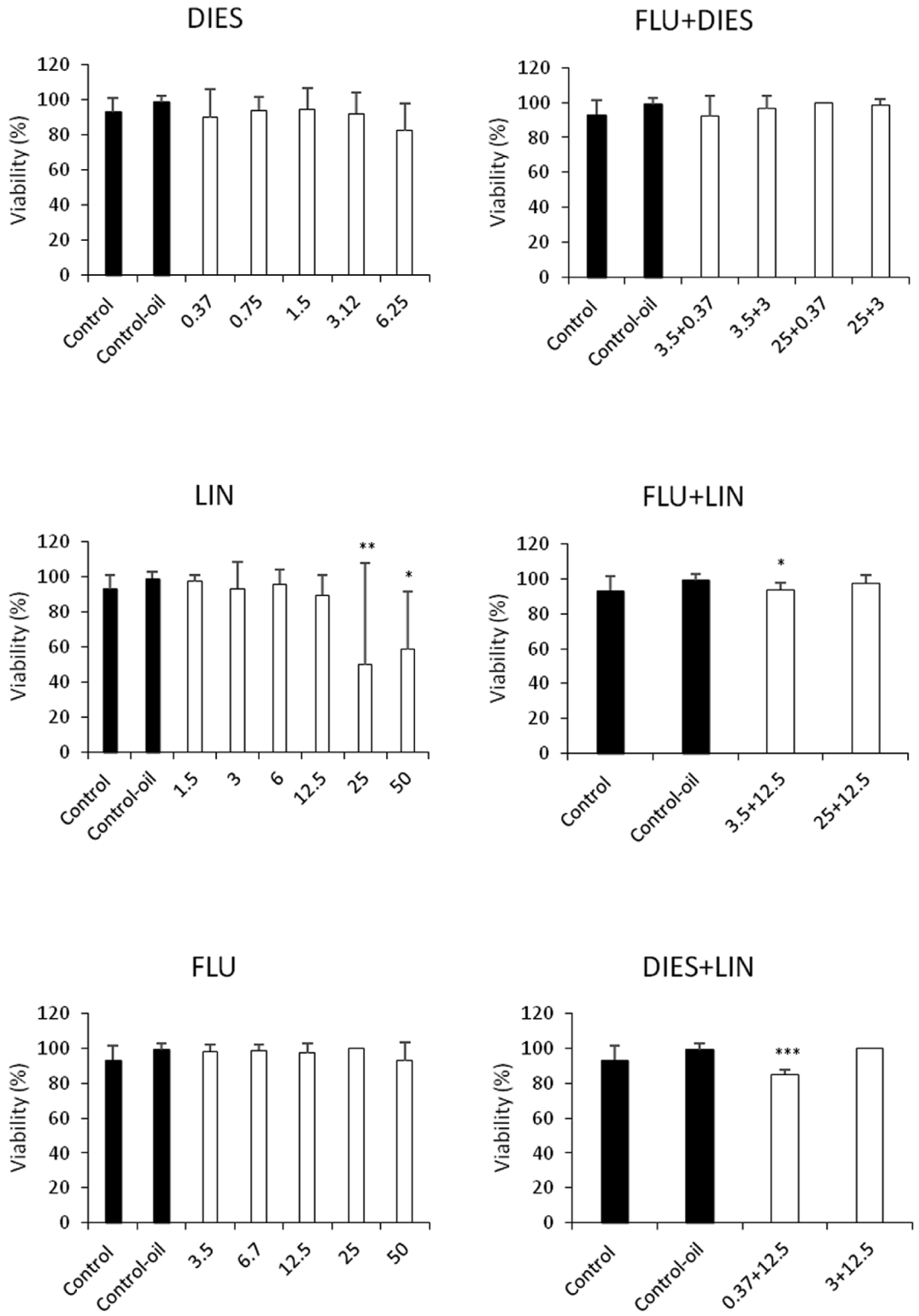

Fig. 2. Viability (%) of pups/litter until PND21 (n = 5, average of 4 male pups/litter). Single doses: DIES (μg/kg/day), dienestrol; LIN (mg/kg/day), linuron; and FLU (mg/kg/day), flutamide. Mixtures doses: FLU + DIES (mg/kg/day + μg/kg/day); FLU + LIN (mg/kg/day + mg/kg/day); and DIES + LIN (μg/kg/day + mg/kg/day). Data are given as means ± SD. * Significantly different from control-oil group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

used in the ANOVAs analysis. A MANOVA was also calculated to analyze body weight pup's data. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Body weights and litter survival

There were no statistical significant differences in the water and food intake of the F0 females (data not shown). The F0 female weight

Table 2
Organs weight and relative organ weight of male rat pups at PND21, from different groups of treatment (n = 5, average of 4 male pups/litter).

	Doses	Weight (g)					
		Liver		Right testis		Left testis	
		mean ± SD	p	mean ± SD	p	mean ± SD	p
Control		1.46 ± 0.37	0.726	0.08 ± 0.01	0.996	0.08 ± 0.02	0.738
Control-oil		1.47 ± 0.32		0.08 ± 0.02		0.09 ± 0.02	
DIES (μg/kg/day)	0.37	1.55 ± 0.36	0.995	0.10 ± 0.02	0.642	0.10 ± 0.02	0.600
	0.75	1.38 ± 0.09	0.984	0.08 ± 0.03	1	0.08 ± 0.02	1
	1.5	1.46 ± 0.25	1	0.08 ± 0.02	0.994	0.08 ± 0.02	0.981
	3.12	1.47 ± 0.23	1	0.07 ± 0.02	0.568	0.07 ± 0.02	0.538
	6.25	1.31 ± 0.40	0.864	0.07 ± 0.02	0.856	0.07 ± 0.01	0.734
LIN (mg/kg/day)	1.5	1.39 ± 0.29	0.996	0.09 ± 0.01	1	0.09 ± 0.01	1
	3	1.55 ± 0.27	0.998	0.09 ± 0.02	0.913	0.09 ± 0.02	0.966
	6	1.40 ± 0.26	0.999	0.09 ± 0.02	0.969	0.09 ± 0.02	0.994
	12.5	1.55 ± 0.38	0.999	0.09 ± 0.02	0.999	0.09 ± 0.02	1
	25	1.56 ± 0.66	0.999	0.08 ± 0.03	1	0.09 ± 0.03	1
	50	1.52 ± 0.33	1	0.10 ± 0.01	0.709	0.10 ± 0.01	0.853
FLU (mg/kg/day)	3.5	1.41 ± 0.15	0.995	0.09 ± 0.01	0.986	0.09 ± 0.01	0.980
	6.7	1.32 ± 0.21	0.716	,	0.290	0.07 ± 0.01	0.282
	12.5	1.38 ± 0.22	0.968	0.07 ± 0.01	0.771	0.07 ± 0.01	0.591
	25	1.32 ± 0.26	0.795	0.07 ± 0.01	0.156	0.07 ± 0.01	0.142
	50	1.40 ± 0.15	0.983	0.07 ± 0.01	0.308	0.07 ± 0.01	0.352
FLU + DIES (mg/kg/day+ μg/kg/day)	3.5 + 0.37	1.28 ± 0.19	0.770	0.07 ± 0.02	0.539	0.07 ± 0.02	0.564
	3.5 + 3	1.40 ± 0.30	0.996	0.09 ± 0.01	0.982	0.09 ± 0.01	0.972
	25 + 0.37	1.71 ± 0.34	0.715	0.08 ± 0.02	1	0.09 ± 0.02	1
	25 + 3	1.60 ± 0.32	0.945	0.07 ± 0.02	0.794	0.08 ± 0.02	0.963
FLU + LIN (mg/kg/day + mg/kg/day)	3.5 + 12.5	1.21 ± 0.05	0.235	0.08 ± 0.01	0.821	0.08 ± 0.01	0.598
	25 + 12.5	1.47 ± 0.47	0.999	0.07 ± 0.03	0.422	0.07 ± 0.02	0.273
DIES + LIN (μg/kg/day + mg/kg/day)	0.37 + 12.5	1.54 ± 0.25	0.961	0.09 ± 0.02	0.979	0.09 ± 0.02	1
	3 + 12.5	1.48 ± 0.32	0.998	0.09 ± 0.02	0.691	0.09 ± 0.02	0.993
		Relative Weight (g/b.w. (g))					
	Doses	Liver mean ± SD	p	Right testis mean ± SD	p	Left testis mean ± SD	p
Control		0.0378 ± 0.0033	0.189	0.0022 ± 0.0002	0.671	0.0022 ± 0.0002	0.500
Control-oil		0.0387 ± 0.0033		0.0022 ± 0.0002		0.0022 ± 0.0002	
DIES (μg/kg/day)	0.37	0.0371 ± 0.0025	0.916	0.0024 ± 0.0002	0.867	0.0024 ± 0.0002	0.857
	0.75	0.0376 ± 0.0029	0.981	0.0022 ± 0.0004	1	0.0022 ± 0.0003	1
	1.5	0.0385 ± 0.0022	1	0.0020 ± 0.0005	0.934	0.0021 ± 0.0005	0.843
	3.12	0.0394 ± 0.0045	0.998	0.0018 ± 0.0005	0.312	0.0019 ± 0.0005	0.274
	6.25	0.0383 ± 0.0054	1	0.0022 ± 0.0001	1	0.0022 ± 0.0001	0.999
LIN (mg/kg/day)	1.5	0.0366 ± 0.0033	0.922	0.0023 ± 0.0001	0.973	0.0023 ± 0.0001	0.995
	3	0.0371 ± 0.0009	0.977	0.0022 ± 0.0002	1	0.0022 ± 0.0001	0.996
	6	0.0378 ± 0.0020	0.999	0.0024 ± 0.0002	0.536	0.0024 ± 0.0002	0.571
	12.5	0.0418 ± 0.0098	0.729	0.0020 ± 0.0004	0.344	0.0020 ± 0.0004	0.104
	25	0.0388 ± 0.0010	1	0.0021 ± 0.0000	0.997	0.0022 ± 0.0001	0.998
	50	0.0372 ± 0.0031	0.994	0.0024 ± 0.0002	0.515	0.0024 ± 0.0001	0.801
FLU (mg/kg/day)	3.5	0.0369 ± 0.0009	0.821	0.0022 ± 0.0001	0.987	0.0023 ± 0.0001	0.885
	6.7	0.0385 ± 0.0028	1	0.0020 ± 0.0002	0.563	0.0021 ± 0.0002	0.525
	12.5	0.0374 ± 0.0026	0.942	0.0020 ± 0.0001	0.557	0.0020 ± 0.0001	0.230
	25	0.0366 ± 0.0031	0.717	0.0018 ± 0.0001	0.017*	0.0019 ± 0.0001	0.013*
	50	0.0321 ± 0.0042	0.002**	0.0019 ± 0.0002	0.044*	0.0019 ± 0.0003	0.032*
FLU + DIES (mg/kg/day+ μg/kg/day)	3.5 + 0.37	0.0369 ± 0.0011	0.807	0.0020 ± 0.0003	0.496	0.0020 ± 0.0003	0.533
	3.5 + 3	0.0371 ± 0.0038	0.868	0.0024 ± 0.0001	0.525	0.0024 ± 0.0000	0.653
	25 + 0.37	0.0415 ± 0.0037	0.605	0.0020 ± 0.0002	0.564	0.0021 ± 0.0002	0.674
	25 + 3	0.0381 ± 0.0027	0.996	0.0017 ± 0.0003	0.007**	0.0019 ± 0.0003	0.028*
FLU + LIN (mg/kg/day + mg/kg/day)	3.5 + 12.5	0.0364 ± 0.0014	0.361	0.0023 ± 0.0002	0.438	0.0023 ± 0.0002	0.739
	25 + 12.5	0.0368 ± 0.0052	0.535	0.0018 ± 0.0003	0.015*	0.0018 ± 0.0002	0.003**
DIES + LIN (μg/kg/day + mg/kg/day)	0.37 + 12.5	0.0383 ± 0.0003	0.976	0.0021 ± 0.0001	0.725	0.0021 ± 0.0002	0.640
	3 + 12.5	0.0360 ± 0.0028	0.202	0.0022 ± 0.0001	0.998	0.0022 ± 0.0001	0.728

DIES, dienestrol; LIN, linuron; FLU, flutamide. All values are expressed as mean ± SD.
* Significantly different from control-oil group (*p < 0.05; **p < 0.01).

during gestation and lactation was measured and no differences in the female weight between all groups at different time-points were found, except a punctual and statistically significant increase at GD6 in the DIES 6.25 (Suppl. Table 1).

A MANOVA with two factors (weight x group) was calculated to analyze body weight pups' data. The body weight change was statistically significant through the observation pup's period (p < 0.001, Samuel Stanley Wilks'). The interaction between body weight and treatment group was not statistically significant (p = 0.474, Samuel Stanley Wilks'), which means that there is not any effect of the treatment group in the body weight change of the pups.

There were also no significant differences between weights of

Table 3
Sex ratio (male:female) of litters at PND0/1 (n = 5, average of 4 male pups/litter).

	Doses	male:female	p
Control		20:25	0.280
Control-oil		43:32	
DIES (μg/kg/day)	0.37	89:56	0.994
	0.75	101:48	0.632
	1.5	16:25	0.630
	3.12	22:15	1
	6.25	29:18	0.992
LIN (mg/kg/day)	1.5	63:74	0.944
	3	13:10	1
	6	89:51	0.985
	12.5	21:20	0.996
	25	25:12	0.930
	50	29:9	0.080
FLU (mg/kg/day)	3.5	71:72	0.933
	6.7	20:31	0.312
	12.5	25:17	0.999
	25	111:97	0.991
	50	36:25	1
FLU + DIES (mg/kg/day + μg/kg/day)	3.5 + 0.37	11:17	0.540
	3.5 + 3	75:53	1
	25 + 0.37	75:50	0.999
	25 + 3	32:31	0.954
FLU + LIN (mg/kg/day + mg/kg/day)	3.5 + 12.5	76:79	0.605
	25 + 12.5	1:1	0.713
DIES + LIN (μg/kg/day + mg/kg/day)	0.37 + 12.5	3:4	0.571
	3 + 12.5	7:5	0.989

DIES, dienestrol; LIN, linuron; FLU, flutamide.

treated pups and control-oil pups at PND0/1, 4, 7, 14, and 21, but at PND4 a reduction (< 20%) was observed in the DIES 0.75 single dose group and the FLU 25 + LIN 12.5 mixture group. These reductions were not maintained at PND7 (Suppl. Table 2).

Supplementary material for body weight of F0 female and male pups were included. No relevant external signs of maternal toxicity were observed (i.e. diarrhea, piloerection, vaginal bleeding, periorbital bleeding, maternal body weight decrease, etc…).

Moreover viability, as the percentage of survival of pups (average of the 5 litters/group) until PND21, was calculated. There was a significant decrease in the viability of pups at both higher doses of LIN, i.e. at 25 and 50. Linuron mixtures also exhibited decreased viability, i.e. in the FLU 3.5 + LIN 12.5 and the DIES 0.37 + LIN 12.5 groups versus the control-oil group. Others groups did not exhibit viability differences (Fig. 2).

3.2. Organ weights

No significant differences between treated and control-oil groups were found in the liver, right testis and left testis weights (all in grams). However, when considering the relative weight (weight of the organ (in grams)/body weight of pup (in grams)), some significant differences were noticed: 1) the FLU 50 group displayed significant lower weights of the liver and both testes, whereas the FLU 25 only showed a lower weight of both testes; 2) the FLU 25 + DIES 3 and FLU 25 + LIN 12.5 showed significant lower values of both testes (Table 2). There were not any other differences in these relative organ weights between the treated, control and control-oil groups.

3.3. Feminization endpoints

The sex ratio of the pups was determined. The results showed that the sex ratio of the pups was not altered by the administration of the administered compounds (Table 3). In order to assess any potential feminization, male pups were examined for AGD, NR and cryptorchidism.

3.3.1. Anogenital distance (AGD)

Both the absolute AGD and relative AGD (AGD/g b.w.) were examined. The absolute and relative AGD in the control and control-oil groups was similar at all time points, excepting at PND14 where AGD was significantly higher in control group with respect to control-oil group. When exposed to single compounds, the absolute AGD was significantly lower in DIES 0.75 group at PND1 and DIES 3.12 group at PND4 and PND7 (compared to the control-oil group). LIN did not affect the AGD at given doses. All FLU groups significantly decreased the AGD, except FLU 3.5 at PND1 and PND21. At PND0/1, AGD of FLU 12.5, 25, and 50 groups have not been measured because it was not possible to differentiate between male and females (Fig. 3, Suppl. Table 3A). Regarding exposure to mixtures, the absolute AGD was significantly lower in the FLU + DIES and FLU + LIN groups at all studied time points (excepting FLU 3.5 + LIN 12.5 at PND0/1), as well as in the DIES + LIN group at PND4. At PND0/1, AGD of FLU 25 + DIES 0.37, and FLU 25 + DIES 3 was not determined because it was also not possible to differentiate between male and females as well as in the highest single FLU dose (Fig. 3, Suppl. Table 3A).

When examining the relative AGD, it was decreased in the DIES 3.12 and 6.25 groups at PND4, and DIES 3.12 at PND21, compared to the control-oil group. LIN did not affect the relative AGD at given doses. Male pups in the FLU groups exhibited a decrease in the relative AGD, at all doses, at PND4 and afterwards time points (except FLU3.5 at PND21) (Fig. 4, Suppl. Table 3A).

The relative AGD was lower in all dose groups of FLU + DIES mixtures at all the time points studied. The FLU 25 + LIN 12.5 mixture group displayed lower relative AGDs also at all the time points studied, whereas the FLU 3.5 + LIN 12.5 mixture group showed lower relative AGDs at PND4 and PND21. The DIES + LIN mixture did not affect the relative AGD (Fig. 4, Suppl. Table 3A). On the other hand, AGD index (AGD/cube root of b.w.) was included in the supplementary material (Suppl. Table 3B).

3.3.2. Nipple retention (NR)

Nipple retention was assessed in all male pups at PND14. The control group did not differ from the control-oil group in any aspect regarding this endpoint. DIES and LIN did not increase the nipples in the male pups compared to these in the control-oil group. There were not any differences in the percentage of pups affected per litter. However, FLU clearly affected NR, showing nipple retention in all treated pups (100%). At higher doses (12.5, 25 and 50), FLU affected all 12 nipples, while in the FLU 6.7 and FLU 3.5 groups, 9 and 7 nipples were respectively affected (on average) (Fig. 5, Suppl. Table 4).

With respect to the mixtures, FLU + DIES affected NR in all male pups (100%). FLU + DIES at doses of FLU 25 + DIES 3, and FLU 25 + DIES 0.37, affected all 12 nipples, FLU 3.5 + DIES 3 affected 10 nipples (on average) and FLU 3.5 + DIES 0.37 affected 8 nipples. The FLU + LIN mixtures also affected NR. FLU 25 + LIN 12.5 affected all 12 nipples while FLU 3.5 + LIN 12.5 affected 6 nipples on average. The DIES + LIN mixture did not affect the NR (Fig. 5, Suppl. Table 4).

3.3.3. Cryptorchidism

Cryptorchidism was assessed at PND21. For statistical analysis, it was recorded as (0) when both testes were nicely descended, as (1) if one testis was not descended, and as (2) if both testes were not descended. Surprisingly, cryptorchidism was observed in the control-oil group: cryptorchidism of one testis (1) in about 15%, and of both testes (2) in about 40% of the male pups. There were no significant differences between the control and control-oil group pups. It is interesting to note that most doses of all compounds (alone or combined) induced an increase in cryptorchidism, but it was not significant when compared to the controls (Fig. 6).

None of the DIES or LIN exposed pups, at any of the doses showed significant effects on cryptorchidism when compared to controls (Fig. 6). However, the FLU treatment at dose 50 increased the

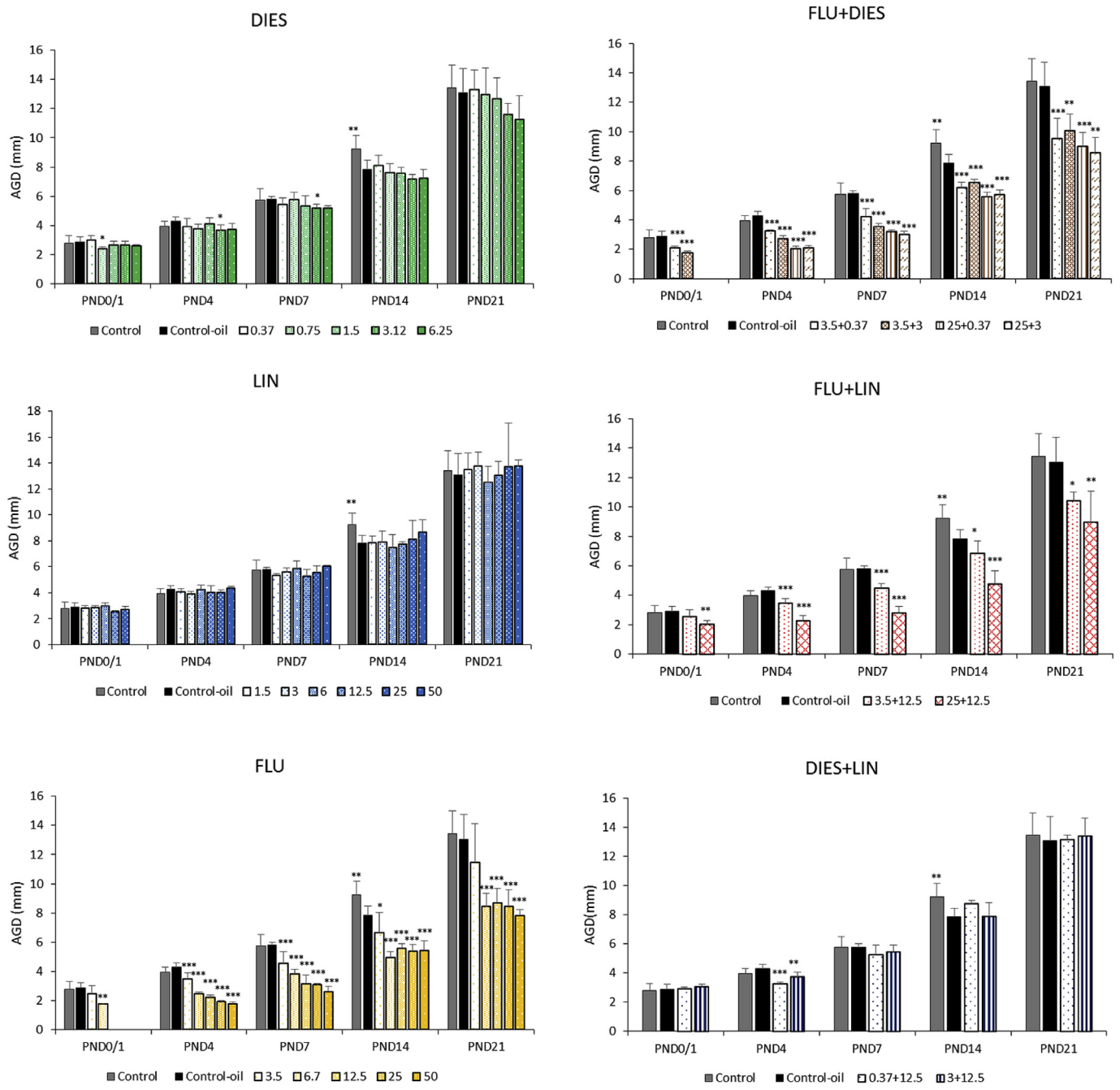

Fig. 3. Anogenital Distance (AGD) of rat pups at PND0/1, PND4, PND7, PND14 and PND21 (n = 5, average of 4 male pups/litter). Single doses: DIES (μg/kg/day), dienestrol; LIN (mg/kg/day), linuron; and FLU (mg/kg/day), flutamide. Mixtures doses: FLU + DIES (mg/kg/day + μg/kg/day); FLU + LIN (mg/kg/day + mg/kg/day); and DIES + LIN (μg/kg/day + mg/kg/day). Data are given as means ± SD. * Significantly different from control-oil group (*p < 0.05; **p < 0.01; ***p < 0.001).

percentage of pups per litter that had one or both testes not descended compared to the control-oil group. Also, FLU at 3.5 dose increased the percentage having one testicle not descended (Fig. 6). In relation to exposure to mixtures, an increase in 1 testis cryptorchidism in the FLU 3.5 + DIES 3 group was found. The FLU 3.5 + LIN 12.5 also showed an increase in cryptorchidism of one testis compared to the control-oil group. No differences with respect to the control-oil group were found in DIES + LIN mixture groups (Fig. 6).

4. Discussion

Although DIES is not currently being used clinically, it seems that in some countries it has not been yet withdrawn from the market (Mei et al., 2015; Socas-Rodríguez et al., 2017). Traces of DIES in the range of ng/kg or μg/kg have been found in yogurt (D'Orazio et al., 2016), milk (Gao et al., 2015; Socas-Rodríguez et al., 2013, 2018; Stypuła-Trębas et al., 2015), cheese and kefir (Socas-Rodríguez et al., 2017). Dienestrol has also been found in bovine muscle (Malone et al., 2009) and even in seawater (He et al., 2016). Nowadays, there is a great lack of in-vivo data on feminizing effects of DIES produced by its administration either in animals or in humans. Although the fetus is not in direct contact with these kinds of foods, the mother could have been exposed to DIES through the diet, and thus also the fetus via the placental route (Stefanidou et al., 2009), moreover, newborns can be

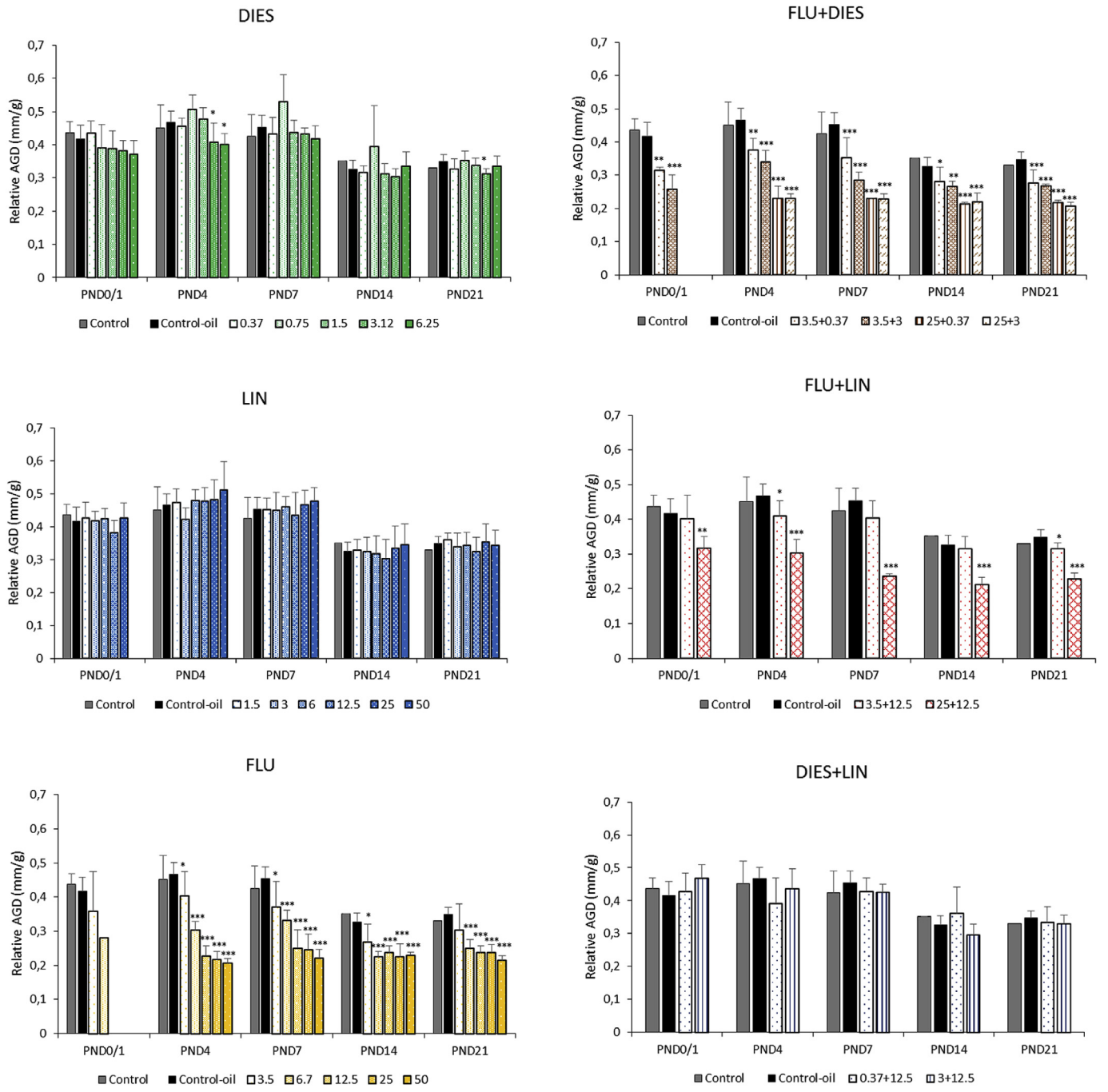

Fig. 4. Relative Anogenital Distance (AGD) of rat pups at PND0/1, PND4, PND7, PND14 and PND21 (n = 5, average of 4 male pups/litter). Single doses: DIES (μg/kg/day), dienestrol; LIN (mg/kg/day), linuron; and FLU (mg/kg/day), flutamide. Mixtures doses: FLU + DIES (mg/kg/day + μg/kg/day); FLU + LIN (mg/kg/day + mg/kg/day); and DIES + LIN (μg/kg/day + mg/kg/day). Data are given as means ± SD. * Significantly different from control-oil group (*p < 0.05; **p < 0.01; ***p < 0.001).

exposed through breast milk. In a recent study in rats, we showed that exposure to DIES during pregnancy induced toxicity on the reproductive system of the male rat offspring, which could affect the capacity of fertilization (Schreiber et al., 2019). Moreover, high DIES concentrations (12.5, 50, and 75 μg/kg/day) caused miscarriage due to its estrogenic potential (Schreiber et al., 2019).

Whit respect to LIN, it has been shown that a long exposure (2-years of dietary administration) to LIN inhibited fetal Leydig cell steroid hormone synthesis in male rats (Cook et al., 1993). An older study reported that LIN was neither teratogenic, nor embryo-toxic, when administered by gavage to pregnant rats from GD6 to GD15 (Khera et al., 1978). Linuron in combination with other EDCs also produced antiandrogenic effects in male pups through maternal exposure (Conley et al., 2018).

Altered junction protein expression after FLU treatment is likely related to disturbed testosterone and estradiol synthesis, which may result from impaired Leydig cell function (Hejmej and Bilinska, 2018). The estimated endocrine no-observed-effect-level (NOEL) for FLU is 0.25 mg/kg/day (Toyoda et al., 2000). Mimicking endogenous hormones is considered to be one of the important mechanisms of EDCs.

Feminizing effects are observed in rat offspring after administration of FLU during the gestation period. At different days of gestation, FLU-induced alterations in AGD and areolae/nipples in early postnatal life, correlated with a reduction in AGD, and retained nipples observed in

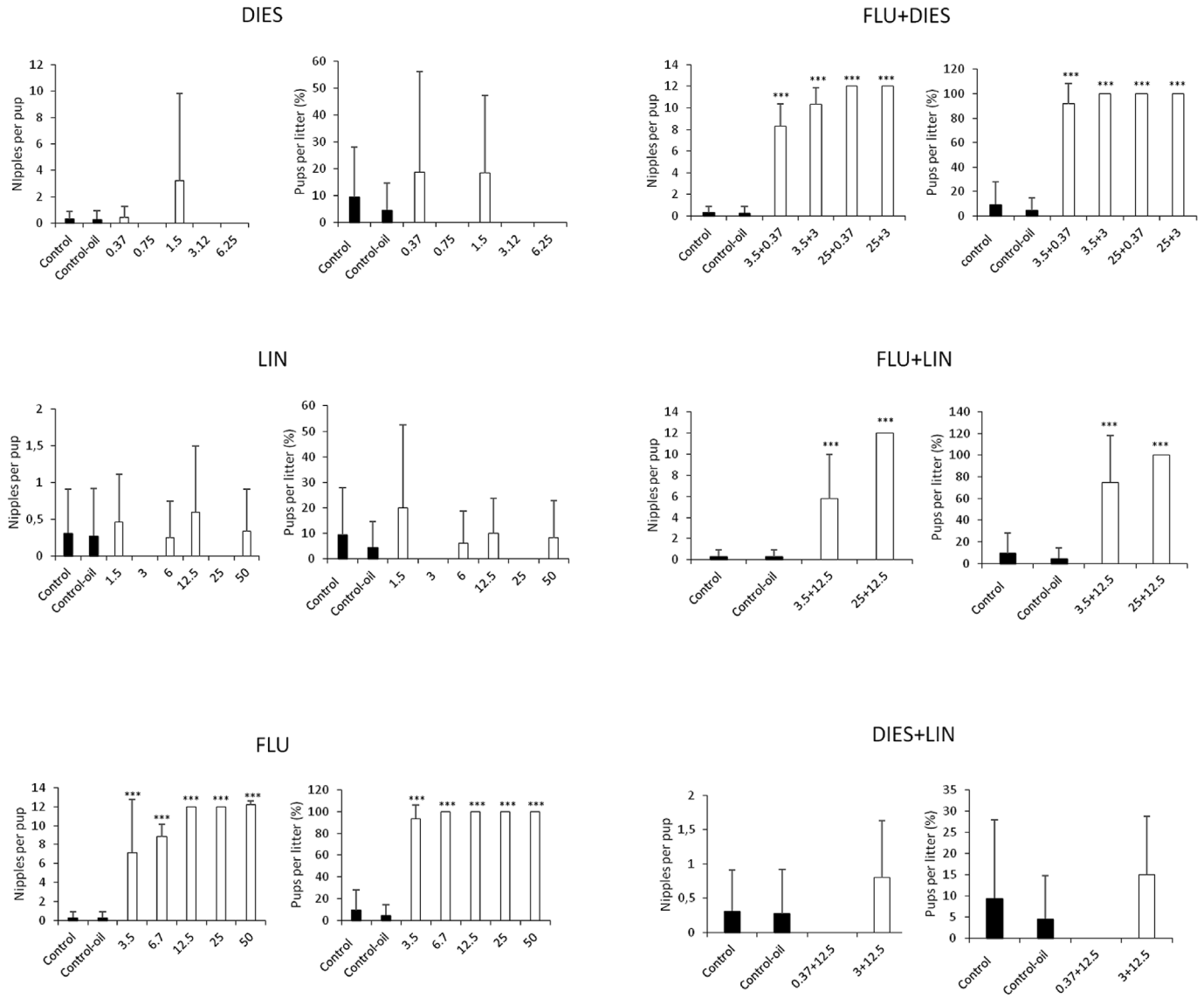

Fig. 5. Nipple Retention (NR) of male rat pups at PND14 (n = 5, average of 4 male pups/litter). Single doses: DIES (μg/kg/day), dienestrol; LIN (mg/kg/day), linuron; and FLU (mg/kg/day), flutamide. Mixtures doses: FLU + DIES (mg/kg/day + μg/kg/day); FLU + LIN (mg/kg/day + mg/kg/day); and DIES + LIN (μg/kg/day + mg/kg/day). Data are given as means ± SD. * Significantly different from control-oil group (***p < 0.001).

the adult (Foster and Harris, 2005; Foster and McIntyre, 2002; McIntyre et al., 2001). Prenatal FLU exposure also resulted in dose-responsive increases in cryptorchidism (Fussell et al., 2015). On the other hand, other authors reported stillborns and dead newborns at birth, hypospadias, penis malformations and small sex organs (McIntyre et al., 2001; Schneider et al., 2017; Yamasaki et al., 2005). These effects have been also observed when FLU is administered in combination with other EDCs (Conley et al., 2018; Hass et al., 2007).

Defects of the male reproductive tract are some of the most common malformations in boys at birth. It is known that exposure to chemicals such as urea-based herbicides (e.g. LIN), estrogens (e.g. DIES), or anti-androgens (e.g. FLU) can interfere with the androgen signaling pathway (Conley et al., 2018). These chemicals can act via several mechanisms including binding to hormone receptors, modifying the production or metabolism of endogenous hormones, or modifying the number of hormone receptors. Considering that humans are often exposed to mixtures of several compounds (EFSA, 2013) in this study we have assessed whether a binary combinations of LIN, DIES and FLU would have effects on AGD, NR and cryptorchidism on male offspring rats, whose mothers were treated with these compounds (single dose or binary mixtures) during pregnancy and subsequent lactation period.

In the offspring male rats, AGD was approximately two-fold higher than AGD of females, being highly correlated with an increase in reproductive tract malformations (Hotchkiss et al., 2004; McIntyre et al., 2002a,b). The analysis of areolae (NR) is free from the effects on body weight changes. Thus, the measurement of both endpoints is useful to determine the impairment of endocrine activity (Hotchkiss et al., 2004). In this way, cryptorchidism is a reproductive health problem that have been reported in males exposed to EDCs (Wilson et al., 2007). Furthermore, alterations in the weight of sex glands may reflect changes in the animal's endocrine status or male testicular function, as they are androgen-dependent (Campion et al., 2012).

In the current investigation, FLU produced a decrease of AGD (except FLU 3.5 at PND1 and 21), an increase of NR (about 100% of pups showed presence of nipples), as well as an increase of cryptorchidism of one testis at FLU 3.5 and total cryptorchidism at 50. In a previous study, Bozec et al. (2004), administered to rats FLU from GD10 to GD22. FLU induced cryptorchid testes at 10 mg/kg/day (the highest dose). Hass et al. (2007), treated rats from GD7 to PND16. The study shows a decreased AGD at different doses of FLU (to 1.0 until 16 mg/kg/day) and

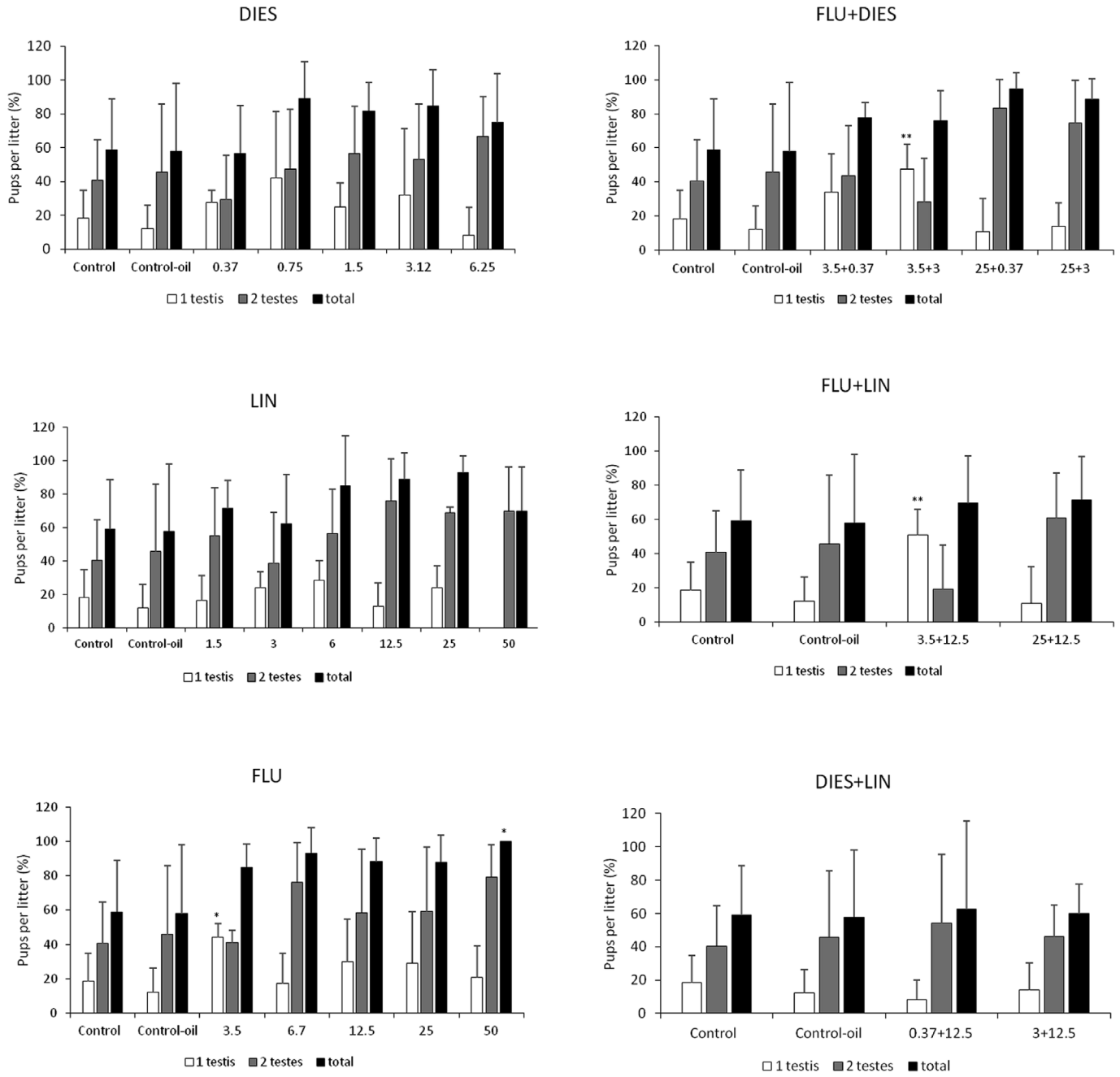

Fig. 6. Cryptorchidism of male rat pups at PND21 (n = 5, average of 4 male pups/litter). Single doses: DIES (μg/kg/day), dienestrol; LIN (mg/kg/day), linuron; and FLU (mg/kg/day), flutamide. Mixtures doses: FLU + DIES (mg/kg/day + μg/kg/day); FLU + LIN (mg/kg/day + mg/kg/day); and DIES + LIN (μg/kg/day + mg/kg/day). Data are given as means ± SD. * Significantly different from control-oil group (*$p < 0.05$; **$p < 0.01$).

NR at all doses tested (0.5–16 mg/kg/day), while the dose of FLU (0.77 mg/kg/day) used in mixture did not affect AGD. However, a dose-depend effect in NR was observed.

Miyata et al. (2002) treated rats from GD14 to PND3. These authors observed a decrease of AGD at 2.5 mg/kg/day (intermediate dose, 0.15–100 mg/kg/day). In turn, Yamasaki et al. (2005) treated rats from GD6 to PND20 at doses of 2 and 10 mg/kg, a reduced AGD and small testes were observed, but there was not effect on NR. In a recent study, Kita et al. (2016) treated females prenatally (GD13 to GD20), postnatally (PND23 to PND53) or the combination of pre- and postnatally with 20 mg/kg/day of FLU. Prenatal exposure decreased AGD in male pups, while postnatal exposure induced slight, but significant reductions, in male AGD with respect to prenatal exposure. On the other hand, McIntyre et al. (2001) administered FLU from GD12 to PND21 to pregnant rats. They observed a decrease in the AGD at birth, an increase of NR in pups, undescended testes, hypospadias, prostate agenesis, and epididymal agenesis and decreased weights of the seminal vesicles. In a previous study, Mylchreest et al. (1999) administered FLU from GD12 to GD21 and also observed a decrease in the AGD, an increase of NR in pups, undescended testes, hypospadias, and sexual organ agenesis. In these studies, the concentrations of FLU were much higher than the above-mentioned doses (Miyata et al., 2002; Yamasaki et al., 2005) even for the lowest dose (from 6.25 to 50 mg/kg/day, and 100 mg/kg/day respectively). According to all studies reviewed, the most sensitive endpoints from the reproductive developmental toxicity were AGD, NR, and sometimes testis descent, along with sex organ weights (Zacharia, 2017).

Although the effects of FLU on the offspring depend basically on the

treatment period (during organogenesis phase, lactation or not) and the doses administered to the females, our results are quite consistent with previous studies of other authors (McIntyre et al., 2001; Miyata et al., 2002; Yamasaki et al., 2005). In all the studies referred above, FLU was orally administered to pregnant rats.

Despite the results of previous studies confirmed that LIN impairs male reproductive tract in rats (Ding et al., 2017; Hotchkiss et al., 2004; Wilson et al., 2009), the current results show that LIN alone did not affect the selected endpoints at given doses (1.5–50 mg/kg/day). Wolf et al. (1999) reported a decrease of AGD in rats treated with 100 mg/kg of LIN from GD14 to GD18. In turn, Lambright et al. (2000) described that offspring of dams orally administered a dose of 50 mg/kg (or greater) of LIN by gavage during androgen-dependent reproductive development exhibited permanent decreases in AGD, presence of areolae and nipples, and histological abnormalities in epididymis and vas deferens. McIntyre et al. (2000, 2002a, b) reported a significant decrease in AGD when pregnant rats were given by gavage, LIN (50 mg/kg/day) from GD12 to GD21. An increase of NR was also observed in male offspring (PND35).

Finally, no effects related with the end-points here evaluated were observed after DIES administration. No data on the effects (AGD, NR and cryptorchidism) of this compound are available in the scientific literature.

In relation to mixtures, the present results showed that the combination of FLU + LIN increased NR and decreased AGD, but no significant effects in cryptorchidism were noticed. FLU + DIES also provoked an increase of NR and a decrease of AGD, but no significant effects in relation to testes descent. The last mixture studied, DIES + LIN, did not produce neither an increase of NR, a decrease of AGD, nor effects in descent of testes. We observed that when combined FLU at the current doses, it induced NR in the male offspring, while both testes had lower weights than those of animals in the control-oil in the FLU 25 + LIN 12.5 group, mainly due to the FLU action.

Rider et al. (2008) also observed NR as well as hypospadias, epididymal agenesis, undescended testes and seminal vesicle weight loss in rats exposed to a mixture containing low doses of LIN plus vinclozolin, procymidone, prochloraz and phthalates during GD14-21. Moreover, these authors stated that these compounds disrupt male reproductive development in a cumulative manner by comparing the observed response and predicted mixture responses (assessed by logistic models). In fact, dose additive effects on male reproductive tract development were also reported by Rider et al. (2010), who tested the exposure of 10-chemical mixtures, including LIN, in prenatal rats (GD14-21) at doses near the individual chemical NOAELs. Furthermore, it has been described that lower doses -than those given by Rider et al. (2010)- of LIN combined with FLU and other 16 antiandrogenic chemicals impaired male development in exposed rats at GD14-18 (Conley et al., 2018). Thus, cumulative low doses exposure of pregnant rats to multiple antiandrogenic chemicals could promote increased risks in male fetuses. On the other hand, Hotchkiss et al. (2004), who administered a high dose of LIN (75 mg/kg/day) to rats, found that prenatal exposure at GD14-18 of LIN alone, as well as combined with benzyl butyl phthalate (BBP at 500 mg/kg/day), resulted in reductions in AGD and ventral prostate. LIN appears to be more potent than BBP in reducing fetal androgen synthesis and in inducing epididymal agenesis. On the other hand, some authors reported that two or more antiandrogens with mixed mechanisms of actions affected more and in a more severe manner than either of the individual treatments, being able to act in cumulative and dose-additive manner, altering sexual differentiation in the rat (Christiansen et al., 2009; Kortenkamp et al., 2007; Rider et al., 2008).

The results of the present investigation conclude that FLU is a chemical able to induce a decrease of AGD, increase of NR and cryptorchidism. The binary combinations with LIN and DIES, at the doses here assessed, did not modify the effects produced by the FLU. Therefore, additional research is needed in order to determine how the mixtures of the studied compounds affect male reproductive tract development. It is known that severe alterations of sexual differentiation can be produced in rat laboratory studies, so the questions of what would be expected in exposed humans to mixture compounds is still unknown.

CRediT authorship contribution statement

Elga Schreiber: Data curation. **Tània Garcia:** Formal analysis, Data curation. **Neus González:** Data curation. **Roser Esplugas:** Data curation. **Raju Prasad Sharma:** Formal analysis. **Margarita Torrente:** Formal analysis, Writing - original draft. **Vikas Kumar:** Formal analysis. **Toine Bovee:** Formal analysis, Writing - original draft. **Efrosini S. Katsanou:** Formal analysis. **Kyriaki Machera:** Formal analysis. **José Luis Domingo:** Formal analysis, Writing - original draft. **Mercedes Gómez:** Writing - original draft, Formal analysis.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2020.111256.

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