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Reproductive toxicity by exposure to low concentrations of pesticides in *Caenorhabditis elegans*

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ABSTRACT

In view of the recurrent applications of pesticides in agricultural producing countries, the increased presence of these substances in the environment raise a demand for the evaluation of adverse effects on non-target organisms. This study assesses the impact of exposure to five pesticides suspected of being endocrine disruptors (atrazine, 2,4-dichlorophenoxyacetic acid, mancozeb, chlorpyrifos and cypermethrin) on the reproductive development of the nematode *Caenorhabditis elegans*. To this end, nematodes in the L4 larval stage were exposed to different concentrations of pesticides for 24 h and the consequences on brood size, percentage of gravid nematodes, expression of reproductive-related genes and vitellogenin trafficking and endocytosis were measured. Moreover, 17β-estradiol was used as an estrogenic control for endocrine disrupting compounds throughout the work. The results showed that all the pesticides disturbed to some extent one or more of the evaluated endpoints. Remarkably, we found that atrazine, 2,4-dichlorophenoxyacetic acid and chlorpyrifos produced comparable responses to 17β-estradiol suggesting that these pesticides may have estrogen-like endocrine disrupting activity. Atrazine and 17β-estradiol, as well as 2,4-dichlorophenoxyacetic acid and chlorpyrifos to a lesser extent, decreased the brood size, affected vitellogenin trafficking and endocytosis, and changed the expression of several reproductive-related genes. Conversely, mancozeb and cypermethrin had the least impact on the evaluated endpoint. Cypermethrin affected the brood size at the highest concentration tested and mancozeb altered the distribution of vitellogenin only in approximately 10% of the population. However, both products overexpressed *hus-1* and *vit-2* genes, indicating that an induction of stress could interfere with the normal development of the nematode. In conclusion, our work proved that *C. elegans* is a useful biological model to identify the effects of estrogen-like endocrine disruptor compounds, and the sublethal endpoints proposed may serve as an important contribution on evaluating environmental pollutants.

1. Introduction

Pesticides are substances (or mixtures of substances) conceived to control pests. They are mainly applied in agriculture to improve crop yields or to protect foods during storage, but they are also used to mitigate organisms that are considered to be harmful or pernicious at home or in public spaces. The term includes, amongst others, herbicides, fungicides and insecticides. The annual global consumption of pesticides is approximately 2 million tons, demonstrating the strong position in the market and the dependence of these products by the current agricultural

Abbreviations: ATR, atrazine; CPF, chlorpyrifos; CTF, corrected total fluorescence; CYP, cypermethrin; DMSO, dimethylsulfoxide; E2, 17β-estradiol; EDCs, endocrine-disrupting chemicals; MCZ, mancozeb; NGM, nematode growth medium; USEPA, U.S. Environmental Protection Agency; VIT, vitellogenin; 2, 4-D, 2,4-dichlorophenoxyacetic acid.

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system (Sharma et al., 2019). Pesticides occupy a unique position among xenobiotics that humans encounter daily. In fact, they have been found in human and animal tissues all over the world connected to dietary, environmental and occupational exposure (Anwar, 1997; Dietz et al., 2013; Kumar and Mukherji, 2018; Kuang et al., 2020). In this context, there is growing concern about the adverse effects that pesticides can cause, creating a demand for the rapid and predictive evaluation of their effects on human health and the environment (Alewu and Nosiri, 2011; Zheng et al., 2016).

The hazardous effects and the modes of toxic action of pesticides on non-target organisms can be diverse, including impairment in normal body functioning, neurotoxicity, carcinogenicity and immune system disorders (Khan and Law, 2005; Mostafalou and Abdollahi, 2017). Furthermore, some pesticides can act like endocrine-disrupting chemicals (EDCs) and affect the homeostasis of organisms by interfering with the normal hormone signaling (Yilmaz et al., 2020). Over recent decades, based on human epidemiological studies and laboratory experiments with animals, a significant increase in reproductive problems have been linked to EDC exposure (Le Moal et al., 2016; Segner et al., 2003). Indeed, many pesticides deemed EDCs were found to impair the animal reproductive system and its functioning, thereby causing negative effects on fertility and population sizes (Bhardwaj and Saraf, 2014; Frazier, 2007; Lushchak et al., 2018). However, even though we know pesticides can induce reproductive toxicity, in many cases their underlying mechanisms are still unclear.

Taking into consideration the ubiquity of pesticides in the environment, the endocrine disruption and the effects on reproduction caused by the exposure to pesticides are high priority issues in toxicology. However, even though EDCs have been studied for 25 years, the methods to identify and regulate them are still under development (Fuhrman and Arnon, 2015; Schug et al., 2016). This is because EDCs elicit complex responses in exposed animals through a wide range of mechanisms of action that can vary by concentration and life stage of the organism. Therefore, in order to reduce the risk associated with the exposure of EDCs, a large number of assays are essential to prove a causal relationship between their exposure and detrimental effects on reproductive functions. Traditionally, the evaluation of human reproductive risk from chemicals includes the extrapolation of data obtained from mammalian models, such as rodents, rabbits and nonhuman primates. Nevertheless, considering the vast amount of new chemicals in the market to be tested, animal experiments would require the sacrifice of millions of individuals (Hecker and Hollert, 2011). The above goes against the global trend to replace the use of vertebrates with alternative models in laboratory tests. In actual fact, in September 2019, the U.S. Environmental Protection Agency (USEPA) announced that it will reduce requests and funding for studies with laboratory mammals by 2025 and will completely eliminate them by 2035 (USEPA, 2019). In this sense, *Caenorhabditis elegans* is an excellent alternative biological model whose use does not raise the ethical issues associated with vertebrate research and that offers several advantages to develop bioassays to estimate the potential risk of reproductive toxicity of xenobiotics (Khabib et al., 2021).

C. elegans is a non-parasitic nematode that inhabits soil and leaf-litter environments with a cosmopolitan distribution (Schulenburg and Félix, 2017). The success of this nematode as a powerful tool for biological research is due to its easy and inexpensive culture in the laboratory. Furthermore, the short life cycle (2.5–3 days at 20 °C) and large number of offspring allow the production of nematodes on a large scale and a short period of time (Hunt, 2017). Its life cycle is divided into the embryonic stage, four larval phases (L1–L4) and adulthood. The small size and transparency allow the use of fluorescent markers to study biological processes *in vivo*. Moreover, *C. elegans* is a promising model that has the potential to link the *in vitro* cell-based toxicological assays and *in vivo* vertebrate studies. This organism adapts well to high-throughput technologies and it also provides physiologically relevant information as a whole animal. In addition, it is a globally accepted model for environmental impact assessment and there are even bioassays validated by international standards (ASTM E 2172–01, 2002; ISO, 2010).

C. elegans has a highly differentiated and simple reproductive system. Under natural conditions, most of the specimens are self-fertilizing hermaphrodites in which sperm and oocytes develop sequentially from a common gonad (Corsi et al., 2015). Hermaphrodites have a female gonad with two symmetrical U-shaped arms connected to a central uterus through the spermatheca (Kimble and Crittenden, 2005). Before reaching adulthood, the functional reproductive system is developed. The gonadal arms, the spermatheca and the uterus are formed from the L3 larval stage and the first part of the last L4 larval stage (Kimble and Hirsh, 1979). Specifically, spermatogenesis initiates at the L3 stage and is completed during the L4, resulting in the production of all sperm cells that are then stored in the two spermathecae (L'Hernault, 2006). After this process, begins the development of female germ cells exclusively. Along each gonad arm these germ cells pass a mitotic zone to continuously generate a pool of potential oocytes. Then, oocytes progress to diakinesis, where they are arrested until meiotic maturation (Laband et al., 2018). In the proximal gonad, fully developed oocytes sequentially migrate to the spermatheca to be fertilized, proceed to the uterus, and finally the eggs are laid within two to three hours after fertilization (Greenstein, 2005; Huelgas-Morales and Greenstein, 2018). Signaling pathways and many genes involved in spermatogenesis and oogenesis have been studied in detail and identified in *C. elegans* (Gumienny et al., 1999; L'Hernault, 2009). Therefore, this alternative model is a valuable tool to study how xenobiotics (including EDCs) could alter the development of the reproductive system, and from there, establish the first scientific bases to approach the consequences of these compounds on the reproduction of higher organisms.

Accordingly, we used *C. elegans* as the model to research the potential adverse effects on reproductive development due to the exposure to five pesticides suspected to be EDCs and widely applied in extensive crop production worldwide (Cecconi et al., 2007; Mnif et al., 2011). In this article, we explore new approaches to further characterize the hazard of pesticide uses, that could contribute to innovative regulatory policy to protect human and environmental health. In order to do that, nematodes in L4 stage were treated with the herbicides atrazine (ATR) and 2,4-dichlorophenoxyacetic acid (2,4-D), the insecticides chlorpyrifos (CPF) and cypermethrin (CYP) and the fungicide mancozeb (MCZ). The effects on brood size, percentage of gravid nematodes, mobilization and endocytosis of vitellogenin and expression of reproductive-related genes were evaluated. Additionally, 17β-estradiol (E2) was used as a positive estrogenic control of EDC throughout all work because it was found that *C. elegans* responds to this vertebrate hormone (Hoshi et al., 2003; Mimoto et al., 2007; Tominaga et al., 2003). Our work demonstrated that the use of *C. elegans* as a biological model allows to identify and characterize the pesticides with estrogenic-like EDCs properties. The data suggest that these bioassays using the nematode *C. elegans* could be included as part of pesticides regulatory policies for environmental and human health protection.

2. Materials and methods

2.1. Chemicals

Commercial formulations of pesticides were purchased as follows: ATR (TRAC 50 FL®, containing 1-chloro-3-ethylamino-6-isopropylamino-s-triazine at 500 g/l as the active ingredient) was from Atanor (Argentina); 2,4-D (DMA®, containing 2,4-dichlorophenoxyacetic acid at 485 g/l) and CPF (LORSBAN® 48E, containing O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate at 480 g/l) were from Dow AgroSciences S.A. (Argentina); CYP (GLEXTRIN®, containing α cyano-3- (3-phenoxyphenyl) cis- trans- 3 (2,2-dichlorovinyl) 2,2-dimethyl cyclopropane carboxylate at 25 g/l) was from GLEBA (Argentina); and MCZ (CANDIL 80®, containing ethylene bis dithiocarbamate combined with zinc ions at 80 g/100 g) was from Agristar (Argentina). The E2 was

supplied by Santa Cruz Biotechnology Inc. (United States of America) and the Trizol Reagent by Thermo Fisher Scientific (United States of America). All other chemicals were of the highest purity available.

Stock solutions of the commercial formulation of ATR, 2,4-D, CPF, CYP and MCZ were freshly made with bidistilled water and E2 with dimethylsulfoxide (DMSO). Different volumes of the stock solution were mixed with M9 buffer (per liter: 3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml of 1 M $MgSO_4$; pH 7.2), or DMSO in the case of E2, to achieve working dilutions expressed in terms of the final active ingredient concentration presented in each assay.

2.2. Organism and culture conditions

The *C. elegans* strains used in the current study were N2 var. Bristol and RT130 [pwls23 (VIT-2::GFP)] obtained from the Caenorhabditis Genetics Center at the University of Minnesota (Minneapolis, MN, USA). *C. elegans* was routinely propagated on nematode growth medium (NGM, per liter: 17 g agar, 2.5 g peptone and 3 g NaCl; with the addition after autoclaving of 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, 25 ml 1 M KH_2PO_4, and 1 ml of a solution containing 5 g/l cholesterol, prepared in ethanol) plates seeded with *Escherichia coli* strain OP50 at 20 °C as described by Brenner (1974). Synchronization of worm culture was achieved by treating gravid hermaphrodites with an alkaline bleach solution (Stiernagle, 2006). The eggs were incubated overnight in a rotor with gentle shaking at 20 °C for hatching and the L1 larvae were then transferred to NGM plates with food to continue their normal development process in a synchronized way until the L4 stage.

2.3. Nematode pesticide acute exposure

Age-synchronized L4 staged nematodes were treated with several concentrations of E2 or commercial formulation of pesticides in M9 buffer for 24 h at 20 °C supplemented with *E. coli* OP50 as a food source to reach a final optical density at 600 nm of 1 unit. The exposure concentrations (µg/l) expressed in terms of the final active ingredient presented in each assay were 0.001, 0.01, 0.1 and 1 of E2; 1, 10, 100 and 1000 of ATR; 1, 10, 100 and 1000 of 2,4-D; 0.1, 1, 10 and 100 of CPF; 0.1, 1, 10 and 100 of CYP; and 0.1, 1, 10 and 100 of MCZ. The worms incubated with M9 buffer without any pesticide or 0.001 g/l DMSO without E2 were considered as a negative control group. A 24-well multiwell plate with a completely randomized design was used for the exposure. After the exposure period each biological response was evaluated as described below.

2.4. Brood size measurement

L4-staged nematodes were treated with E2 or commercial formulation of pesticides as described above. To determine the offspring of the treated nematodes, 15 hermaphrodites were randomly selected per treatment and each one placed on a plate with NGM medium seeded with *E. coli*. The parental individuals were transferred daily to fresh plates with food and the eggs in the old plates were incubated at 20 ºC for the hatching and growth of the offspring. The count of the offspring was performed by quantifying the number of larvae until the end of the egg-laying period of the treated nematodes. Five independent biological replicates were carried out and the data is expressed as mean ± SD.

2.5. Determination of the percentage of gravid nematodes

L4-staged nematodes were treated with E2 or commercial formulation of pesticides as described in 2.3. Percentage of gravid nematodes was measured by microscopic observation of 100 nematodes per treatment, considering that a hermaphrodite is pregnant when it has one or more eggs inside the uterus (Höss et al., 2009). Three independent biological replicates were performed per treatment and the data is expressed as mean ± SD.

2.6. Reverse transcriptase real-time PCR analysis

L4 staged N2 strain nematodes were treated as described in 2.3. Then, 6000 nematodes were harvested, washed five times with M9 buffer, resuspended into 200 µl of Trizol Reagent and kept at − 80 °C until further use. Total RNA was isolated with Trizol Reagent protocol and was quantified with a qubit RNA HS assay kit using the qubit fluorometer (Thermo Fisher Scientific, United States of America). Complementary DNA (cDNA) synthesis was performed using the Superscript II Reverse Transcriptase (Thermo Fisher Scientific, United States of America) according to the manufacturer's recommendations. cDNA was diluted 1:4 and 1 µl of the resulting cDNA preparation was used for quantitative real-time PCR using the Fast-Plus EvaGreen® Master Mix (Biotium, United States of America) and an Applied Biosystems 7500 Fast Real-Time PCR system. The oligonucleotide sequences of all the primers were designed according to the sequences retrieved from www.wormbase.org and using Primer 3 Plus software and checked for specificity with NCBI BLAST (Johnson et al., 2008; Untergasser et al., 2007). Table 1 shows the list of primers. Gene expression was assessed relative to its non-pesticide respective control and a stable transcript, *cdc-42*, was used as reference gene (Hoogewijs et al., 2008). The relative quantification was calculated using the software LinRegPCR (Ramakers et al., 2003). For each replicate every gene was amplified three times. The results represent the mean ± SEM of three independent biological replicates.

2.7. Vitellogenin distribution and endocytosis

L4-staged nematodes of strain RT130 were exposed for 24 h at 20 °C to pesticides as described in 2.3 and subsequently were washed three times with M9 buffer. The animals were anesthetized using 20 mM sodium azide, were mounted on 20 g/l agarose pads and were photographed using a Nikon Eclipse 50i optical microscope with fluorescence (excitation wavelength 460–495 nm, emission wavelength 510–550 nm), 40X magnification and equipped with a Nikon CoolPix S10 digital camera. The distribution of the VIT-2::GFP was observed and the percentage of animals possessing abnormal distribution was counted through the number of fluorescent agglutination. At least 100 nematodes per replicate were analysed. Three independent replicates were performed per treatment and the data is expressed as mean ± SD.

The fluorescence of intrauterine embryos of two-cell stage from photographed adult nematodes was quantified to estimate vitellogenin endocytosis. For this end, the ImageJ software was used and the corrected total fluorescence (CTF) was calculated with the following equation: CTF = Egg fluorescence - (Egg area x Mean background fluorescence) (Fitzpatrick, 2014). The results were expressed as the fluorescence of each treatment relative to its control and represent the mean of three independent biological replicates, for which 20 embryos were analysed in each one.

2.8. Statistical analysis

Statistical analysis and all the graphs generated in this work were carried out using Graphpad Prism 6.0 software (GraphPad Software, San Diego, CA). Statistical significance was assessed using ANOVA followed by Dunnett's multiple comparison test to compare the means of brood size, percentage of gravid nematodes, gene expression and vitellogenin trafficking with respect to the control. Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. Effect of exposure to ED-putative pesticides on brood size and on the percentage of gravid nematodes

To analyse the reproductive toxicity of different pesticides in

Table 1
Gene name, function and primer sequences of genes used in reverse transcriptase real-time PCR analysis.

Gene	WormBase ID	Gene function	Primer pair (5'−3')
vit-2	WBGene00006926	Vitellogenin structural protein, precursor of egg yolk (Perez y Lehner, 2019)	AGAAGGACACCGAGCTCATCC TCTCGACTTCTTGGATTTGCTC
cpb-3	WBGene00000772	Cytoplasmic polyadenylation element-binding protein enriched in oogenic cells and required for normal oogenesis (Hasegawa et al., 2006)	TGGGCATTGGATCACGGAAA CGCCGACGATGATAGGGAAA
hus-1	WBGene00002042	Nuclear protein expressed in adult germ lines in response to DNA damage (Hofmann et al., 2002)	AATTCGTACTCCAGGCCGTC GCTGTGAGCTTCACAACTCG
spe-11	WBGene00004965	Perinuclear protein involved in the dynamic morphology of sperm pseudopods and required for egg activation after fertilization (Nishimura and L'Hernault, 2010)	AGGGATTCTTCAACTGCGGG CCATTGTCGGGTTACCTGCT
spe-6	WBGene00004960	Serine/threonine protein kinase needed for sperm activation, progression through late prophase of meiosis I and assembly of the major sperm protein (Varkey et al., 1993)	TGGCTTTGTCGAGACGTGAA CGGGATGATTTTGCCCTCCT
glp-1	WBGene00001609	Member of LIN-12/Notch receptor family that modulates oocyte growth when sperm are available, essential for both germline maintenance and proliferation during gonad development (Nadarajan et al., 2009).	CCAACAAGTGCAGCATCGTC GGTGGTGCCATTTCATGGTT
fem-3	WBGene00001413	Essential protein for hermaphroditic germline sperm/oocyte switch, positive regulator of spermatogenesis in the germ line (Kimble and Crittenden, 2007)	AGGGCTTCCACGTATTGAGC GCGAGAGATTTCAAACGGCG
cdc-42	WBGene00000390	RHO GTPase, used as housekeeping gene (Hoogewijs et al., 2008)	CTGCTGGACAGGAAGATTACG CTCGGACATTCTCGAATGAAG

C. elegans, age-synchronized nematodes were exposed during 24 h to a concentration range of the pesticides from L4 larval stage to adulthood and several reproductive endpoints were quantified. This life period coincides with the last stage of sexual development of *C. elegans*, when the germ cells reach their maximum number and the reproductive organs mature, both hormonally regulated processes (Hall et al., 2017). Pesticides were used in commercial formulation considering that active ingredients are accompanied by adjuvants, resulting in products that can be more toxic than their active compounds alone (Mesnage et al., 2014). The tested concentrations of the pesticides were selected including values lower than the levels allowed in the Guidelines for the quality of drinking water as well as considering concentrations found in the environment (Flores-García et al., 2011; Gagneten et al., 2020; Marino and Ronco, 2005; WHO, 2017; Wijnja et al., 2014). Likewise, the analysis of the steroid hormone E2, the major female sex endocrine regulator of vertebrates, was included as a positive control for the screening of estrogenic-like effects of EDCs. It was already reported that E2 affected the number of germ cells and reproduction of *C. elegans* (Hoshi et al., 2003; Tominaga et al., 2003).

Fig. 1 shows the effect of pesticides treatment on the brood size of the nematode. Compared with each respective negative control, E2, as well as ATR, 2,4-D and CPF significantly affected the size of the nematode offspring at almost all the concentrations tested. ATR was the pesticide analysed that most affected the brood size, causing decreases close to 30% at the highest tested concentrations (10, 100 and 1000 μg/l). In the case of 2,4-D, the decrease in the number of the nematode progeny was between 12% and 21% at all concentrations, while with CPF the effect was of 12–13% at the concentrations 0.1, 10 and 100 μg/l. Besides that, the results showed that MCZ had no effect on the brood size of the hermaphrodites exposed at any of the concentrations, while CYP only affected at the highest concentration assessed (100 μg/l). It is important to note that by bright field microscopic observation, neither physical abnormality in the treated hermaphrodites nor embryonic lethality have been detected as a result of the pesticides treatments performed (data not shown).

To determine if the pesticides treatments cause a delay in reaching sexual maturation, a bioassay to establish the percentage of gravid worms after the exposition of L4 larval nematodes during 24 h was performed. As shown in Fig. 2, the worms treated with E2 or ATR showed a decrease in the percentage of gravid nematodes at all the concentrations evaluated, while the worms treated with 2,4-D did so only at 10 μg/l of the herbicide. CPF, MCZ and CYP did not have any significant effect on the percentage of gravid worms within the range of concentrations studied. These results suggest that treatment with ATR and 2,4-D could be causing a delay in the maturation of the nematode reproductive system as well as E2 positive control.

3.2. Changes in the expression patterns of reproductive-related genes upon exposure to ED-putative pesticides

It is known that the sexual maturation of *C. elegans* depends upon the switch in expression of hormonally regulated genes between L4 stage and the young adult. To study if the female and/or male reproductive system could be affected upon exposure to the ED-putative pesticides, a gene expression analysis on selective genes after the pesticide treatment of the L4 stage nematodes during 24 h was performed. Once again, the

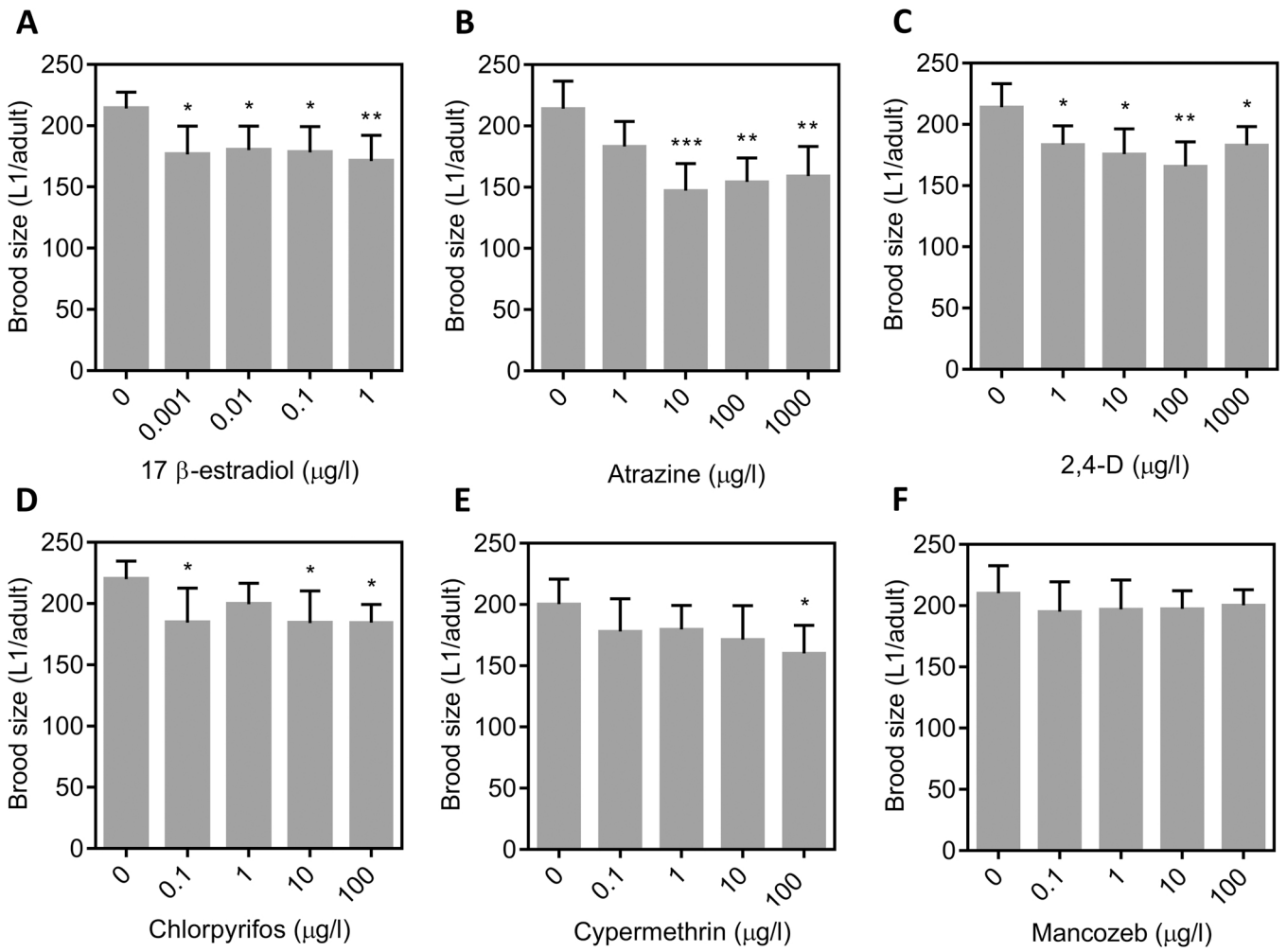

Fig. 1. Brood size of nematodes exposed to E2 (A), ATR (B), 2,4-D (C), CPF (D), CYP (E) and MCZ (F) for 24 h at 20 °C as described in Material and methods. The values represent means ± SD of 5 independent biological replicates. *p < 0.05, **p < 0.01 and ***p < 0.001 based on ANOVA followed by Dunnett's multiple comparisons test.

vertebrate hormone E2 was used as a positive control. Using the expression database WormBase (2021), genes related to the reproductive system of *C. elegans* whose transcription was relevant during the last stage of the sexual development (L4 - young adult), were selected to be included in the gene expression analysis. Thus, *cpb-3* and *glp-1* genes were chosen to analyse alteration in oogenesis of the hermaphrodites, while *spe-6*, *spe-11* and *fem-3* were used to study the effect on the spermatogenesis. Furthermore, the expression of *hus-1* was assessed to identify DNA damage response and the one of *vit-2* to detect disturbance in the process of supplying nutrients to the embryo.

Fig. 3 shows the gene expression analyses for the selected genes after 24 h of exposure to pesticides and the hormone E2. The treatment with ATR significantly reduced the expression levels of *cpb-3* gene at 10 μg/l and of *glp-1* gene at 10 and 100 μg/l. Regarding the genes involved in the spermatogenesis, the expression of the *spe-11* and *fem-3* genes was significantly decreased in nematodes exposed to 100 μg/l of ATR. Additionally, a significant increase in the *vit-2* gene transcription was revealed at all ATR concentrations evaluated. Remarkably, a similar gene expression pattern was observed in E2-treated animals, indicating that both the herbicide ATR and steroid hormone could trigger a common signaling pathway in reply to treatment.

Treatment with the herbicide 2,4-D caused a significant reduction in the gene expression of *cpb-3* at 10 μg/l, *spe-6* at 10 and 100 μg/l, and *fem-3* at the three concentrations tested (Fig. 3). Regarding the transcription of the *vit-2* gene, a significant increase was observed for all 2,4-D treatments, while no significant differences were found in the *glp-1*, *spe-11* and *hus-1* genes. When the animals were exposed to CPF, there was a significant decrease in the expression of the *glp-1* at all concentrations evaluated and *spe-11* gene at 10 and 100 μg/l. In addition, all the concentrations showed an increase in the expression of the *vit-2* gene and in the case of *hus-1* gene, at 10 μg/l. No changes were detected in the expression of the *cpb-3*, *spe-6* and *fem-3* genes with respect to the control. Finally, the fungicide MCZ and the insecticide CYP induced a higher expression of the *hus-1* and *vit-2* genes at the three concentrations assessed and a decreased expression of the *fem-3* at some of the treatments. The *cpb-3* gene expression showed an increase when the nematodes were treated at the highest concentration of CYP (100 μg/l).

3.3. Influence of ED-putative pesticide exposure in vitellogenin trafficking and endocytosis

In *C. elegans*, vitellogenins are synthesized in the intestine of adult hermaphrodites where they recruit lipids and other nutrients to produce yolk complexes. Then, the yolk complexes are secreted into the pseudocoelom, from where they pass into the ovary to be absorbed by the three oocytes most proximal to the uterus through receptor-mediated endocytosis (Grant and Hirsh, 1999). Based on the discovery that all pesticides tested increased *vit-2* gene expression (Fig. 3), the distribution and uptake of the VIT-2 protein in *C. elegans* upon exposure to pesticides were further investigated. For this purpose, transgenic nematodes

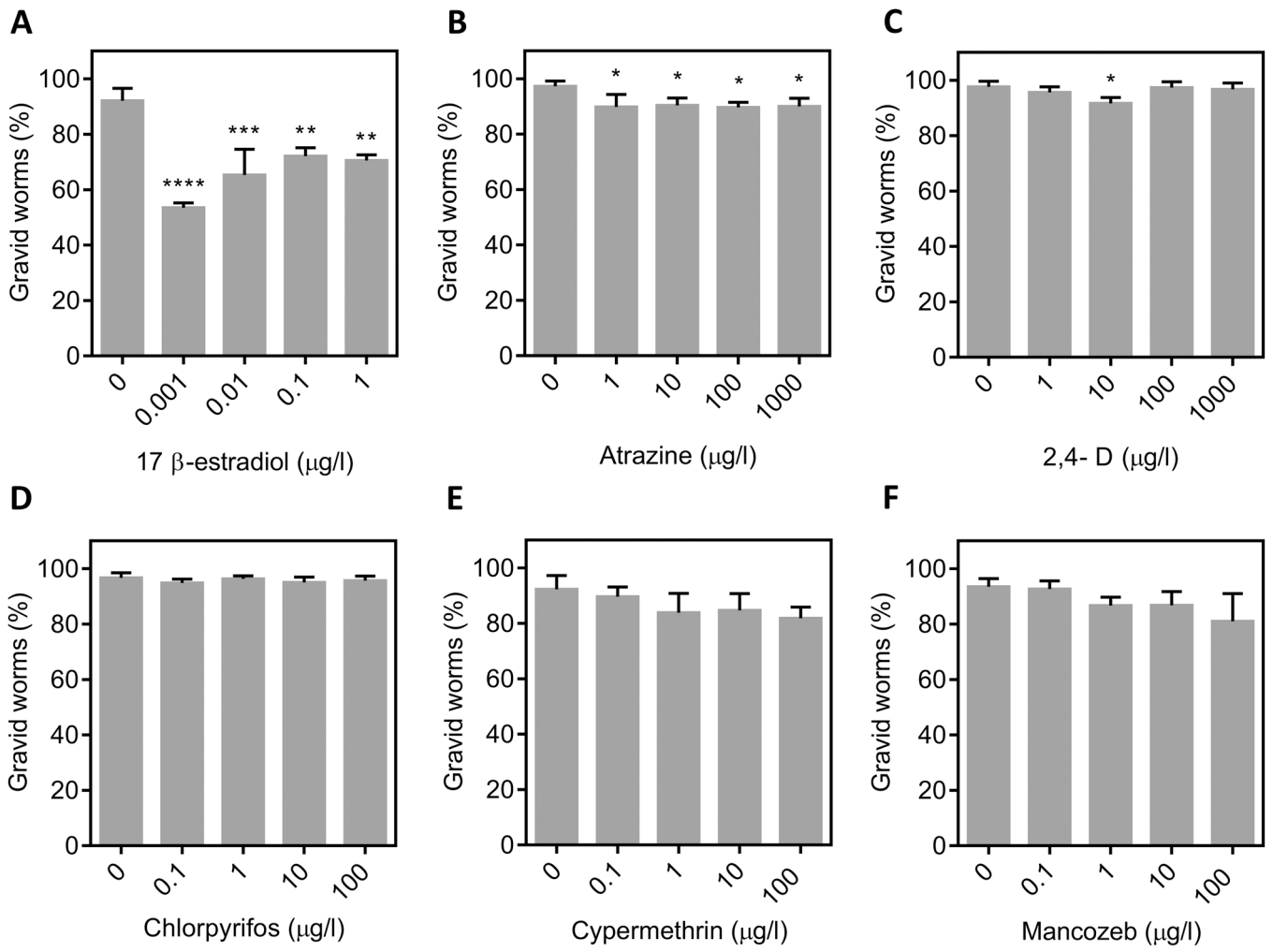

Fig. 2. Percentage of gravid nematodes exposed to E2 (A), ATR (B), 2,4-D (C), CPF (D), CYP (E) and MCZ (F) for 24 h at 20 °C as described in Material and methods. The values represent means ± SD of 3 independent biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 based on ANOVA followed by Dunnett's multiple comparisons test.

expressing a GFP-tagged VIT-2 protein (VIT-2::GFP) were treated with the pesticides or E2 in L4 stage during 24 h and the yolk accumulation was visualized by fluorescence microscopy. VIT-2::GFP was visible mainly in oocytes and intrauterine eggs of the negative control group, but in treated nematodes it often accumulated as enlarged yolk agglutination throughout the body cavity (Fig. 4). It should be noted that these abnormal yolk agglutinations were found distributed throughout the body in both gravid and non-gravid nematodes.

In order to quantify the anomaly yolk observed, the percentage of animals presenting more than 5 agglutinations throughout the body was determined for each treatment. Compared with each negative control, the steroid hormone E2, as well as the pesticides, significantly influenced the percentage of the nematodes with yolk agglutination in more than one of the concentrations evaluated (Fig. 5). However, the magnitude of the effect observed in these percentages varied widely. While E2, ATR, 2,4-D and CPF produced an increase in the average percentage of animals with abnormal yolk agglutination between 37% and 50%, MCZ did to 11% for the highest concentrations used. In addition, the results showed that the treatment with CYP only affected approximately 2,3% of the nematodes at 1 and 10 μg/l.

To study if the pesticide treatment could affect the vitellogenin endocytosis of the oocytes, the fluorescence of intrauterine embryos of one cell division from adult nematodes of the same transgenic strain was quantified. Embryos of one cell division were selected for fluorescence determination since they have been fertilized recently and their yolk consumption has not been significant (Grant and Hirsh, 1999). Therefore, the VIT-2::GFP content inside these embryos could be considered comparable with that of the oocyte closest to the spermatheca. Fig. 6 shows that the intrauterine embryos contained a lower amount of fluorescence when the nematodes were exposed to E2, ATR, 2,4-D and CPF suggesting that these chemicals could also be affecting the yolk endocytosis of the oocytes.

4. Discussion

Annually, approximately two million tons of pesticides are pulverized into the environment around the world (Sharma et al., 2019). The ubiquity of these substances in the air, land and water raises global concerns regarding adverse health effects on humans and non-target animals. Particularly, the interference with the normal functioning of the endocrine system of exposed organisms is a controversial aspect among these impacts. In the last two decades, several government agencies from the European Union and United States, as well as non-government organizations such as the Endocrine Society, have been developing framework strategic programs to understand the mechanism of action and deal with these substances (Ho et al., 2022). Hence, EDCs detailed further research are still required to provide considerable evidence, particularly associated with mechanisms, non-monotonic dose-response and critical windows of development to environmentally relevant concentrations (EC, 2016). Moreover, it is necessary to develop

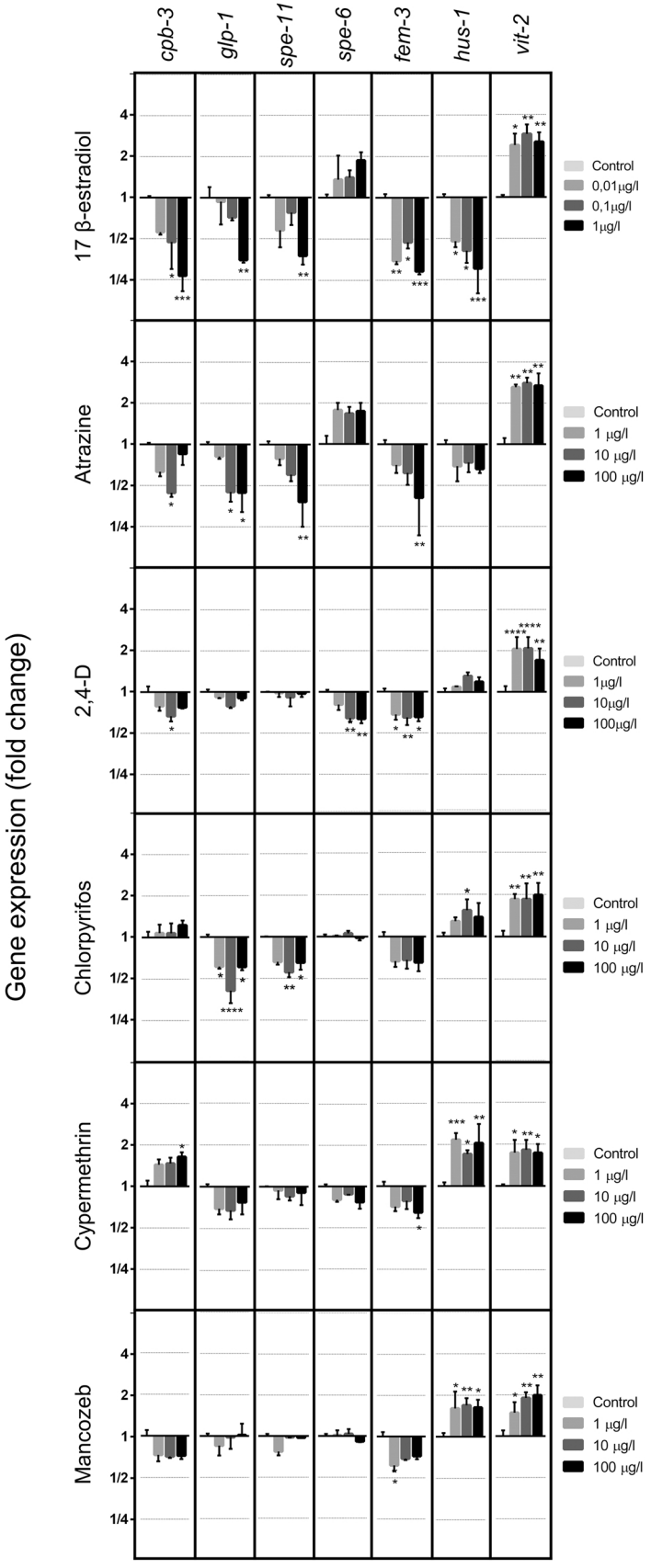

Fig. 3. Quantitative RT-PCR analysis of genes involved in the reproductive response in nematodes *C. elegans* exposed to E2, ATR, 2,4-D, CPF, CYP and MCZ for 24 h at 20 °C as described in Material and methods. The expression level of each target PCR product was normalized to that of the housekeeping gene, *cdc-42*, and related to the control treatment. A base-2 log scale is used for the Y axis. The values represent means ± SEM of 3 independent biological replicates. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ based on ANOVA followed by Dunnett's multiple comparisons test.

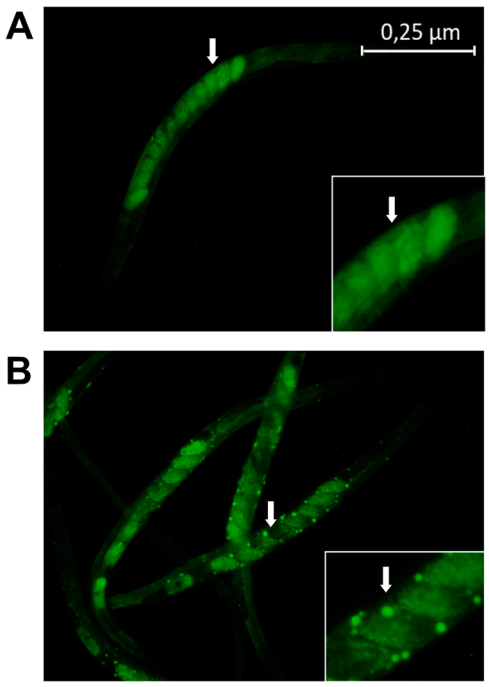

Fig. 4. Representative images of transgenic nematodes expressing a GFP-tagged VIT-2 protein (VIT-2::GFP) exposed to pesticides for 24 h at 20 °C as described in Material and methods. Hermaphrodite nematodes with normal yolk distribution (A) or with abnormal agglutinations (B) were visualized by fluorescence microscopy 24 h after the treatments. Scale bars, 0.25 µm.

new alternative models of endocrine disruption that allow fast and predictive evaluation of the effects on non-target organisms. Here, we evaluated the reproductive toxicity of five EDC-suspected pesticides (ATR, 2,4 D, CPF, CYP and MCZ) in the model organism *C. elegans* and we compared the responses with those obtained by treatment with the E2 positive estrogenic control. All the pesticides disturbed to some extent one or more of the evaluated endpoints. Singularly, ATR, 2,4-D and CPF induced comparable responses to E2 suggesting that these pesticides may have estrogen-like endocrine disrupting activity.

Herbicide ATR is the most known of the triazines and it is a very widely used herbicide that has been associated with oxidative stress, cytotoxicity, reproductive toxicity and delays in sexual maturation in animals (Nicolopoulou-Stamati et al., 2016). Our results showed that ATR and E2 caused similar disturbances in all endpoints: decreased the brood size and the percentage of gravid nematodes, and altered the VTG trafficking and endocytosis. Likewise, the expression pattern of reproductive-related genes was similar with both chemicals. It is possible that the reproductive disturbances provoked by E2 or ATR were caused by a delay in the development and sexual maturity of the nematodes. This assumption arises from the fact that 12 h after the end of the treatment, the percentage of gravid animals in the treated population equaled the control group (data not shown). Likely, the treatment of nematodes during the L4 stage would produce a hormonal imbalance that leads to a developmental delay in this last larval molt. It was already shown that this stage, which is required to reach sexual maturity, is accurately regulated by neuroendocrine signally (Russel et al., 2011). In particular, between L4 and adult, germ cell meiosis begins and sperm maturation occurs. According to this observation, *C. elegans* treated with the EDC tributyltin during this stage showed negative effects in reproduction (Cheng et al., 2014). Furthermore, this delay in reproductive development observed in *C. elegans* was also reported in male and female rodents exposed to ATR (Breckenridge et al., 2015; Stoker et al., 2002).

Regarding the expression analysis of genes involved in reproductive development, it was found that ATR, and similarly E2, produced a significant underexpression of the spermatogenesis-associated genes *spe-11* and *fem-3*. *Spe-11* gene encodes a soluble protein localized in the perinuclear region of sperm that is involved in the dynamic morphology of sperm pseudopods and is required for egg activation after fertilization, while *fem-3* gene is essential for hermaphroditic germline sperm/oocyte switch and acts as a positive regulator of spermatogenesis in the germline (Kimble and Crittenden, 2007; Nishimura and L'Hernault, 2010). Our results suggest that both ATR and E2 could affect the development of male gametes in *C. elegans*, which would explain at least partially the decrease of the brood size in treated hermaphrodites. Moreover, the genes associated with oogenesis *cpb-3* and *glp-1*, showed be underexpressed with both compounds, indicating that the development of the female germline would also be contributing to the observed decrease of the progeny. The *cpb-3* gene encodes a cytoplasmic polyadenylation element-binding protein that is enriched in oogenic cells and seems to play a key role in the early phases of germ cell sex determination and meiosis (Hasegawa et al., 2006). Meanwhile, *glp-1* gene encodes a member protein of the conserved LIN-12/Notch receptor family that functions in the germline to modulate oocyte growth when sperm are available for fertilization and it is essential for both germline stem cell maintenance and germ cell proliferation during gonad development (Nadarajan et al., 2009).

The nematode *C. elegans* has been documented to respond to vertebrate steroids and alters the expression patterns of VTG genes (Custodia et al., 2001; Fischer et al., 2012; Kohra et al., 1999). Our results showed that ATR, like E2, caused overexpression of *vit-2* gene and induced an abnormal distribution of VTG in exposed nematodes. In addition, both compounds caused a decrease of VTG content in treated embryos compared to those of the control group, suggesting a lower endocytosis of VTG in female gametes. Consequently, the higher expression of *vit-2* and the lower VTG endocytosis in oocytes, could be the cause of the generation of VTG agglutination in the body of the exposed nematodes. In fact, it was already shown that VIT-2::GFP nematodes that fail to be taken up by oocytes accumulate VTG as large aggregates in the pseudocoelom (Grant and Hirsh, 1999; Kim et al., 2011). Although the physiological consequences of these abnormal VTG agglutination are uncertain. A higher frequency of nematodes with sterility has been found in those whose embryos were depleted in VTG, obtained from mothers without VTG or from null mutants of the yolk receptor-mediated endocytosis *rme-2* (Jordan et al., 2019; Perez et al., 2017). This suggests that the abnormal VTG synthesis and trafficking could be one of the reasons for the negative impact on the reproductive toxicity described in the present study. In addition, other physiological functions of VTG have been described, such as a protective role during aging, antioxidant action and pathogen defense, that could be affecting the homeostasis of the treated nematodes (Fischer et al., 2012; Lynn et al., 2015; Nakamura et al., 1999; Perez and Lehner, 2019; Sornda et al., 2019).

2,4-D is currently the main ingredient of over 1500 commercially available products and is one of the most widely distributed pollutants in the environment (Akpan and Hameed, 2011). In recent years, transgenic agricultural crops resistant to 2,4-D have been released, highlighting a possible global increase in the volume of application of this herbicide (Islam et al., 2018). Several studies in different organisms show that 2, 4-D could have a potencial effect as EDC, however, there is no evidence to date that it interferes with hormonal signaling in *C. elegans* (Islam et al., 2018; Stürtz et al., 2010). Our results show that the treatment of L4 stage nematodes with 2,4-D decreased the percentage of gravid animals, the brood size, the VTG endocytosis, and increased the number of animals with VTG aggregates, similarly to E2 and ATR treatments. Data suggests that 2,4-D could induce an estrogenic-like effect. Accordingly, Xie et al. (2005) found a putative estrogenic activity for 2,4-D in rainbow trout. As discussed above for ATR and E2 results, these negative effects on the reproduction could be explained by a delay in the maturation of the gonads due to an imbalance in the endocrine systems during the L4-adult stage. In fact, 2,4-D not only reduced the percentage of gravid, but it also decreased the number of eggs inside the uterus of

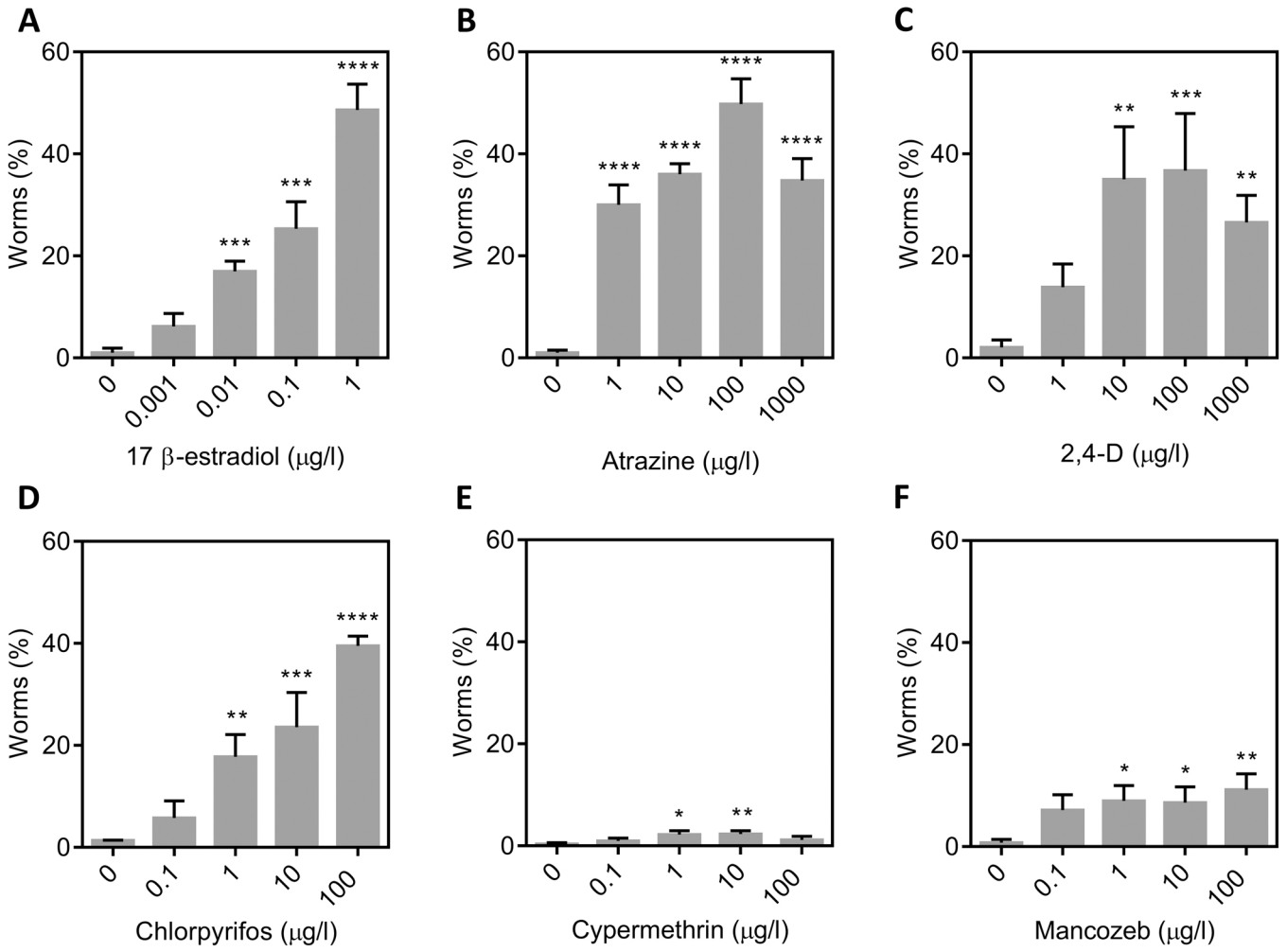

Fig. 5. Percentage of nematodes expressing a GFP-tagged VIT-2 protein with abnormal yolk agglutinations exposed to E2 (A), ATR (B), 2,4-D (C), CPF (D), CYP (E) and MCZ (F) for 24 h at 20 °C as described in Material and methods. The values represent means ± SD of 3 independent biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001 based on ANOVA followed by Dunnett's multiple comparisons test.

treated hermaphrodites, indicating a delay in the maturation of the reproductive system (data not shown). One possible explanation for the decrease in the brood size could be a disturbance of male and female germ cell development. The results of the gene expression analysis show that the 2,4-D reduced the expression of *cpb-3*, *spe-6* and *fem-3* genes (Fig. 3). The subexpression of *cpb-3* would indicate an imbalance in the sperm-oocyte switching signal that would lead to a defect in oogenesis, while the lower expression of *fem-3* and *spe-6* would indicate a defect in spermatogenesis (Barton et al., 1987; Hodgkin, 1986; Varkey et al., 1993). In line with our results, several publications report that 2,4-D produces a genotoxic effect in mammalian cells, inhibits the development and blocks the maturation of *X. laevis* oocytes and it impaired normal spermatogenesis in mice and rats (González et al., 2005; Harada et al., 2016; LaChapelle et al., 2007; Maire et al., 2007; Stebbins-Boaz et al., 2004; Tayeb et al. 2010; Zhang et al., 2017). Furthermore, a decrease in sperm counts and an increase in sperm abnormalities have been observed in agricultural workers exposed to 2,4-D (Islam et al., 2018).

CPF was one of most frequently used organophosphate insecticides due to its effective and cost-competitive broad-spectrum activity (Hites, 2021). Due to its reported hazardous impacts on human and animal health, many countries are gradually restricting its use. CPF toxicity was already associated with neurological dysfunctions and reproductive abnormalities, and it is currently considered EDC, as it can imitate hormone actions both *in vitro* and *in vivo* (Lasagna et al., 2021). Interestingly, Yu et al. (2015) and Mishra and Singh (2021) found evidence of a possible estrogenic effect in fish. In the present study, CPF treatments produced a decrease in the brood size and abnormalities in the distribution of VTG and in the expression of some genes (*glp-1*, *spe-11*, *fem-3*, *vit-2*), similarly to the effects of E2. The results in the brood size were in a good agreement with the results reported in *C. elegans* by Ruan et al., 2012 and in other alternative models as well (Gupta et al., 2007; Jager et al., 2007). The decrease in the number of gravid nematodes exposed to the CPF could be due to negative effects on gametes development caused either by a hormonal imbalance and/or by the generation of DNA damage. Interestingly, we found that the genes related to gametogenesis *spe-11* and *glp-1* were underexpressed by the effect of this insecticide (Fig. 3). Besides that, CPF produced an overexpression of the *hus-1* gene, that encodes a nuclear protein that is expressed in adult germ lines in response to DNA damage and it is considered a genotoxicity biomarker (Hofmann et al., 2002). Supporting this discovery, Gupta et al., (2007) reported evidence about CPF induced DNA damage and apoptosis in *Drosophila melanogaster* larvae and these effects were attributed to the generation of ROS (reactive oxygen species). Furthermore, this insecticide has been reported to induce oxidative stress and tissue damage in rats (Goel et al., 2005; Jett and Navoa, 2000).

CYP is a broad-spectrum insecticide with an enormous global use and it is one of the most studied pyrethroids worldwide due to the concern about its detrimental effects on human and animal health. Increasing

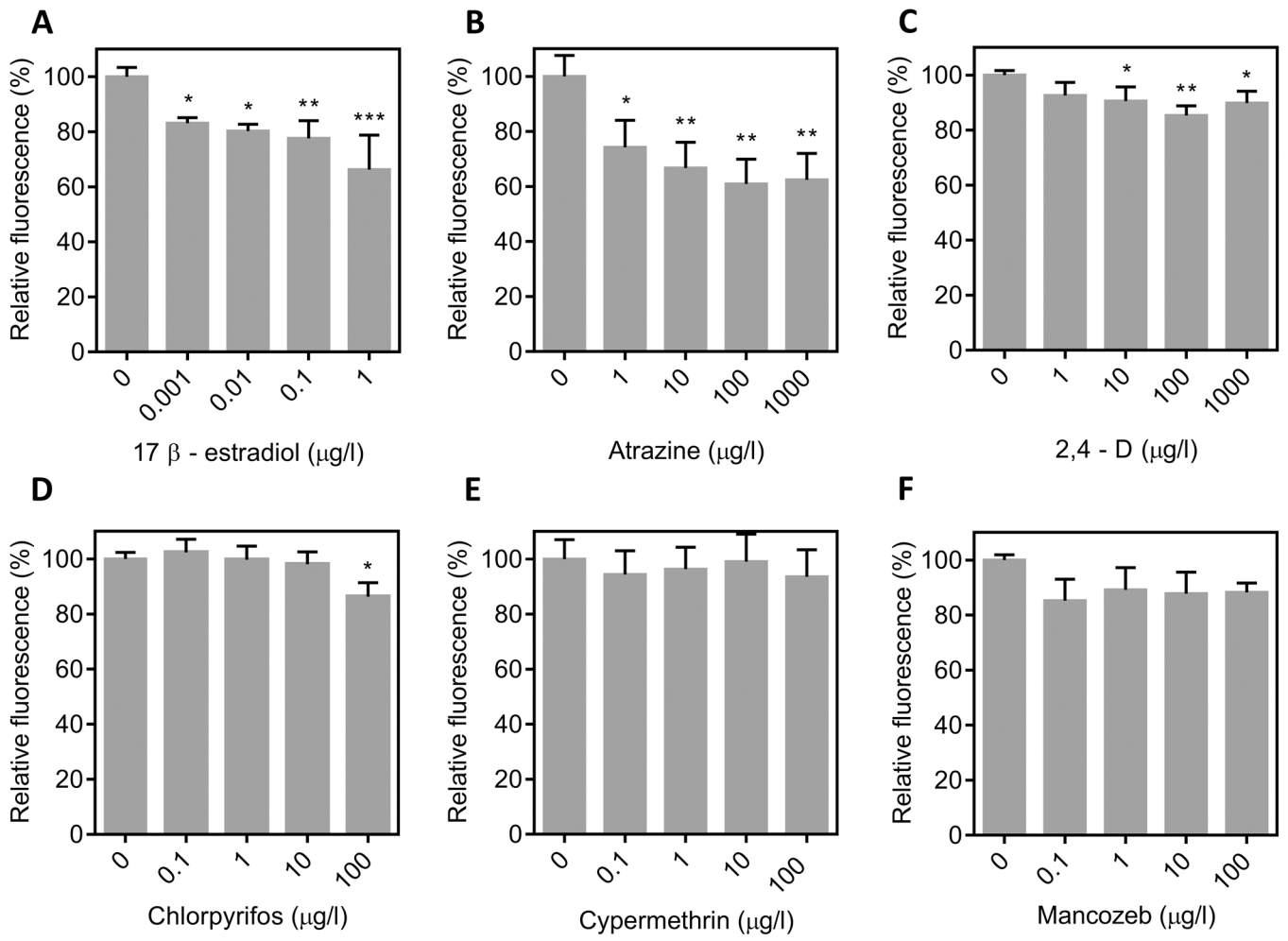

Fig. 6. Relative fluorescence of *C. elegans* intrauterine one-cell stage embryos from hermaphrodites expressing GFP-tagged VIT-2 protein (VIT-2::GFP) exposed to E2 (A), ATR (B), 2,4-D (C), CPF (D), CYP (E) and MCZ (F) for 24 h at 20 °C as described in Material and methods. The values represent means ± SD of 3 independent biological replicates. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ based on ANOVA followed by Dunnett's multiple comparisons test.

evidence has suggested the endocrine disrupting feature of CYP in these years (Ha et al., 2021; Liu et al., 2021; Wang et al., 2020). Besides that, MCZ is a fungicide intensively used to protect fruit and vegetables and its production is expected to increase due to its low price, its broad-spectrum efficiency and the increased global demand for fresh food (Asparch et al., 2019; Market F., 2019). However, this fungicide has been linked to thyroid hormone disruption and neurotoxic effects among others (Cecconi et al., 2007; EPA, 2005; Runkle et al., 2017). The results of our study showed that CYP and MCZ exposure had little impact on the reproductive endpoint tested in *C. elegans* at the evaluated concentrations. CYP affected the brood size at the highest concentration assessed and MCZ altered the distribution of VTG within the body of the nematodes only in approximately 10% of the population. However, it is important to note that both products overexpressed the *hus-1* gene, suggesting a possible genotoxic activity. MCZ and CYP demonstrated to produce oxidative stress and DNA damage on diverse cellular models and non-target animals (Bano and Mohanty, 2020; Dall'Agnol et al., 2021; Jin et al., 2011; González et al., 2021; Srivastava et al., 2012; Ravula and Yenugu, 2021). In *C. elegans*, it was shown that both pesticides induce oxidative stress in treated worms, nevertheless, the concentrations used were considerably higher than those used in the present study (Bailey et al., 2016; Shashikumar and Rajini, 2010). Hence, further investigations are needed to confirm the generation of oxidative stress and its consequent genotoxicity with the concentrations tested in our study.

VTG is the major precursor of egg-yolk proteins conserved in all oviparous and whose role is to facilitate the transport of lipids, carbohydrates and other nutrients for embryonic development (Sappington and Raikhel, 1998; Tufail and Takeda, 2008). So, it is not surprising that VTG synthesis is strongly regulated hormonally at transcriptional level (Tufail and Takeda, 2008). In this study, it was observed that all the pesticides assayed, as well as E2, increased the expression of the *vit-2* gene and affected the VTG distribution to some extent, highlighting that this protein may be an excellent biomarker of EDC action. However, even if the expression of VTG may be an indicator of an estrogenic-like effect, a cautious interpretation of the results is necessary. Moreover, this kind of study should be conducted in an integrative way, performing diverse as complementary bioassays in order to guarantee the correct reading of data. In fact, in *C. elegans*, there have been described multiple physiological and environmental factors that modulate VTG synthesis, such as oxidative stress, nutrient availability or iron overdose (Perez and Lehner, 2019). Nevertheless, due to the high evolutionary conservation of VTGs in invertebrates and vertebrates, their study is not only of great interest to understand the impact of estrogenic EDC in the nematode *C. elegans*, but also in other species. Indeed, VTGs are similar to human low-density lipoproteins which allow correlating *C. elegans* results with cholesterol transport in mammals (Perez and Lehner, 2019).

To summarize, in the present study the reproductive toxicity of the pesticides ATR, 2,4-D, CPF, CYP and MCZ was evaluated using diverse *C. elegans* endpoints like the brood size, the percentage of gravid

nematodes, the expression of reproductive-related genes and VTG trafficking and endocytosis. The results showed that ATR induced a similar response that E2 in all responses analysed. CPF and 2,4-D decreased the brood size, caused abnormal VTG distribution and disturbed the expression of some reproductive-related genes like ATR and E2, suggesting that CPF and 2,4-D could also induce an estrogenic action. Regarding MCZ and CYP, the reproductive responses evaluated in *C. elegans* did not show any pattern similar to E2. The influence on reproduction of ATR, CPF and 2,4-D points them out as potential health and environmental risks. As some responses were observed at concentrations below values detected in the environment, *e.g.* in watercourses or rainfall, the reproductive endpoints evaluated in this study could be used as biomarkers to assess the ecological risks of pesticides suspected to be EDC in order to protect the environment and the ecosystem.

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.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.tox.2022.153229.

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