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Direct effects of pesticides and pesticide metabolites on the CatSper Ca2+-channel in human sperm - A novel test method for endocrine disrupting effects

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Authors: Michala R. Birch[1,2],
Mathias Johansen[1,2],
Niels E. Skakkebæk[1,2],
Anna-Maria Andersson[1,2],
Anders Rehfeld[1,2]

[1]Department of Growth and Reproduction, Copenhagen University Hospital, Rigshospitalet, Denmark.
[2]International Center for Research and Research Training in Endocrine Disruption of Male Reproduction and Child Health (EDMaRC), Rigshospitalet, University of Copenhagen, Denmark.

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Contents

1. Preface and Acknowledgements

The presented investigation “Direct effects of pesticides and pesticide metabolites on the CatSper Ca^{2+}-channel in human sperm - A novel test method for endocrine disrupting effects” was a study elucidating the effects of 53 different pesticides and pesticide metabolites on Ca^{2+}-signaling in human sperm cells and Ca^{2+}-regulated sperm cell functions important for successful fertilization.

The project was financed by the Danish Environmental Protection Agency (Miljøstyrelsen) and was carried out in the period July 2019 to June 2022 at Department of Growth and Reproduction, Rigshospitalet. The overall progress of the project was coordinated by Anna-Maria Andersson. Niels Erik Skakkebæk assisted Anna-Maria Andersson in the overall project management and Anders Rehfeld was responsible for the daily coordination of the project as well as the day-to-day laboratory work. Michala Rosa Birch assisted Anders Rehfeld with data analysis and drafts for publishing in scientific journals. Due to delays of the laboratory work during the global corona pandemic data with satisfying quality from the computer assisted microscopic analysis of sperm cell motility have not been yet obtained.

This project would like to acknowledge our lab technician Sissel Marie Bredesen for the enormous experimental work she has done during all three years of the project. Likewise, we would like to thank Mathias Johansen for assisting with the data treatment during his employment at Department of Growth and Reproduction, Rigshospitalet as well as Kristian Almstrup. Their involvement ensured the progress of the project during the corona lockdown in 2020.

Furthermore, the project would like to acknowledge the advisory board for “Sundhed og Pesticider” (“Health and pesticides”) for very constructive discussions of the project, and in particular senior researcher Jorid Birkelund Sørli from Det Nationale Forskningscenter for Arbejdsmiljø (NFA) and Stine Jensen from the Danish EPA for their thorough reading of this report, constructive comments and suggestions. Finally, we would like to acknowledge Henrik Frølich Brødsgaard at Danish EPA for the good collaboration during the project period.

The advisory group and the Danish Environmental Protection Agency have had no influence on the presentation and interpretation of the results and the report's conclusion.

2. Abstract

During the last decades human fertility rates have been declining globally (Skakkebaek *et al.*, 2015; Skakkebæk *et al.*, 2022) and one in six couples need fertility treatment to conceive. Ca^{2+}-signaling mediated by the sperm specific CatSper channel controls sperm cell functions necessary for successful fertilization and males with mutations in CatSper-subunits are sterile. Multiple endocrine disrupting chemicals have been found to interfere with normal Ca^{2+}-signaling in human sperm cells through an activation of the promiscuous CatSper channel, thereby affecting sperm cell functions such as the acrosome reaction and sperm penetration. Therefore, the exposure of human sperm cells to different endocrine disrupting chemicals has been suspected to be a contributing factor to sperm cell dysfunction and the widespread male infertility.

In this study we investigated 53 pesticides for their ability to interfere with CatSper mediated Ca^{2+}-signaling and function in human sperm cells. The effects of the pesticides on Ca^{2+}-signaling in human sperm cells were evaluated using a Ca^{2+}-fluorometric assay. Effects via CatSper were assessed using the specific CatSper inhibitor RU1968. Effects on human sperm function and viability were assessed using an image cytometry-based acrosome reaction assay and the modified Kremer's sperm–mucus penetration assay. We found that 28 of 53 pesticides induced Ca^{2+}-signals in human sperm cells at 10 µM. The majority of these 28 active pesticides induced Ca^{2+}-signals through the CatSper channel and interfered with subsequent Ca^{2+}-signals induced by the two endogenous CatSper ligands progesterone and prostaglandin E_1. Multiple active pesticides were found to affect Ca^{2+}-mediated sperm functions and viability at 10 µM. Since 10 µM is a rather high concentration for a single compound in a physiological context, we investigated the effects of low nM dose mixtures of the active pesticides alone or in combination with other environmental chemicals. These mixtures were found to significantly induce Ca^{2+}-signals and inhibit Ca^{2+}-signals induced subsequently by progesterone and prostaglandin E_1. Our results show that pesticides, both alone and in low nM dose mixtures, interfere with normal Ca^{2+}-signaling in human sperm cells *in vitro* at concentrations that could be of physiological relevance. However, biomonitoring of pesticides in relevant matrices such as blood and reproductive fluids is very limited and the effects of real time human pesticide exposure on human sperm cells and fertility thus remains largely unknown. To which extent current levels of human pesticide exposure affect the chances of a successful fertilization in humans *in vivo* needs further research.

3. Introduction

Pesticidal products are frequently used in agriculture for plant protection, but pesticides can also occur in biocidal products used to control organisms harmful to human or animal health (European Commission, 2021a, b). The environmental pesticide pollution is well documented, and pollution has been reported in water, soil and indoor as well as outdoor air (Stephenson and Solomon, 2007; Rathore and Nollet, 2019). Furthermore, the widespread use of pesticidal products in agriculture has resulted in pesticide residues being present in numerous food products today (Cabrera and Pastor, 2021). Therefore, most humans are likely to experience a continuous pesticide exposure mainly through the dietary intake of fruits, vegetables, and grains (Oates and Cohen, 2011; Nougadère *et al.*, 2012; Jensen *et al.*, 2015; Winter, 2015; Chiu *et al.*, 2018).

3.1 Human pesticide exposure

Several commonly used as well as banned or severely restricted pesticides have been detected in human matrices in numerous biomonitoring studies all across Europe (Heudorf *et al.*, 2006; Ye *et al.*, 2008; Saoudi *et al.*, 2014; Dereumeaux *et al.*, 2016; Koureas *et al.*, 2016; Ramos *et al.*, 2017; Béranger *et al.*, 2018, 2020; Norén *et al.*, 2020; Hardy *et al.*, 2021). These biomonitoring studies include the measurement of organophosphorous and pyrethroid insecticide metabolites in urine specimens of the general population in Germany (Heudorf *et al.*, 2006) and Sweden (Norén *et al.*, 2020) and measurement of the serum distribution of banned organochlorine pesticides of the general population in France (Saoudi *et al.*, 2014), Greece (Koureas *et al.*, 2016), and Spain (Ramos *et al.*, 2017). Furthermore, both banned and contemporary pesticides have been detected in hair samples from pregnant women (Béranger *et al.*, 2018, 2020; Hardy *et al.*, 2021). Pesticide metabolites from contemporary pesticides have similarly been detected in urine samples from pregnant women (Ye *et al.*, 2008; Hardy *et al.*, 2021). However, few biomonitoring studies measuring the concentration of contemporary pesticides and their metabolites in blood plasma have been conducted (Huen *et al.*, 2012; Shi *et al.*, 2021) and only one study has measured the concentration of a few selected pesticide metabolites in male reproductive fluids (Wang *et al.*, 2021).

3.2 Adverse effects on time-to-pregnancy

Studies from the 1970s and 1980s showed that workers exposed to the pesticide DBCP became oligo- or azoospermic and had skewed sex ratio of offspring following cessation of exposure (Whorton *et al.*, 1979; Potashnik *et al.*, 1984). Though contemporary pesticides are designed to be nonpersistent and less toxic than the first generation pesticides currently replaced or severely restricted (World Health Organisation, 1990) they are still suspected to have adverse effects on human reproductive function (Sanborn *et al.*, 2007; Kim *et al.*, 2017). Recent studies found no association between pesticide exposure and altered semen parameters (Abell and Ernst, 2000; Oliva *et al.*, 2001), but several studies found an increased time-to-pregnancy among males exposed to pesticides (De Cock *et al.*, 1994; Curtis *et al.*, 1999; Petrelli and Figà-Talamanca, 2001; Sallmén *et al.*, 2003; Bretveld *et al.*, 2008). These findings could indicate negative effects on

sperm function, which are not related to standard semen parameters such as sperm count and morphology. Among females exposed to pesticides while trying to conceive, inconsistent results on time-to-pregnancy have been described (Larsen *et al.*, 1998; Thonneau *et al.*, 1999a, b; Abell *et al.*, 2000; Harley *et al.*, 2008). However, a recent study found that females consuming shellfish twice a week had a longer time-to-pregnancy, which increased with increasing serum levels of the pesticide metabolite p,p'-DDE among other environmental pollutants (Chevrier *et al.*, 2013).

3.3 CatSper mediated Ca^{2+}-signaling and male fertility

The last decades human fertility rates have been declining globally (Skakkebaek *et al.*, 2015; Skakkebæk *et al.*, 2022). One in six couples need fertility treatment to conceive (Agarwal *et al.*, 2015) and intracytoplasmic sperm injection (ICSI) where a sperm cell is injected directly into the egg cell is increasingly being used for *in vitro* fertilization (IVF) indicating a rise in human sperm dysfunction (Jain and Gupta, 2007; Kupka *et al.*, 2014; Okhovati *et al.*, 2015). Presently, the etiology of male infertility remains unknown in many cases (Olesen *et al.*, 2017), but sperm defects or dysfunction, in particular impaired motility, are important contributing factors (Brown *et al.*, 2019). For natural fertilization of the egg cell to occur, it is crucial that sperm cell functions are precisely controlled and triggered at the correct time in the correct order within the female reproductive tract (Publicover *et al.*, 2007, 2008). Ca^{2+}-signaling is a central regulator of sperm cell function in the transcriptionally silent sperm cell (Publicover *et al.*, 2007). The sperm specific CatSper Ca^{2+}-channel facilitates all channel mediated Ca^{2+}-influx in human sperm cells (Publicover, 2017), where it is activated by the endogenous ligands progesterone and prostaglandins, prostaglandin E_1 (PGE_1) being the most potent (Lishko *et al.*, 2011; Strünker *et al.*, 2011). Progesterone and PGE_1 employ distinct binding sites to activate CatSper (Strünker *et al.*, 2011). Progesterone activates CatSper though α,β-hydrolase domain-containing protein 2 (ABHD2), whereas PGE_1 is believed to stimulate CatSper directly (Miller *et al.*, 2016). Upon activation the intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) increases rapidly through a CatSper mediated Ca^{2+}-influx (Lishko *et al.*, 2011; Strünker *et al.*, 2011) regulating important sperm cell functions such as capacitation, sperm motility, chemotaxis towards the egg, and the acrosome reaction (Eisenbach and Giojalas, 2006; Publicover *et al.*, 2007; Lishko *et al.*, 2012). The CatSper channel is also weakly voltage dependent and can be activated by alkalization of the intracellular pH (pH_i) (Brown *et al.*, 2019; Wang *et al.*, 2020). Studies have shown that impaired progesterone-induced Ca^{2+}-signaling is associated with lower rates of IVF success (Williams *et al.*, 2015; Luo *et al.*, 2019) and that males with mutations in CatSper-subunits are sterile (Williams *et al.*, 2015; Luo *et al.*, 2019), highlighting the importance of CatSper for normal male fertility.

Recent studies have found that human CatSper is promiscuous and can be activated by a variety of compounds including steroids (Rehfeld, 2020), signaling molecules (Brenker *et al.*, 2012), small molecules (Martins Da Silva *et al.*, 2017), and environmental chemicals including the banned pesticides lindane, p,p'-DDT, and its metabolite p,p'-DDE (Schiffer *et al.*, 2014), which was found to be associated with a longer time-to-pregnancy in Chevrier *et al.*, 2013. Several of these compounds have been found to activate CatSper through the binding site of progesterone (Schiffer *et al.*, 2014; Rehfeld *et al.*, 2016) or PGE_1 (Schiffer *et al.*, 2014; Birch *et al.*, 2021) on CatSper, act like endocrine disrupting chemicals (EDCs), and interfere with the normal CatSper mediated Ca^{2+}-signaling. In line with this, several studies have found that such interference with

the normal CatSper mediated Ca^{2+}-signaling affects functional sperm responses such as the acrosome reaction and sperm motility (Schiffer *et al.*, 2014; Rehfeld *et al.*, 2018, 2020; Birch *et al.*, 2021). Therefore, exposure of human sperm cells to these EDCs is suspected to be a contributing factor to sperm cell dysfunction and the widespread male infertility.

3.4 Aim of the study

The purpose of this study was to investigate contemporary pesticides and pesticide metabolites for their ability to interfere with CatSper mediated Ca^{2+}-signaling and function in human sperm cells *in vitro*. As two currently banned pesticides lindane, p,p'-DDT, and its metabolite p,p'-DDE previously were found to interfere with the normal CatSper mediated Ca^{2+}-signaling in our previous study (Schiffer *et al.*, 2014) we found it relevant to investigate a much larger group of pesticides and pesticide metabolites used currently.

In this study 53 pesticides, and pesticide metabolites were investigated for their effect on Ca^{2+}-signaling in human sperm and their pharmacological mode of action was characterized. The 53 pesticides and pesticide metabolites (Table 1 and Table S1) were chosen due to their occurrence in hair samples of pregnant woman in a recent environmental biomonitoring study (Béranger *et al.*, 2018) and/or due to the priority of the Danish EPA. In the following, both the pesticides and pesticide metabolites will be referred to as the "pesticides".
Pesticides and pesticide metabolites, which induced a significant Ca^{2+}-signals at a 10 µM concentration in the initial screening were further investigated regarding their effects on human sperm motility and acrosome reaction. Finally, the cooperative action of the "active" pesticides, and pesticide metabolites in low concentration chemical mixtures were investigated.

4. Materials and methods

4.1 Reagents and chemicals

All pesticides were purchased from Sigma-Aldrich (MO, USA), except milbemectin A4 and milbemectin A3, which were purchased from Bionordika (Herlev, Denmark); metofluthrin, which was purchased from LGC Standards (Wesel, Gemrany) and CL2CA, 3-Methyl-4-Nitrophenol (3Me4NP), dimethyl phosphate and diethyl phosphate, which were purchased from VWR (Søborg, Denmark). All pesticides were dissolved in DMSO at a stock concentration of 10 mM. PGE_1, progesterone and ionomycin were obtained from Sigma-Aldrich (MO, USA) and dissolved in DMSO at stock concentrations of 20 mM (PGE_1 and progesterone) and 1 mM (ionomycin). RU1968, a selective CatSper inhibitor, was obtained from Professor Timo Strünker upon request and dissolved in DMSO at a stock concentration of 10 mM. Additionally, three different chemical mixtures were prepared for the assessment of cooperative effects. The first two pesticide mixtures contained respectively 300 nM and 1000 µM of the 28 different pesticides in DMSO (Table 1). The third larger mixture contained 322.59 µM of the 28 pesticides from this study and 44 other chemicals known to affect Ca^{2+}-signaling in human sperm cells in DMSO (Table S2) (Schiffer *et al.*, 2014; Rehfeld *et al.*, 2016, 2020). All the chemicals and chemical mixtures mentioned above were stored in aliquots at -20 ˚C until use. The fluorophores Fluo-4 AM and BCECF AM were purchased from Invitrogen (CA, USA) and kept at -20 ˚C until they were dissolved in DMSO to a concentration of 10 µM on the day of the experiments. Fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (FITC-PSA) was purchased from Sigma–Aldrich (MO, USA) and propidium iodide (PI) and Hoechst-33342 (Hoechst) were obtained from ChemoMetec A/S (Allerød, Denmark).

4.2 Semen samples and ethical approval

Healthy human volunteers, with a history of delivering normal semen samples, donated the semen samples after their prior written consent. The semen samples were produced by masturbation and ejaculated into wide-mouthed plastic containers. No data on the fertility status or the general health of the donors was provided, and the semen samples were anonymized. Each donor received a compensation of 500 DKK (about 75 US dollars) per sample for their inconvenience. All samples were used for experiments on the day of delivery and destroyed immediately after the laboratory analyses were conducted. The study was approved by the regional scientific ethical committee of the Capital Region of Denmark with permit number H-19089581.

4.3 Purification of motile sperm cells

After delivery the semen sample was allowed to liquefy for 15-30 min at 37 °C. The fraction of viable and motile sperm cells was isolated from the semen by the swim-up method (Rehfeld *et al.*, 2019) using human tubular fluid (HTF^+) medium with the following composition: 97.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 4 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH. The cell concentration was determined by image cytometry (Egeberg *et al.*,

2013) and adjusted to 10×10^6 sperm cells in HTF^+-medium with human serum albumin (3 mg/mL). In experiments using capacitated sperm cells, the semen samples were resuspended to a concentration of 10×10^6 sperm cells in a capacitating medium with the following composition: 76.8 mM NaCl, 4.69 mM KCl, 0.2 mM $MgSO_4$, 0.37 mM KH_2PO_4, 2.04 mM $CaCl_2$, 0.33 mM Na-pyruvate, 21.4 mM Na-lactate, 2.78 mM glucose, 21 mM HEPES, and 25 mM $NaHCO_3$, adjusted to pH 7.3–7.4 with NaOH, with human serum albumin (3 mg/mL) and the sperm cells were incubated for >3 h at 37 °C in a 5% CO_2 atmosphere.

4.4 Measurement of changes in $[Ca^{2+}]_i$

Changes in $[Ca^{2+}]_i$ in human sperm cells were measured in 384 multi-well plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30 °C as described in Schiffer *et al.*, 2014. The sperm cells were incubated with the fluorescent Ca^{2+} indicator Fluo-4 AM (10 µM) for 45 min at 37 °C and excess dye was subsequently removed by centrifugation (700 x g, 10 min, RT). The sperm pellet was resuspended in HTF^+-medium to 5×10^6 sperm cells/mL. Aliquots of 50 µL were loaded to the wells of a 384 multi-well plate. Fluorescence was excited at 480 nm and emission was recorded at 520 nm with bottom optics. The fluorescence was recorded before and after injection of 25 µL (1:3 dilution) of pesticides, negative buffer control (HTF^+ with vehicle), and positive control (progesterone) to duplicate wells. In the initial screening a final pesticide concentration of 10 µM was used as this concentration allowed the best identification of compounds inducing Ca^{2+}-influxes in the screening performed by Schiffer *et al.*, 2014 and progesterone was used at a concentration of 5 µM for the positive control as in Rehfeld *et al.,* 2016. Changes in Fluo-4 fluorescence are shown as $\Delta F/F_0$ (%), indicating the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before addition of pesticides, positive and negative controls. The changes in $[Ca^{2+}]_i$ induced by the pesticides (final concentrations listed in Table S3) in the presence of the selective CatSper inhibitor RU1968 (Rennhack *et al.*, 2018) were measured as outlined above with or without preincubation for 5 minutes with 30 µM RU1968, as in Rehfeld *et al.*, 2020. The inhibitory effect of RU1968 was examined at 0-70 seconds after injection of the compounds to duplicate wells corresponding to the timeframe of a complete progesterone response. For the mixture experiment the changes in $[Ca^{2+}]_i$ induced by the pesticides alone (100 nM, final concentration) and in mixture (100 nM of each pesticide, final concentration) were measured as outlined above.

4.5 Assessment of dose-response relations

To assess the dose-response curves 10 serial dilutions with fixed ratios were conducted from a high concentration of each pesticide (100 µM or 200 µM, initial concentration of the serial dilution) to induce saturating responses. The 11 concentrations of the pesticides were added to the sperm cells together with a negative buffer control (HTF^+ with vehicle) and the changes in $[Ca^{2+}]_i$ was measured as mentioned above. The $\Delta F/F_0$ of the negative control was subtracted from the $\Delta F/F_0$ of the serial dilution to remove the dilution and pipetting artefacts. The dose-repose curves were calculated from the maximal values of the $\Delta F/F_0$ peaks, using the “log(agonist) vs. response - Variable slope (four parameters)” nonlinear regression analysis in GraphPad Prism 9. Since addition of hexachlorophene, cypermethrin and deltamethrin induced slowly rising Ca^{2+}-signals, the maximal peak values recorded until 120 seconds after addition of the compounds were used in the calculation of the dose-response curves. To be able to display all dose-response curves in a single figure (Figure 3) each dose-response curve was normalized to the

highest $\Delta F/F_0$ value of the individual curve. For the inhibition studies the mean basal fluorescence (F_0) was defined as the last 40 seconds prior to the subsequent addition of 100 nM progesterone or PGE_1. The dose-inhibition curves were calculated from the maximal $\Delta F/F_0$ peaks, using the "log(inhibitor) vs. response - Variable slope (four parameters)" nonlinear regression analysis in GraphPad Prism 9. For the assessment of the mixture dose-response curves serial dilutions of each of the two mixtures (10 µM, initial concentration of the pesticide mixture and 1.075 µM initial concentration of the larger mixture) were conducted and experiments performed as mentioned above.

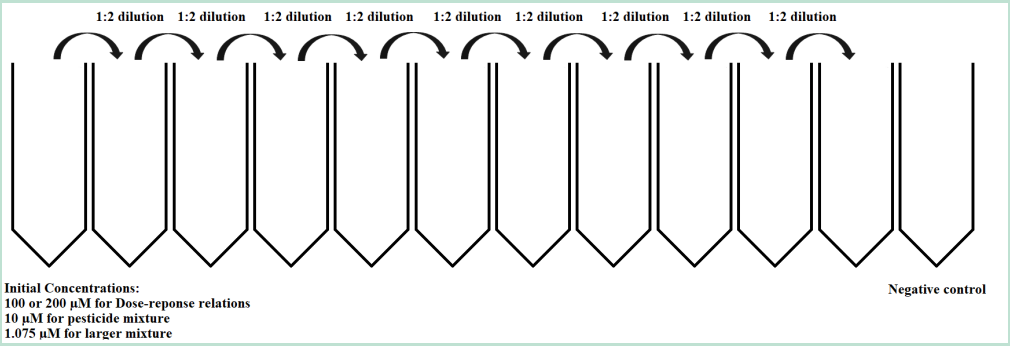


FIGURE 4.1. Illustration of serial dilutions used in this study. The Initial concentrations for the serial dilutions used in the assessment of the dose-response relations for each individual pesticide was either 100 or 200 µM. The initial concentration used in the assessment of the pesticide mixture dose-response relations was 10 µM. The initial concentration used in the assessment of the larger mixture dose-response relations was 1.075 µM.

4.6 Measurement of changes in pH_i

Changes in pH_i in human sperm cells were measured in 384 multi-well plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30 °C as in Schiffer *et al.*, 2014. Sperm cells were loaded with the fluorescent pH indicator BCECF AM (10 µM) for 15 min at 37 °C. Fluorescence was excited at 440 and 480 nm (dual excitation) and emission was recorded at 520 nm with bottom optics. Fluorescence was recorded before and after injection of 25 µL (1:3 dilution) of the pesticides (final concentrations listed in Table S3), negative buffer control (HTF^+ with vehicle) and positive control (NH_4Cl, 30 mM final concentration) to duplicate wells. Changes in the ratio of BCECF fluorescence between the 440 and 480 nm excitation are shown as $\Delta R/R_0$ (%) and indicates the percentage change in the ratio of fluorescence between the two modes of excitation (ΔR) with respect to the mean basal ratio of fluorescence between the two modes of excitation (R_0) before addition of pesticides and controls.

4.7 Assessment of acrosome reaction

Capacitated sperm cells were stained with an HTF^+-solution containing 5 µg/mL FITC-PSA, 0.5 µg/mL PI and 10 µg/mL Hoechst as in Rehfeld *et al.,* 2016. The pesticides (10 µM, final concentration), the two positive controls (ionomycin and progesterone, 10 µM final concentrations) and the negative buffer control (HTF^+ with vehicle) was added to separate aliquots of stained sperm sample and incubated for 30 minutes in a mixing heating plate at 37˚C. After incubation, the

sperm cells were immobilized with a solution consisting of 0.6 M $NaHCO_3$ and 0.37% (v/v) formaldehyde in distilled water. The samples were then transferred to a NC-slide A2 (ChemoMetec A/S, Allerød, Denmark) and analysed by image cytometry in a NC-3000 (ChemoMetec A/S, Allerød, Denmark) using the protocol described in Rehfeld *et al.*, 2018. PI intensity was plotted as a function of FITC-PSA intensity on bi-exponential scales and PI-negative, FITC-PSA-positive cells were categorized as viable acrosome reacted sperm cells. Only experiments with at least a 1.6-fold increment in viable acrosome reacted sperm cells after treatment with progesterone (10 µM) and a 3-fold increment in viable acrosome reacted sperm cells after treatment with ionomycin (10 µM) were included similar to in Birch *et al.*, 2021. The mean increment in viable acrosome reacted sperm cells after treatment with progesterone relative to the negative control was 2.3, which is comparable with the results obtained in our previous study with this assay (Egeberg Palme *et al.*, 2018). To visualize data from multiple experiments in a single figure, the data is shown as increase in viable acrosome-reacted sperm cells relative to the negative control.

4.8 Assessment of penetration into viscous medium

Sperm penetration was assessed in the modified Kremer's sperm–mucus penetration assay with 4000 cP methylcellulose (1% w/v) as the artificial viscous medium as described in detail in Rehfeld *et al.*, 2018. In brief, sperm samples containing pesticides (10 µM, final concentration), a positive control (progesterone, 5 µM final concentration) and a negative buffer control (HTF^+ with vehicle) were allowed to penetrate into the methylcellulose solution for 1 hour at 37˚C. The methylcellulose medium was freshly made before each experiment. After 1 hour the sperm cells were immobilized by exposure to UV radiation (ChemiDoc XRS^+ imaging system, Bio-Rad) for five minutes as in Rehfeld *et al.*, 2020. The amount of sperm cells penetrating 2 cm from the base of the glass tube were assessed using phase contrast on an Olympus BX45 microscope at a total magnification of x200 (Olympus, Denmark).

4.9 Assessment of sperm viability

The sperm viability of uncapacitated sperm cells was determined by image cytometry on a NC-3000 (ChemoMetec A/S, Allerød, Denmark) as described in Birch *et al.*, 2021. In brief, sperm cells were incubated for 21 hours at 37 °C with each pesticide (10 µM, final concentration), a positive control (0.5% Triton X, final concentration) and a negative buffer control (HTF^+ with vehicle) before the viability was assessed. To visualize data from multiple experiments in a single figure, the data is normalized relative to the positive control. The sperm viability of capacitated sperm cells was measured after incubation for 30 mins at 37 °C with each pesticide (10 µM, final concentration) concurrently with the assessment of the acrosome reaction.

4.10 Statistical analysis

The data from the three assays for acrosome reaction, sperm penetration into viscous medium, and viability assessment were analyzed using linear mixed models with donorID and experimentID inserted as random factors. This allows taking into account the considerable inter-donor and inter-experiment variation. Data from the assessment of sperm viability and acrosome reaction were log transformed prior to analysis. Data from the assessment of sperm penetration were square root transformed prior to analysis. The transformations ensured that the model residuals

were approximately Gaussian distributed. A Dunnett's correction was applied to p-values to adjust for multiple testing. The analyses were done in R (R for Windows version 4.2.0).

5. Results

5.1 Assessment of changes in $[Ca^{2+}]_i$ in human sperm after pesticide exposure

In this study we examined 53 different pesticides for their ability to induce Ca^{2+}-signals in human sperm cells using a well-established Ca^{2+}-fluorometric assay (Schiffer *et al.*, 2014). The pesticides were tested at 10 µM along with a positive control (5 µM progesterone) and a negative buffer control (HTF^+ with vehicle). The Ca^{2+}-signals were recorded for 208 seconds after injection of the compounds to duplicate wells (Figure 2).

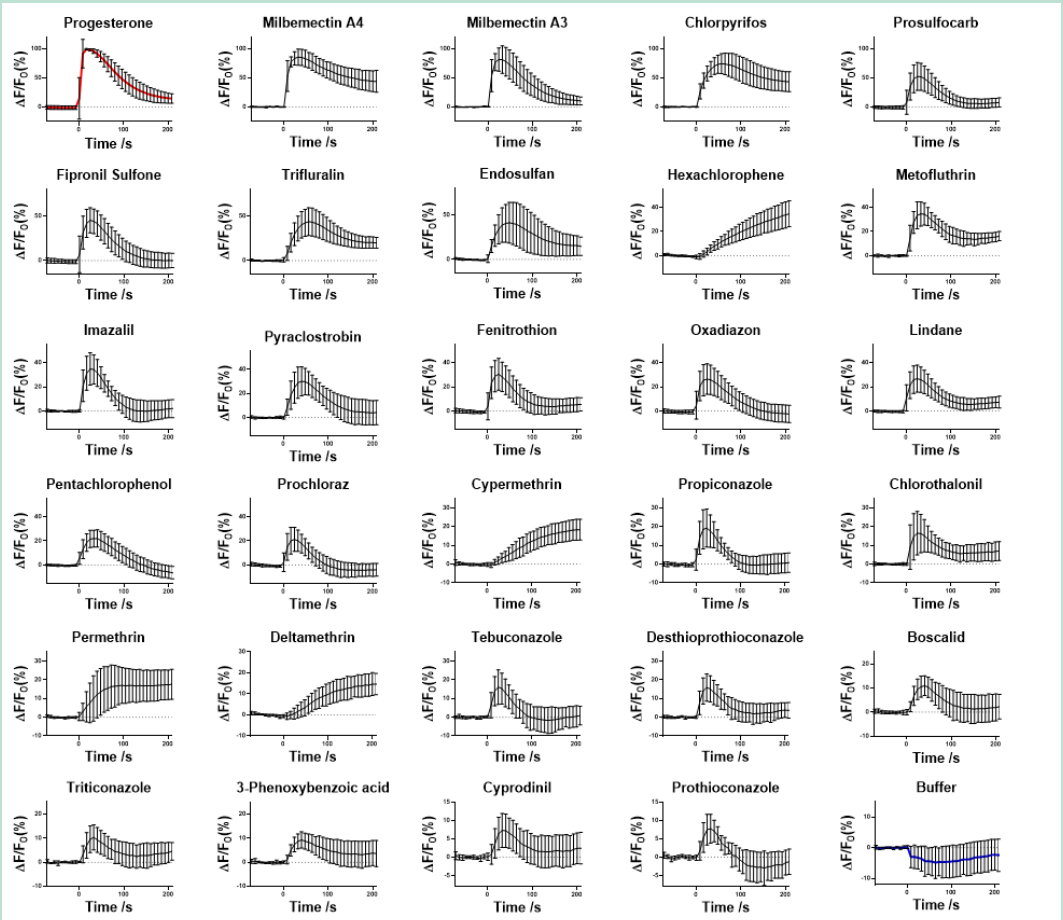
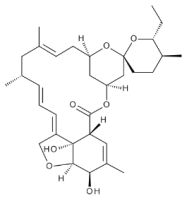
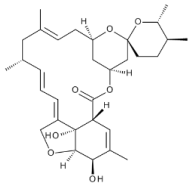
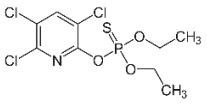
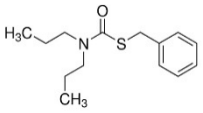
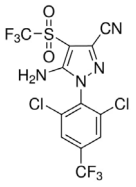
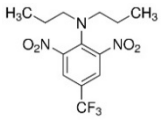


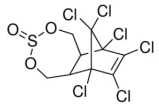
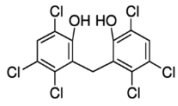
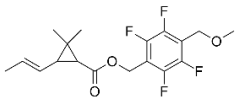
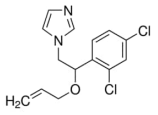
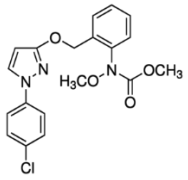
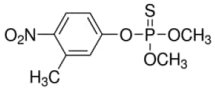
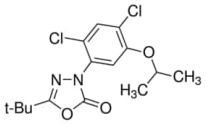
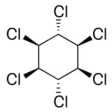
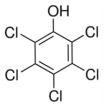
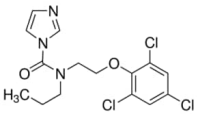
FIGURE 5.1. Ca2+-signals induced by 5 µM progesterone, 10 µM of each active pesticide, and the negative buffer control relative to progesterone (mean ± SD, n ≥ 6, with ≥ 3 different donors). Note the decrease in the y-axis range from the upper left corner (progesterone) toward the lower right corner (negative buffer con-trol).

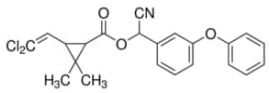
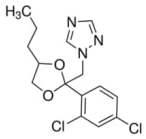
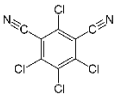
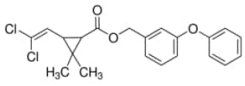
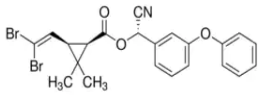
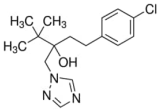
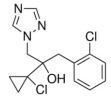
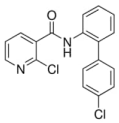
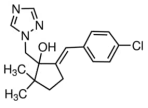
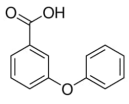
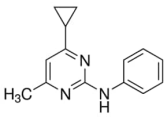
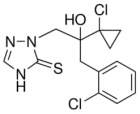
To compare results between experiments and donors, relative peak Ca^{2+}-signals were calculated by dividing the peak Ca^{2+}-signal induced by a pesticide with that of the paired positive control in a given experiment. Of the 53 pesticides tested 28 pesticides induced a mean relative

peak Ca^{2+}-signal larger than the mean relative peak Ca^{2+}-signal induced by the negative buffer controls ± 3 SD (0.458% ± 3 × 2.608%, giving a maximal value of 8.282%) (Table 1). These 28 pesticides were chosen for further investigation and are in the following referred to as the “active” pesticides. No further investigation of the remaining 25 “inactive” pesticides was conducted (Table S1).

TABLE 5.1. The 28 pesticides categorized as active pesticides* ranked according to their efficaciousness at 10 µM in the Ca2+-fluorometric assay. CAS numbers and chemical structures are also listed in the table.

Rank:	Compound:	CAS #:	Mean relative peak Ca^{2+}-signal at 10uM (in % of paired progesterone induced response) (n ≥ 6):	Chemical structure**:
1	Milbemectin A4	51596-11-3	87.76	
2	Milbemectin A3	51596-10-2	85.61	
3	Chlorpyrifos	2921-88-2	76.29	
4	Prosulfocarb	52888-80-9	53.05	
5	Fipronil Sulfone	120068-36-2	46.36	
6	Trifluralin	1582-09-8	44.19	

7	Endosulfan	115-29-7	42.37	
8	Hexachlorophene	70-30-4	41.53	
9	Metofluthrin	240494-70-6	37.10	
10	Imazalil	35554-44-0	36.56	
11	Pyraclostrobin	175013-18-0	32.35	
12	Fenitrothion	122-14-5	31.16	
13	Oxadiazon	19666-30-9	26.92	
14	Lindane	58-89-9	26.79	
15	Pentachlorophenol	87-86-5	24.58	
16	Prochloraz	67747-09-5	22.26	

17	Cypermethrin	52315-07-8	19.96	
18	Propiconazole	60207-90-1	19.31	
19	Chlorothalonil	1897-45-6	17.75	
20	Permethrin	52645-53-1	17.72	
21	Deltamethrin	52918-63-5	16.66	
22	Tebuconazole	107534-96-3	16.63	
23	Desthioprothioconazole	120983-64-4	15.62	
24	Boscalid	188425-85-6	11.81	
25	Triticonazole	131983-72-7	10.62	
26	3-Phenoxybenzoic acid	3739-38-6	9.48	
27	Cyprodinil	121552-61-2	9.24	
28	Prothioconazole	178928-70-6	8.74	

*: Based on their ability to induce Ca2+-signals, the pesticides were categorized as “active” if they induced a mean relative peak Ca2+-signal above that of the negative control (HTF+ with vehicle) ± 3 × SD (0.458% ± 3 × 2.608% = 8.282%) (n ≥ 6, with ≥ 3 different donors) **: The chemical structure for each pesticide was obtained from the supplier website.

5.2 Dose-response relations

We investigated the dose-response relations for each of the active pesticides to examine whether these pesticides could induce Ca^{2+}-signals at µM or nM concentrations, which most likely could be of physiological relevance. All active pesticides except hexachlorophene, lindane, boscalid and 3-phenoxybenzoic acid (Figure S1), produced saturating dose-response curves with mean EC_{50} values within the concentration range 0.901 - 43.87 µM and mean EC_{05} values within the concentration range 0.036 - 6.48 µM (Table 2 and Figure 3).

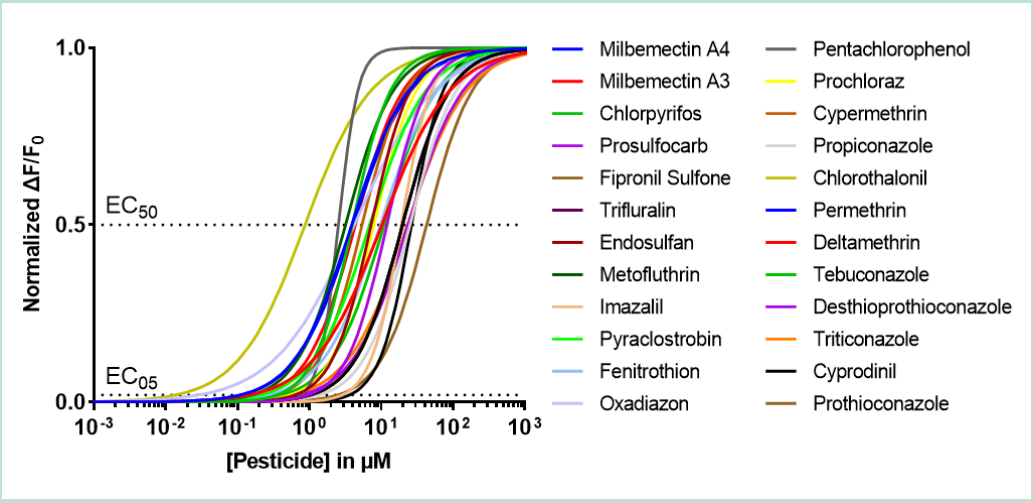


FIGURE 3. Normalized dose-response curves for the 24 pesticides producing saturating responses. The dose-response curves were generated using the mean log(EC50) and hillslope values and normalized between 0 and 1. All dose-response curves shown in this figure were normalized between 0 and 1. Note that the efficacy of the pesticides cannot be deduced from this figure, but was found to differ at 10 µM as seen in Table 1.

TABLE 5.2. Table 2. For each active pesticide the EC50 and EC05 values were calculated from the dose response curves. The IC50 values for Ca2+-signals induced by subsequent addition of progesterone or PGE1 (100 nM) in the presence of each active pesticide were calculated from the dose inhibition curves.

	EC_{50}, µM		EC_{05}, µM		IC_{50} Prog., µM		IC_{50} PGE_1, µM	
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
Milbemectin A4	4.68	2.60	0.40	0.37	6.74	2.11	9.13	0.89
Milbemectin A3	12.37	7.40	0.65	0.75	10.50	2.51	16.10	1.18

Chlorpyrifos	4.15	1.33	0.65	0.20	6.28	2.56	4.67	0.55
Prosulfocarb	12.24	2.69	1.88	0.97	19.09	2.28	16.83	1.93
Fipronil Sulfone	21.54	9.97	2.19	1.26	37.99	18.11	32.47	7.86
Trifluralin	2.57	0.64	0.31	0.15	-	-	-	-
Endosulfan	7.51	1.95	1.17	0.35	7.21	0.56	7.82	1.71
Hexachlorophene	-	-	-	-	-	-	1.41	0.19
Metofluthrin	3.39	1.32	0.37	0.21	3.89	0.24	3.82	1.48
Imazalil	21.68	11.90	5.51	5.02	24.03	4.92	124.10	84.26
Pyraclostrobin	7.26	2.47	0.49	0.25	11.75	4.58	8.54	0.64
Fenitrothion	10.02	3.15	0.62	0.28	18.46	6.76	-	-
Oxadiazon	4.58	2.26	0.09	0.04	6.76	2.62	-	-
Lindane	-	-	-	-	-	-	-	-
Pentachlorophenol	2.70	1.01	0.90	0.25	-	-	9.29	2.25
Prochloraz	9.39	6.38	0.94	0.38	-	-	-	-
Cypermethrin	8.71	8.07	0.80	0.49	-	-	-	-
Propiconazole	26.57	9.40	3.26	1.87	43.41	17.14	78.95	15.53
Chlorothalonil	0.95	0.49	0.04	0.02	3.02	0.59	-	-
Permethrin	3.80	1.40	0.33	0.20	-	-	-	-
Deltamethrin	5.16	3.59	0.41	0.13	-	-	-	-
Tebuconazole	11.25	2.86	0.93	0.27	-	-	-	-
Desthioprothioconazole	24.06	5.02	1.81	1.26	33.16	8.32	93.59	22.45
Boscalid	-	-	-	-	-	-	-	-
Triticonazole	23.99	6.93	1.38	0.35	-	-	-	-
3-Phenoxybenzoic acid	-	-	-	-	-	-	-	-
Cyprodinil	35.22	21.86	6.48	3.43	30.65	7.22	43.55	7.95
Prothioconazole	43.87	9.21	5.16	2.91	-	-	-	-

EC50 and EC05: mean and SD were calculated from EC50 and EC05 values of the individual dose response curves (n ≥ 3, with ≥ 3 different donors). IC50: mean and SD were calculated from IC50 values of the individual dose inhi-bition curves generated from the Ca2+-signals induced by subsequent addition of 100 nM progesterone or PGE1 after preincubation of human sperm cells with various concentrations of the active pesticides (n = 3, with 3 differ-ent donors).

5.3 Effect on CatSper

To test whether the active pesticides induced Ca^{2+}-influxes through CatSper, we used the specific CatSper inhibitor RU1968 (Rennhack *et al.*, 2018). The peak Ca^{2+}-signals induced by near EC_{100}-concentrations of the active pesticides (Table S3) in the presence and absence of 30 µM RU1968 were compared. Similar to progesterone, Ca^{2+}-signals induced by all pesticides, except hexachlorophene and deltamethrin, were strongly inhibited in the presence of 30 µM RU1968 (Figure 4). Furthermore, these data show that no interactions between the pesticides and the fluorophore Fluo-4 AM occur as the signals are eliminated.

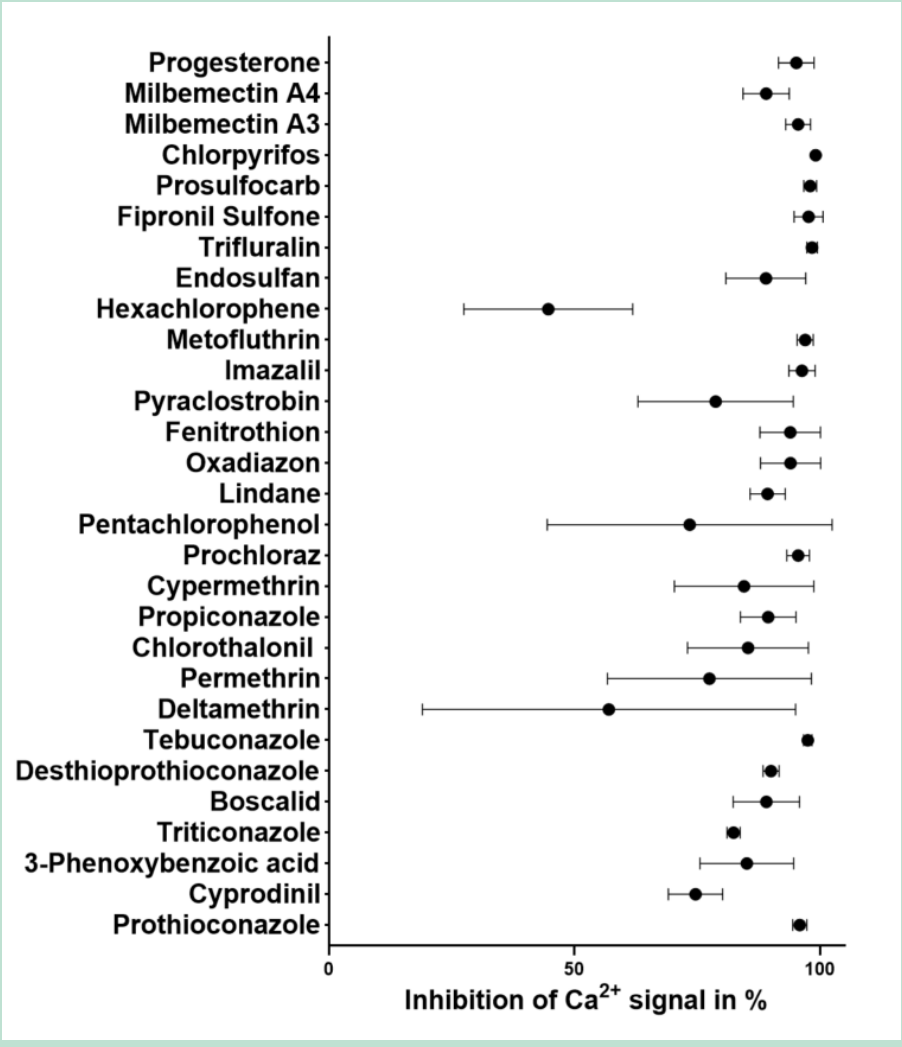


FIGURE 4. Percentage inhibition of peak Ca2+-signals induced by the active pesticide (at near EC100-concentrations) and progesterone (5 µM) in the presence of 30 µM RU1968 (mean inhibition ± SD, n ≥ 4, with ≥ 3 differ-ent donors).

Since the CatSper channel can also be activated by an alkalization of pH_i we investigated whether the active pesticides alkalized the pH_i at concentrations near the EC_{100} (Table S3). No alkalization of the pH_i was found for the pesticides, except for hexachlorophene that was found to slowly increase the pH_i from 174 seconds after injection to duplicate wells (Figure 5).

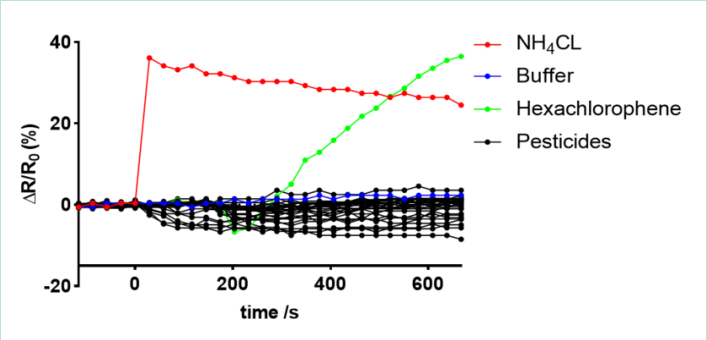


FIGURE 5. Changes in pHi induced by each active pesticide (near EC100-concentrations), the negative buffer con-trol, and the positive control (NH4Cl, 30 mM) (mean ± SD, n ≥ 4, with ≥ 3 different donors).

5.4 Inhibition of progesterone and PGE_1-induced Ca^{2+}-signals

To clarify if the active pesticides interfered with subsequent progesterone and PGE_1-induced Ca^{2+}-signals, human sperm cells were preincubated for 5 minutes with serially diluted doses of the active pesticides or the negative buffer control (HTF^+ with vehicle). The amplitude of the Ca^{2+}-signal induced by a subsequent addition of 100 nM progesterone or PGE_1 was examined. 15 of the 28 active pesticides inhibited either progesterone or PGE_1-induced Ca^{2+}-signals, or both, in a dose dependent manner (Figure 6 and Figure S2). Inhibition of both progesterone and PGE_1-induced Ca^{2+}-signals was seen for milbemectin A4 and prosulfocarb (Figure 6A, B) among 10 other compounds (Figure S2). However, for some of the active pesticides a dose dependent inhibition was only observed for one of the two endogenous ligands. For instance, for fenitrothion and pentachlorophenol a dose dependent inhibition was found for only progesterone and only PGE_1, respectively (Figure 6C, D). The mean IC_{50} values for the active pesticides inhibiting progesterone and PGE_1-induced Ca^{2+}-signals were within the concentration range of 1.83-168.7 µM and 3.82-687.5 µM, respectively (Table 2).

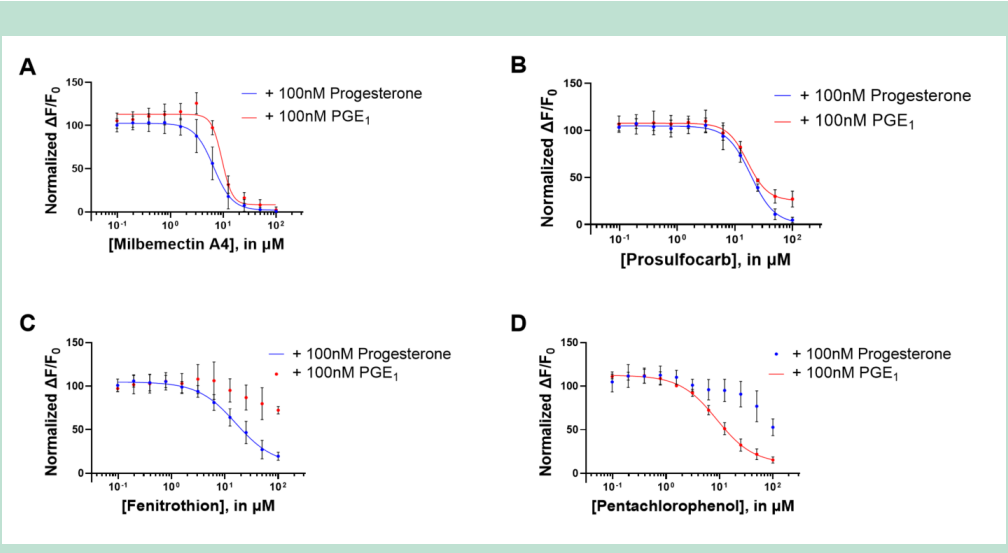


FIGURE 6. Normalized dose-inhibition relations (n ≥ 3, with ≥ 3 different donors) of the Ca2+-signals induced by 100 nM progesterone and PGE1 after 5 minutes of preincubation with different concentrations of the pesti-cides: (A) Milbemectin A4, (B) Prosulfocarb, (C) Fenitrothion,

and (D) Pentachlorophenol. IC50 values are listed in Table 2. The results for the remaining 22 active pesticides are shown in Figure S2.

5.5 Effects on Ca^{2+}-mediated sperm functions and viability

We next investigated whether an effect on the Ca^{2+}-mediated sperm functions, acrosome reaction (Figure 7), and penetration into viscous medium (Figure 8) could be observed for the active pesticides at 10 µM. Milbemectin A4 ($p < 0.001$), milbemectin A3 ($p < 0.001$), metofluthrin ($p = 0.026$), and cyprodinil ($p = 0.038$) were all found to significantly increase the amount of viable acrosome reacted sperm cells in a manner similar to the positive control, 10 µM progesterone ($p < 0.001$) (Figure 7). Fipronil sulfone ($p = 0.005$) and pyraclostrobin ($p = 0.024$) were found to significantly decrease the amount of viable acrosome reacted sperm cells (Figure 7).

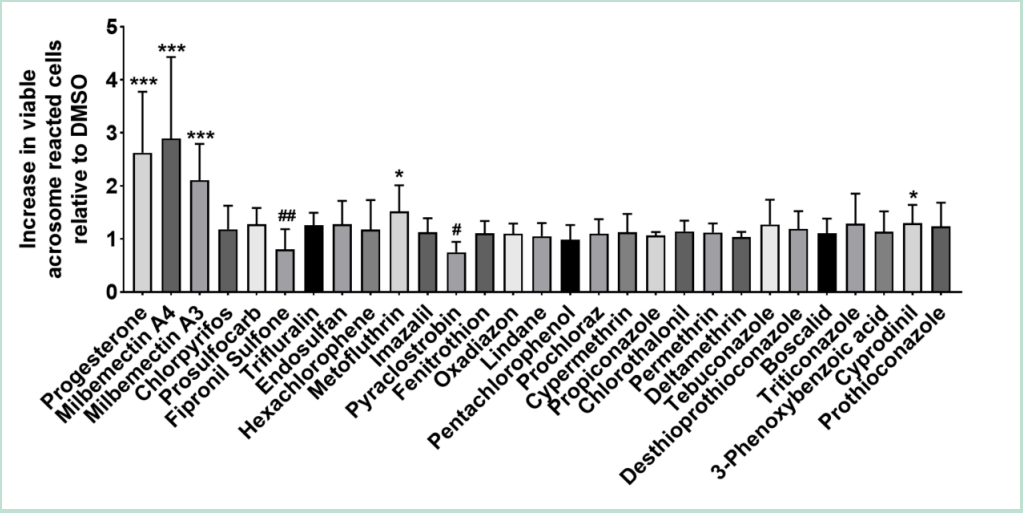


FIGURE 7. Increase in viable acrosome-reacted sperm cells relative to in in the negative 0.2% DMSO control (mean ± SD, n ≥ 5, with ≥ 3 different donors) after 30-min incubation with the positive control (10 µM progester-one), the negative 0.2% DMSO control, or the active pesticides (10 µM). *: $p < 0.05$ and ***: $p < 0.001$ indicates a significant increase in viable acrosome reacted sperm cells. #: $p < 0.05$ and ##: $p < 0.01$ indi-cates a significant decrease in viable acrosome reacted sperm cells.

Effects on sperm penetration into viscous medium (Figure 8) was observed for chlorpyrifos, trifluralin, metofluthrin, and propiconazole. Chlorpyrifos ($p < 0.001$), trifluralin ($p = 0.046$), and metofluthrin ($p = 0.019$) significantly increased the amount of sperm penetration similar to what is observed for progesterone ($p < 0.001$), whereas propiconazole ($p = 0.002$) significantly inhibited sperm penetration.

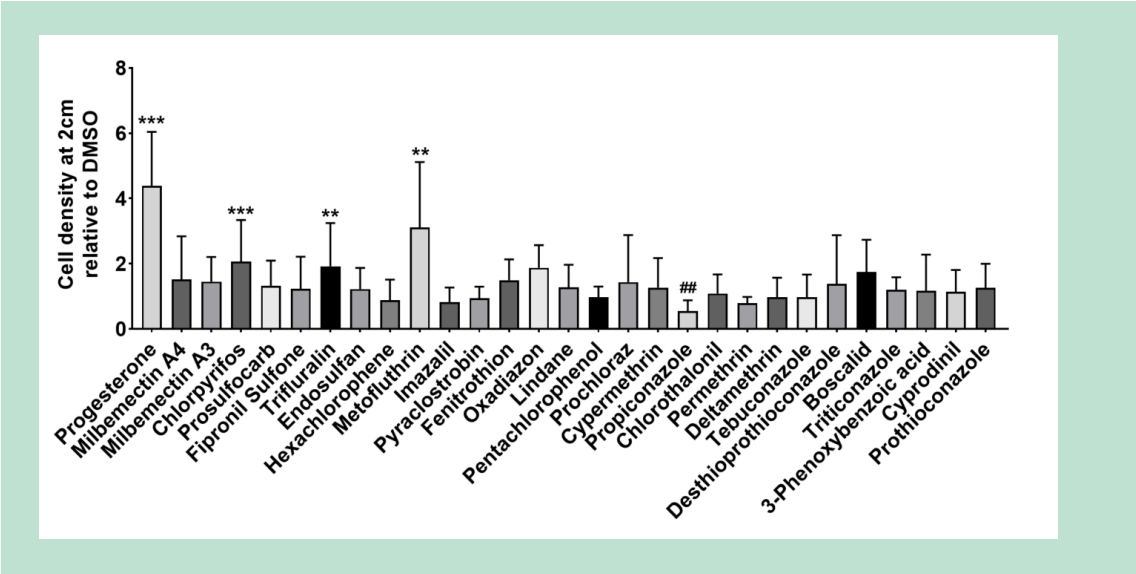


FIGURE 8. Cell density at 2 cm into a viscous medium relative to the cell density in the negative 0.2% DMSO control (mean ± SD, n ≥ 4, with ≥ 3 different donors) after 1 h incubation with the positive control (5 µM proges-terone), the negative 0.2% DMSO control, or the active pesticides (10 µM). **: $p < 0.01$ and ***: $p < 0.001$ indicates a significant increase in sperm penetration. ##: $p < 0.01$ indicates a significant decrease in sperm penetration.

The active pesticides were also investigated for their effect on sperm viability at 10 µM. Only milbemectin A4 ($p < 0.001$) and milbemectin A3 ($p < 0.001$) were found to significantly affect the sperm viability of uncapacitated sperm cells after 21 hours of incubation (Figure 9A). In capacitated sperm cells we investigated if the changes in the sperm cells arisen during the capacitation process could render them more sensitive towards pesticide exposure. Boscalid ($p = 0.019$), cyprodinil ($p = 0.015$), prothioconazole ($p = 0.007$), triticonazole ($p = 0.008$), and desthioprothioconazole ($p = 0.019$) all significantly induced cell death in capacitated sperm cells after 30 minutes of incubation (Figure 9B).

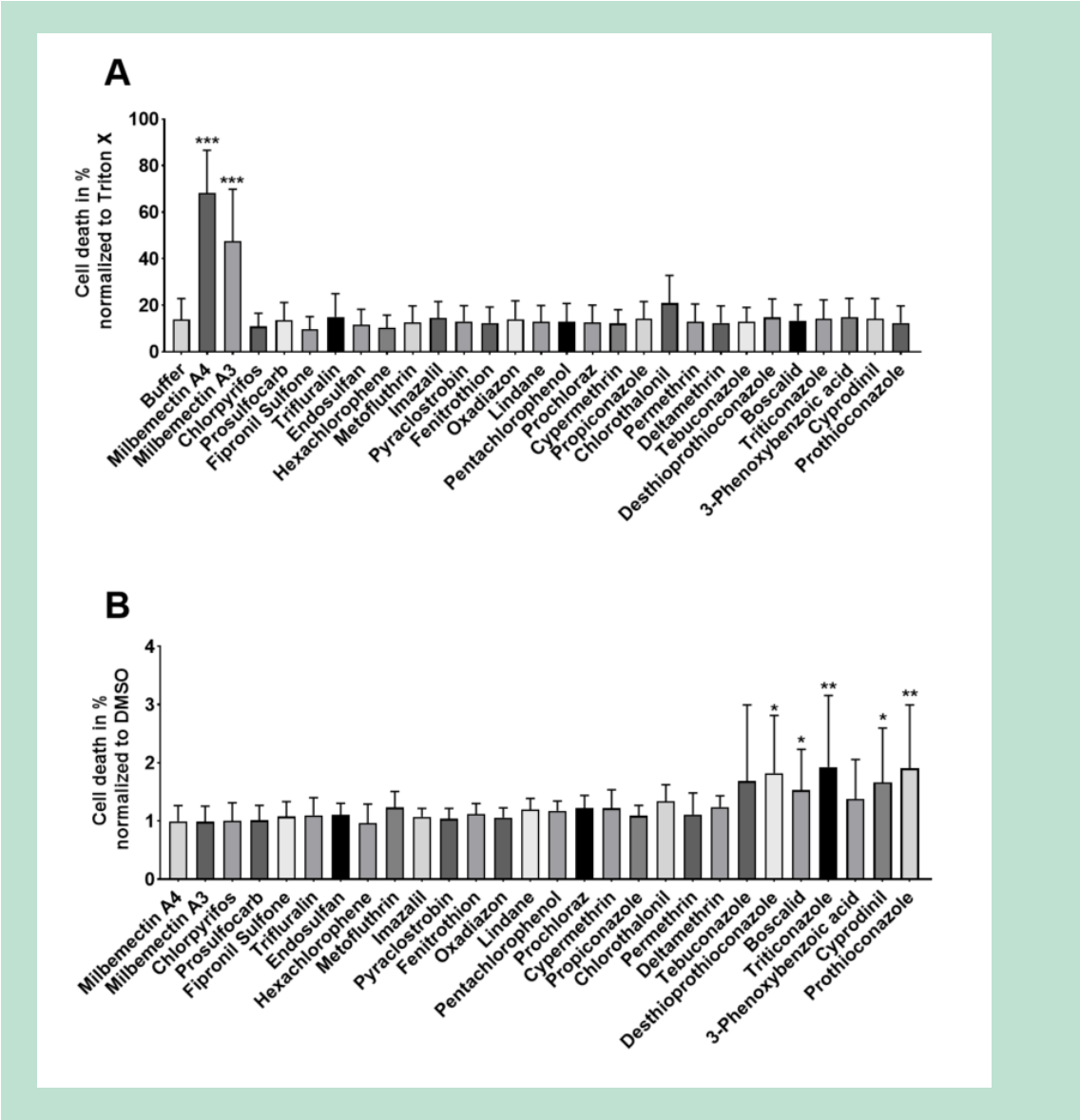


FIGURE 9A.: Cell death in % in uncapacitated sperm cells relative to the positive 0.5% Triton X control after 21 hours of incubation with the negative control (0.1% DMSO), the positive control, or the active pesticides (10 µM) (mean ± SD, n = 3, with 3 different donors). ***: $p < 0.001$ indicates a significant increase in cell death compared to the negative buffer control (0.1% DMSO). **B**: Cell death in % in capacitated sperm cells relative to the negative 0.2% DMSO control after 30 minutes of incubation with the active pesticides (10 µM) or the negative buffer control (0.2% DMSO) (mean ± SD, n ≥ 5, with ≥ 3 different donors). *: $p < 0.05$ and **: $p < 0.01$ indicates a significant increase in cell death compared to the negative buffer control (0.2% DMSO).

5.6 Mixture effect of the active pesticides

Human sperm cells most likely encounter a complex mixture of different chemicals within the female reproductive system (Di Renzo *et al.*, 2015). We therefore examined whether pesticides in a low dose mixture could cooperate to induce Ca^{2+}-signals. The pesticide mixture containing 100 nM of each active pesticide induced a Ca^{2+}-signal smaller than that of the positive control (progesterone, 5 µM), but significantly larger than the Ca^{2+}-signals induced by the single compounds at 100 nM and the negative buffer control ($p = 0.002$) (Figure 10A). The mean peak Ca^{2+}-signal induced by the pesticide mixture was found to be 23.44% of the mean peak Ca^{2+}-

signal induced by the positive control (Figure 10B). We also investigated whether the low dose pesticide mixture could exert a cooperative inhibitory effect on the Ca^{2+}-signal induced subsequently by 10 nM progesterone. The pesticide mixture was found to significantly inhibit the Ca^{2+}-signal induced by 10 nM progesterone by 29.47% (p = 0.013) (Figure 10C), whereas no inhibitory effect of the individual pesticides at 100 nM was observed (Figure 10D).

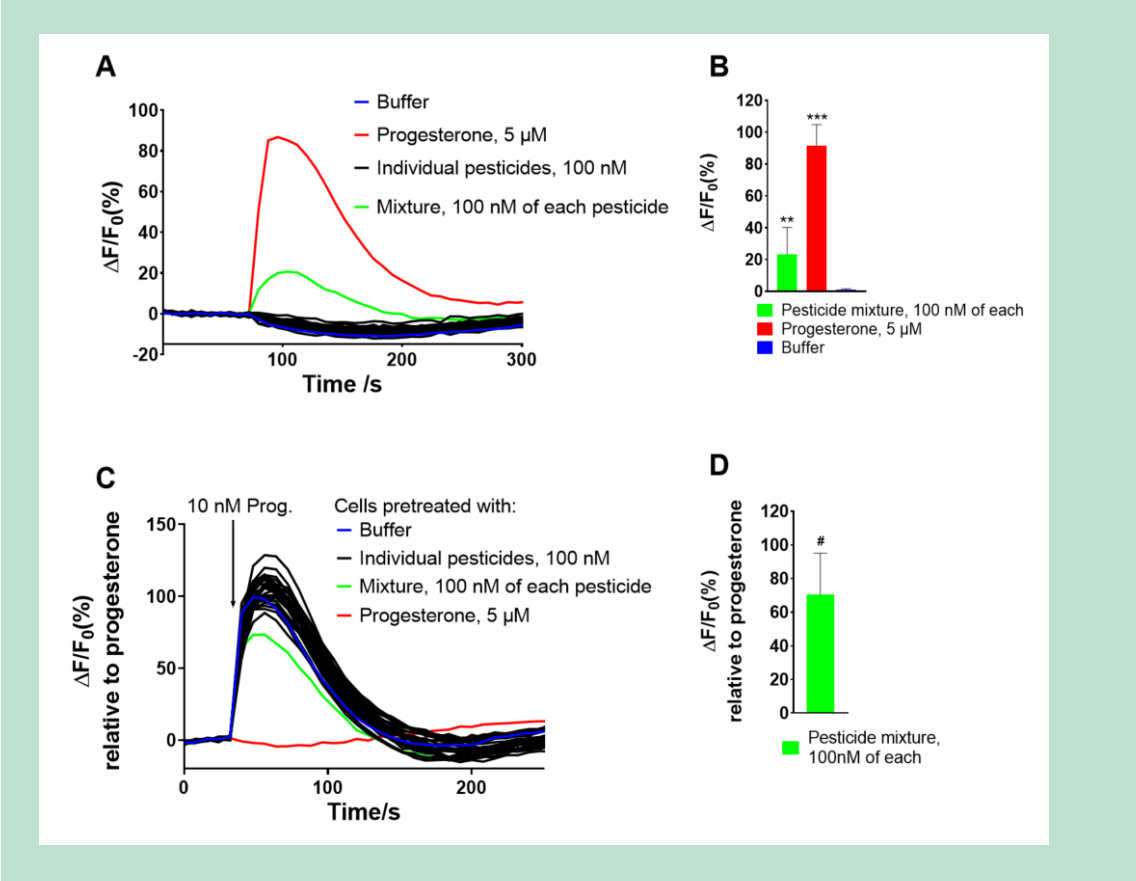


FIGURE 10. Figure 10. **A**, Ca2+-signals induced by the individual pesticides (100 nM), progesterone (5 µM, positive control), negative buffer controls, and the pesticide mixture (100 nM of each pesticide). **B**, Mean peak Ca2+-signals induced by the pesticide mixture, 5 µM progesterone, and the negative buffer control (mean ± SD, n = 10, with 8 different donors). **: $p < 0.01$ and ***: $p < 0.001$ indicates a mean peak Ca2+-signal significantly larger than in the negative buffer control. **C**, Ca2+-signals induced by 10 nM progesterone in the presence of the individual pesticides (100 nM), progesterone (5 µM), the negative control buffer (set to 100%), and the pesticide mixture (100 nM of each pesticide). **D**, Mean inhibition of the peak Ca2+-signal induced by 10 nM progesterone in the presence of the pesticide mixture relative to the Ca2+-signal in-duced by 10 nM progesterone in buffer (set to 100%) (mean ± SD, n = 10, with 8 different donors). #: $p < 0.05$ indicates a significant inhibition of the progesterone-induced Ca2+-signals compared to in the buffer alone. Please note that 10 nM progesterone does not induce any Ca2+-signal after pretreatment with 5 µM progesterone as the CatSper channel is desensitized (Blackmore et al., 1990).

To investigate the physiological relevance of the pesticide mixture, dose-response curves were conducted (Figure 11). The pesticide mixture was found to yield a saturating dose response curve with a mean EC_{50} of 329.3 nM and a mean EC_{05} of 69.72 nM (Figure 11A). Furthermore, the pesticide mixture was found to inhibit Ca^{2+}-signals induced by subsequent addition of both

100 nM progesterone and PGE_1 (Figure 11B, C) with mean IC_{50} values of 286.2 nM and 559.3 nM, respectively (Figure 11D).

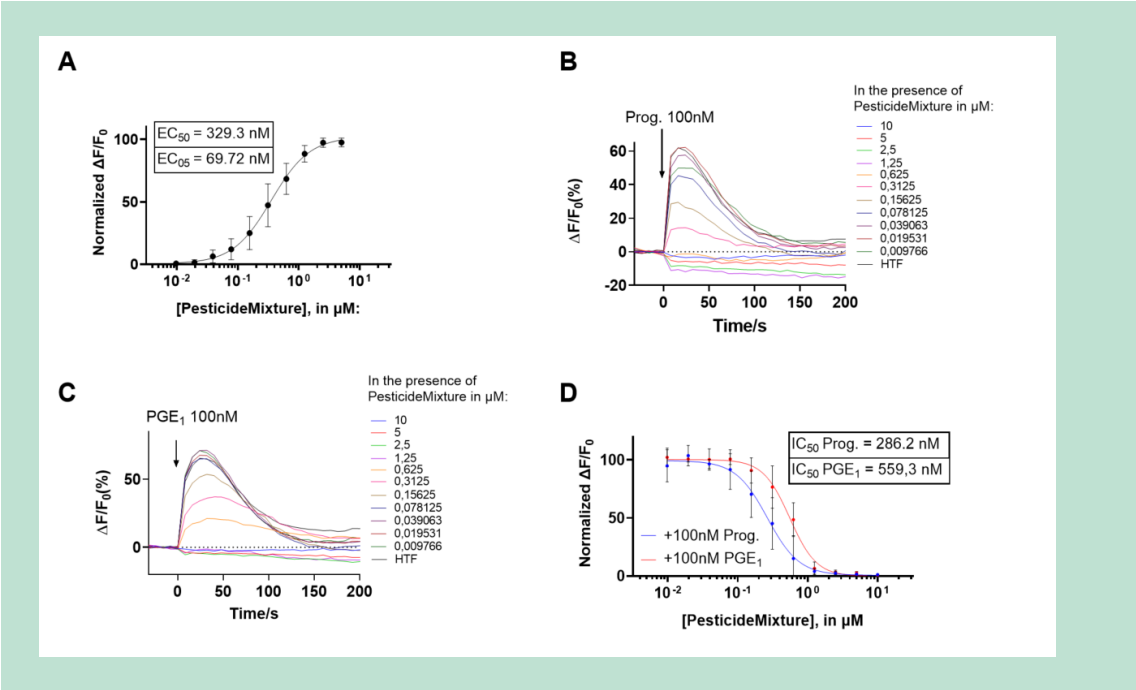


FIGURE 11. A, Normalized dose-response curve (mean ± SD, n = 6, with 3 different donors) of the pesticide mixture. Note that EC50 and EC05 values are both in the nM range. **B**, Inhibition of Ca2+-signals induced by 100 nM progesterone after 5 minutes of preincubation with the negative buffer control and different concen-trations of the pesticide mixture. **C**, Inhibition of Ca2+-signals induced by 100 nM PGE1 after 5 minutes of preincubation with the negative buffer control and different concentrations of the pesticide mixture. **D**, Normalized dose-inhibition relations of the Ca2+-signals induced by 100 nM progesterone and PGE1 in the presence of the pesticide mixture (mean ± SD, n = 3, with 3 different donors). Note that both IC50 values are in the nM range.

We also examined the effect of the pesticide mixture in combination with other types of environmental chemicals to simulate a more realistic human exposure. In addition to the 28 active pesticides, 44 different chemicals (Table S2) known to affect Ca^{2+}-signaling in human sperm cells (Schiffer *et al.*, 2014; Rehfeld *et al.*, 2016, 2020) were included in this larger mixture. The larger mixture was found to yield a saturating dose response curve with a mean EC_{50} of 47.72 nM and a mean EC_{05} of 4.60 nM (Figure 12A). Furthermore, the larger mixture was found to inhibit Ca^{2+}-signals induced by subsequent addition of both 100 nM progesterone and PGE_1 (Figure 12B, C) with mean IC_{50} values of 44.76 nM and 62.71 nM respectively (Figure 12D).

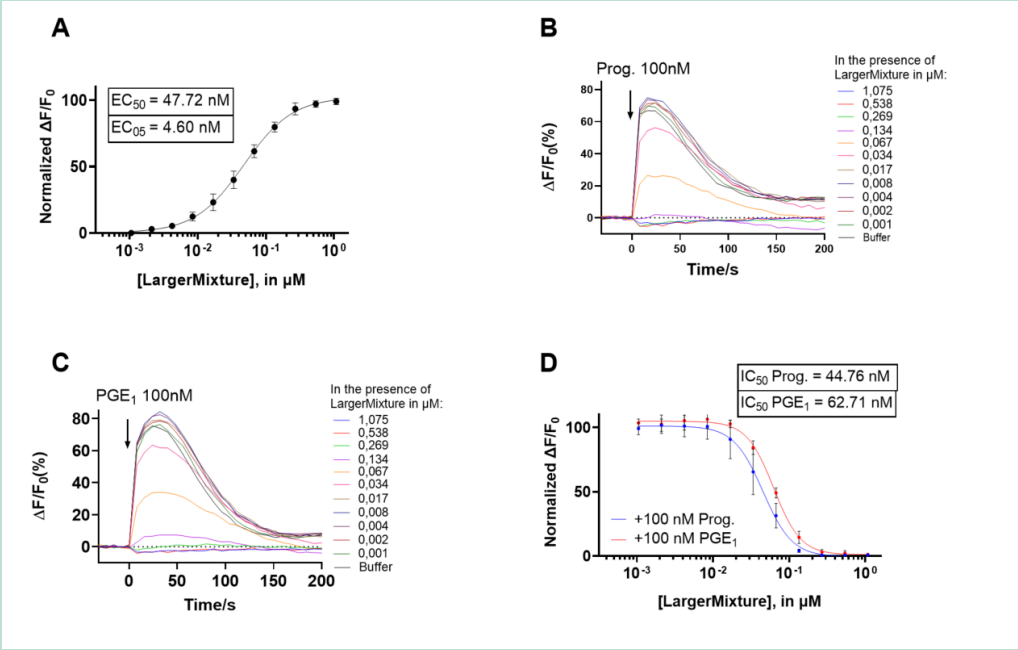


FIGURE 12. A, Normalized dose-response curve (mean ± SD, n = 6, with 3 different donors) of the larger chemical mixture. Note that EC50 and EC05 values are both in the low nM range. **B**, Inhibition of Ca2+-signals in-duced by 100 nM progesterone after 5 minutes of preincubation with the negative buffer control and different concentrations of the larger mixture. **C**, Inhibition of Ca2+-signals induced by 100 nM PGE1 after 5 minutes of preincubation with the negative buffer control and different concentrations of the larger mixture. **D**, Normalized dose-inhibition relations of the Ca2+-signals induced by 100 nM progesterone and PGE1 in the presence of the larger mixture (mean ± SD, n = 3, with 3 different donors). Note that both IC50 values are in the low nM range.

6. Discussion

We found that 28 of 53 tested pesticides induced Ca^{2+}-signals in human sperm cells at 10 µM (Figure 2). Using the specific CatSper inhibitor RU1968 we showed that 26 of these 28 pesticides induced Ca^{2+}-signals through an activation of the CatSper channel (Figure 4). These pesticides thereby mimic the effect of progesterone in human sperm cells, act as EDCs, and can be added to the still growing list of environmental chemicals that interfere with the normal Ca^{2+}-signaling in human sperm (Tavares *et al.*, 2013; Schiffer *et al.*, 2014; Rehfeld *et al.*, 2016, 2018, 2020; Shannon *et al.*, 2016; Zou *et al.*, 2017; Yuan *et al.*, 2020; Birch *et al.*, 2021), highlighting the promiscuous activation of CatSper in human sperm cells. The chemical structure of the pesticides investigated in this study (Table 1) vary a lot and do not resemble the chemical structure of either progesterone or PGE_1. Thus, from the structure alone it is difficult to predict through which of the ligand binding sites these pesticides may activate the CatSper channel. Even the structurally similar pyrethroids, cypermethrin and deltamethrin, which apparently induce Ca^{2+}-signals with similar slowly increasing kinetics are inhibited to quite different degrees in the presence of CatSper inhibitor RU1968 (Figure 4), indicating that even such structurally very similar pesticides induce Ca^{2+}-signals in human sperm cells through different modes of action.

Three of the active compounds were metabolites of pesticides that were also investigated in this study. Fipronil sulfone was the fifth most efficacious compound at inducing Ca^{2+}-signals at 10 µM (Table 1), whereas the mother compound fipronil was inactive (Table S1). Desthioprothioconazole, the metabolite of prothioconazole, was also found to be more efficacious at inducing Ca^{2+}-signals than the mother compound at 10 µM, whereas 3-phenoxybenzoic acid, which is a nonspecific metabolite of a variety of pyrethroid insecticides (Norén *et al.*, 2020), was found to be less efficacious at inducing Ca^{2+}-signals than its mother compounds cypermethrin, permethrin, and deltamethrin at 10 µM. In addition, TCPy the metabolite of chlorpyrifos, which was the third most efficacious pesticide at inducing Ca^{2+}-signals at 10 µM, was not among the 28 active compounds. This further emphasizes that small molecular changes can have a large impact on the effect on Ca^{2+}-signaling in human sperm cells (Table 1) and that endogenous metabolism of the pesticides may both increase and decrease the ability of pesticides to induce Ca^{2+}-signals in human sperm cells.

All the active pesticides except hexachlorophene, lindane, boscalid and 3-phenoxybenzoic acid produced a saturating dose response curve (Figure 3) with a mean EC_{50} ranging from 0.901 - 43.87 µM and a mean EC_{05} ranging from 0.036 - 6.48 µM (Table 2). The EC_{05} can be used as an indicator of the lowest effective concentration of the individual pesticide. With the majority of pesticides having EC_{05} values in the nM range it is possible that human sperm cells may be affected at physiologically relevant pesticide concentrations. However, biomonitoring data from relevant matrices such as follicular fluids or seminal plasma is limited in the literature and only few of the compounds investigated in this study has been measured in human blood plasma or serum (Ulsamer *et al.*, 1973; Huen *et al.*, 2012; Shi *et al.*, 2021).

The Ca^{2+}-signals induced by all the active pesticides were strongly inhibited in the presence of the specific CatSper-inhibitor RU1968 except hexachlorophene and deltamethrin (Figure 4). This indicates that the majority of the active pesticides investigated in this study induce Ca^{2+}-signals in human sperm cells through an opening of the CatSper channel. Only hexachlorophene was found to alkalize the pH_i of the sperm cells (Figure 5), which can lead to an activation of CatSper. With a pK_a of 4.95 a large portion of hexachlorophene can be expected to be in the anion form at physiological pH, which could induce changes in pH. However, the Ca^{2+}-signal induced by hexachlorophene was evident within seconds after addition of hexachlorophene (Figure 2) and did not follow the increasing alkalization evident 174 seconds after addition (Figure 5). Furthermore, the Ca^{2+}-signal induced by hexachlorophene was only slightly inhibited in the presence of RU1968 (Figure 4). Taken together, this indicates that hexachlorophene, despite the ability to alkalize the pH_i of the sperm cells, uses another unknown mode of action to induce Ca^{2+}-signals not involving CatSper.

We investigated whether the active pesticides would interfere with subsequent Ca^{2+}-signals induced by the two endogenous ligands of the CatSper channel, progesterone and PGE_1. Half of the pesticides inhibited Ca^{2+}-signals induced by a subsequent addition of 100 nM progesterone or PGE_1 after 5 minutes of preincubation (Figure 6 and Figure S2). Some of the pesticides exerted a dose dependent inhibition on only one of the two endogenous ligands as seen for fenitrothion and progesterone and pentachlorophenol and PGE_1 (Figure 6C, D). This could indicate that these compounds compete for the binding sites of progesterone and PGE_1, respectively. However, fenitrothion, oxadiazon, and chlorothalonil, which were the only pesticides to selectively inhibit progesterone-induced Ca^{2+}-signals have very different molecular structures (Table 1). Whether this is an indication of the promiscuity of the progesterone binding site of ABHD2 or an indication of different inhibitory effects on ABHD2 remains unknown. The majority of the pesticides were found both to inhibit progesterone- and PGE_1-induced Ca^{2+}-signals. This indicates that these pesticides may directly interact with unknown allosteric sites on the CatSper channel or interact with both the progesterone and PGE_1 binding sites.

Of the pesticides tested in this study nine were found to affect Ca^{2+}-mediated human sperm function. The two most efficacious pesticides at 10 µM milbemectin A4 and milbemectin A3 significantly induced acrosome reaction (Figure 7) but showed no effect on sperm penetration (Figure 8), whereas chlorpyrifos, the third most efficacious pesticides at 10 µM, showed no effect on the acrosome reaction (Figure 7), but significantly induced sperm penetration (Figure 8). Both sperm functions are known to be regulated by CatSper (Lishko *et al.*, 2012), but the downstream mechanisms controlling each sperm function are yet to be fully understood. Our results indicate that milbemectin and chlorpyrifos may affect the sperm cells on other parameters unrelated to Ca^{2+}-signaling, which could influence the results in the acrosome reaction and penetration assays, respectively. Metofluthrin, ranked nine out of 28 in efficaciousness at 10 µM, but was the only pesticide which exerted a significant effect on both the acrosome reaction and sperm penetration, indicating that this pesticide at a concentration of 10 µM may act agonistically on CatSper without negatively affecting the sperm cells in other ways. Several pesticides were found to inhibit sperm function at 10 µM. Both fipronil sulfone and pyraclostrobin significantly decreased the amount of viable acrosome reacted sperm cells (Figure 7), while propiconazole significantly inhibited sperm penetration (Figure 8). Importantly, both stimulatory and inhibitory

effects could potentially disturb the timing and order of the sperm cell functions necessary for natural fertilization to occur (Publicover *et al.*, 2007, 2008).

Significantly decreased viability in uncapacitated sperm cells was found only for milbemectin A4 and milbemectin A3 after 21 hours of incubation (Figure 9A), which could perhaps explain the lack of effect of these two pesticides in the penetration assay. Long term exposure to these pesticides might affect the viability of sperm cells *in vivo*. Significantly decreased viability in capacitated sperm cells were found after 30 minutes of incubation for boscalid, cyprodinil, prothioconazole, triticonazole, and desthioprothioconazole (Figure 9B), all of which had no effect on viability in uncapacitated sperm cells. During the capacitation process several changes in the sperm cell membrane occurs (Molina *et al.*, 2018), which might result in an increased sensitivity towards these five pesticides. Interestingly, short term exposure to milbemectin A4 and milbemectin A3 in capacitated sperm cells did not affect the viability of the sperm cells, indicating that their effect on viability first arises after 30 minutes of incubation.

Studies have shown that environmental chemicals can cooperate to activate CatSper both additively (Schiffer *et al.*, 2014; Rehfeld *et al.*, 2016) and synergistically (Brenker *et al.*, 2018). We found that a low dose mixture of the 28 active pesticides both induced a larger Ca^{2+}-signal and exerted a larger inhibition of progesterone-induced Ca^{2+}-signals than each pesticide alone at 100 nM (Figure 10). Furthermore, the EC_{05} of this low dose mixture was found to be 69.72 nM which is much lower than the EC_{05} of each individual pesticide (Figure 11A and Table 2). This emphasizes the relevance of considering the cooperative effect of low dose mixtures as humans in the industrialized part of the world are exposed to thousands of environmental chemicals (Di Renzo *et al.*, 2015). Next, we constructed a theoretical larger mixture of 72 different environmental chemicals containing an additional 44 different chemicals (Table S2) known to affect Ca^{2+}-signaling in human sperm cells (Schiffer *et al.*, 2014; Rehfeld *et al.*, 2016, 2020). This larger mixture was found to induce Ca^{2+}-signals with EC_{50} and EC_{05} in the low nM range (Figure 12A) and to inhibit Ca^{2+}-signals induced by the two endogenous ligands, progesterone and PGE_1, with IC_{50} values in the low nM range (Figure 12D). The cooperative effect of the chemicals in different mixtures investigated in this study could be a result of additive effects of chemicals acting on the same binding site, synergistic effects of chemicals acting via both the progesterone and PGE_1 binding sites, and/or through effects on other unknown sites and proteins regulating CatSper, e.g, the KSper channel (Lishko *et al.*, 2012). These findings stress that pesticides may act in concert with other environmental chemicals in the reproductive fluids to interfere with the normal Ca^{2+}-signaling in human sperm cells at concentrations well below the EC_{05} of each individual chemical.

Several of the active pesticides have been detected in human matrices such as hair (Béranger *et al.*, 2018; Peng *et al.*, 2020; Hardy *et al.*, 2021) or urine (Norén *et al.*, 2020; Hardy *et al.*, 2021) (Table S4), but human exposure data for the pesticides tested in this study are generally scarce. The pesticide milbemectin, which is a mixture of milbemectin A3 and A4, has to our knowledge not been monitored in a human matrix. No biomonitoring data or residues present on food products was available in the literature for metofluthrin, triticonazole, and the metabolite desthioprothioconazole, while the exposure to chlorothalonil has so far only been estimated as the chronic dietary exposure in the general public in the United States (Table S4) (Winter, 2015).

Unfortunately, few reports on human plasma- and serum levels of the pesticides investigated in this study exist. Only three of the pesticides from this investigation, the organophosphorus insecticide chlorpyrifos (Huen et al., 2012), the organochlorine disinfectant hexachlorophene (Ulsamer et al., 1973), and the pesticide metabolite fipronil sulfone (Shi et al., 2021) have been measured in human blood plasma with concentrations of 1.141 nM, 49.15 pM, and 3.11 nM respectively.

Chlorpyrifos is still widely used in agriculture and pesticide residues has been detected in numerous different food products in the European Union's report on pesticide residues in food from 2019 (Cabrera and Pastor, 2021). Chlorpyrifos is known to be metabolized rapidly and extensively (ATSDR, 1997) with the specific main metabolite TCPy not showing an effect in our study. TCPy concentrations in urine have been found to vary form 216 nM (Norén *et al.*, 2020) to 1525 nM (Hardy *et al.*, 2021). This confirms human exposure to chlorpyrifos in the period 2000-2017 with an increasing exposure over the time period (Norén *et al.*, 2020).

It is difficult to directly translate the results of this study as real time biomonitoring of pesticide levels in relevant human matrices such as blood or reproductive fluids is very limited (Table S4). Similarly, this makes it difficult to estimate both the concentrations and types of pesticides the sperm cells may encounter during spermatogenesis and maturation in the male reproductive tract as well as after ejaculation within the female reproductive tract. However, multiple pesticides from our study were detected in hair samples from three different populations of females in the reproductive age (Béranger *et al.*, 2018; Peng *et al.*, 2020). Therefore, there is evidence in the presented literature confirming that humans either have been exposed to or are likely to be exposed to many of the pesticides investigated in our study. To definitively link the effects of pesticide exposure to human fertility *in vivo* exposure studies would have to be conducted. However, since no suitable non-primate animal model for human CatSper is currently known (Schiffer *et al.*, 2014; Liu *et al.*, 2021), the use of animal models to obtain such *in vivo* data is currently not an option. Until a suitable non-primate animal model is discovered time-to-pregnancy cohorts with measurements of multiple pesticides in relevant matrices, such as female reproductive tract fluids and/or seminal plasma, could be used to help identifying possible *in vivo* effects on fertility driven by the actions of pesticides on CatSper. Therefore, using the data presented here from *in vitro* based methods in the evaluation or regulation of pesticides may first be relevant in the future, depending on the specific regulatory requirements.

7. Conclusions

Our study showed that several pesticides, either alone or in complex low-dose mixtures, may interfere with human sperm function through effects on the sperm-specific and steroid-activated CatSper Ca^{2+}-channel. The concentrations at which each pesticide alone exerted effects on Ca^{2+}-signaling in human sperm cells may not be of physical relevance since the concentration of most of the studied pesticides in relevant human matrices is unknown. However, humans are exposed to a large variety of different environmental chemicals (Di Renzo *et al.*, 2015) and this study emphasizes a possible putative effects on human fertility of pesticide exposure in low-dose mixtures together with other environmental chemicals. In fact, several decades of research in the field of mixture toxicology have shown that the thresholds set for individual chemicals do not necessarily provide protection against the combined effects from the very same chemicals in mixtures (Kortenkamp, 2014). Therefore, further research concerning effects on human fertility should aim at providing a better understanding of the putative risk of exposure to mixtures of pesticides and their metabolites alone and together with other ubiquitous environmental chemicals. As real time data on pesticide exposure from biomonitoring directly from blood or reproductive fluids is very limited, biomonitoring data from relevant matrices would be an important contribution when seeking to link the effects of pesticide exposure to human fertility. Importantly, in risk assessment and regulation, *in vitro* approaches alone are not sufficient in linking the effects of pesticide exposure to human fertility. However, as CatSper present on human sperm have different natural ligands compared to CatSper in most common laboratory animals (e.g., mouse and rat), and therefore also is likely have different susceptibility to exogenous compounds, no good *in vivo* animal models currently exist to study chemical effects on human sperm function via interaction with human CatSper. Thus, until a relevant animal model may be identified or developed for *in vivo* exposure studies, using human sperm as in the presented *in vitro* model is currently the best (and only) option for a high-through put screening method. While *in vitro* data alone may not be sufficient for legal action and regulation it can be used to guide which chemicals/chemical mixtures to focus on in human epidemiological studies (e.g., time-to-pregnancy cohorts).

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Appendix 1 – Supplemental material

Appendix 1.1 - Table S1

TABLE S1. Inactive pesticides ranked according to the mean relative peak Ca2+-signal* induced at 10 µM

Rank:	Compound:	CAS #:	Mean relative signal at 10 µM (in % of paired progesterone induced response) (n=3):
29	3-(2,2-Dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (CL2CA)	55701-05-8	7.96
30	3,5,6-Trichloro-2-pyridinol (TCPy)	6515-38-4	5.75
31	Bifenthrin	82657-04-3	5.41
32	Pyrimethanil	53112-28-0	5.31
33	Dichloroprop-P	15165-67-0	4.96
34	Metolachlor	51218-45-2	4.63
35	Diuron	330-54-1	4.01
36	1,2,4-triazol	288-88-0	3.96
37	Azoxystrobin	131860-33-8	3.88
38	Imidacloprid	138261-41-3	3.64
39	Glyphosate	1071-83-6	3.30
40	3-Methyl-4-Nitrophenol (3Me4NP)	2581-34-2	3.06
41	Propamocarb	24579-73-5	2.71
42	Dimethyl phosphate	813-78-5	2.70
43	Terbutryn	886-50-0	2.64
44	p-Nitrophenol	100-02-7	2.57
45	Fludioxonil	131341-86-1	2.23
46	Carbendazim	10605-21-7	2.11
47	MCPA	94-74-6	1.93
48	Mecoprop	93-65-2	1.77
49	Fipronil	120068-37-3	1.70
50	2,4-Dichlorophenoxyacetic acid (2,4-D)	94-75-7	1.36
51	Diethyl phosphate	598-02-7	1.33
52	2-Isopropyl-6-methyl-4-pyrimidinol (IMPy)	2814-20-2	1.30
53	Thiabendazole	148-79-8	0.97

*the peak Ca2+-signal induced by the pesticides at 10 µM divided by the peak Ca2+-signal induced by pro-gesterone at 5 µM in the same experiment.

Appendix 1.2 - Table S2

TABLE S2. The additional 44 environmental chemicals included in the larger mixture.

Compound:	CAS #:	Author (Year):
Benzylparaben	94-18-8	Schiffer et al. (2014)
Hexylparaben	1083-27-8	Schiffer et al. (2014)
Homosalate	118-56-9	Schiffer et al. (2014)
Nonylparaben	38713-56-3	Schiffer et al. (2014)
Padimate O	21245-02-3	Schiffer et al. (2014)
4-methylbenzophenone	134-84-9	Schiffer et al. (2014)
Benzophenone-3 / oxybenzone	131-57-7	Schiffer et al. (2014)
Benzophenone-7	85-19-8	Schiffer et al. (2014)
DDT dehydrochloride	72-55-9	Schiffer et al. (2014)
Chlorophenothane	50-29-3	Schiffer et al. (2014)
Dieldrin	60-57-1	Schiffer et al. (2014)
Methoxy-DDT	72-43-5	Schiffer et al. (2014)
Triclosan	3380-34-5	Schiffer et al. (2014)
Vinclozoline	50471-44-8	Schiffer et al. (2014)
3-(4-methylbenzylidene) camphor	36861-47-9	Schiffer et al. (2014)
3-benzylidene camphor; benzal camphor	15087-24-8	Schiffer et al. (2014)
α-zearalenol	36455-72-8	Schiffer et al. (2014)
Octinoxate	5466-77-3	Schiffer et al. (2014)
Octocrylene	6197-30-4	Schiffer et al. (2014)
Perfluorooctanoic acid	335-67-1	Schiffer et al. (2014)
Tributyltin acetate	56-36-0	Schiffer et al. (2014)
Tributyltin chloride	1461-22-9	Schiffer et al. (2014)
2-phenylphenol	90-43-7	Schiffer et al. (2014)
4-nonylphenol	104-40-5	Schiffer et al. (2014)
4-octylphenol	1806-26-4	Schiffer et al. (2014)
4-phenylphenol	92-69-3	Schiffer et al. (2014)
4-tert-octylphenol	140-66-9	Schiffer et al. (2014)
Diethylstilbestrol / stilbestrol	56-53-1	Schiffer et al. (2014)
Benzyl butyl phthalate	85-68-7	Schiffer et al. (2014)
Dibutyl phthalate / di-n-butyl phthalat	84-74-2	Schiffer et al. (2014)
Di-iso-butyl phthalat	84-69-5	Schiffer et al. (2014)
Dipentyl phthalate / di-n-pentyl phthalat	131-18-0	Schiffer et al. (2014)
Methyl anthranilate	134-09-8	Rehfeld et al. (2016)
Isoamyl P-methoxycinnamate	71617-10-2	Rehfeld et al. (2016)
Ethylhexyl salicylate	118-60-5	Rehfeld et al. (2016)
Benzylidene camphor sulfonic acid	56039-58-8	Rehfeld et al. (2016)
Butyl methoxydibenzoylmethane	70356-09-1	Rehfeld et al. (2016)
Diethylamino hydroxybenzoyl hexyl benzoate	302776-68-7	Rehfeld et al. (2016)
Bisphenol G	127-54-8	Rehfeld et al. (2020)

Bisphenol AF	1478-61-1	Rehfeld et al. (2020)
Bisphenol C	79-97-0	Rehfeld et al. (2020)
Bisphenol A diglycidyl ether	1675-54-3	Rehfeld et al. (2020)
Bisphenol B	77-40-7	Rehfeld et al. (2020)
Bisphenol BP	1844-01-5	Rehfeld et al. (2020)

Appendix 1.3 - Table S3

TABLE 0.1. The concentrations for each active pesticide used in the investigations including the specific CatSper inhibitor RU1968 and the investigations of changes in pHi.

Compound:	Concentration:	Compound:	Concentration:
Milbemectin A4	10 µM	Pentachlorophenol	50 µM
Milbemectin A3	10 µM	Prochloraz	50 µM
Chlorpyrifos	50 µM	Cypermethrin	50 µM
Prosulfocarb	50 µM	Propiconazole	50 µM
Fipronil Sulfone	50 µM	Chlorothalonil	25 µM
Trifluralin	50 µM	Permethrin	50 µM
Endosulfan	25 µM	Deltamethrin	50 µM
Hexachlorophene	10 µM	Tebuconazole	50 µM
Metofluthrin	25 µM	Desthioprothioconazole	50 µM
Imazalil	50 µM	Boscalid	50 µM
Pyraclostrobin	50 µM	Triticonazole	50 µM
Fenitrothion	50 µM	3-Phenoxybenzoic acid	50 µM
Oxadiazon	50 µM	Cyprodinil	50 µM
Lindane	50 µM	Prothioconazole	50 µM

These concentrations are close to the EC100 for each individual pesticide.

Appendix 1.4 - Table S4

TABLE 0.2. Human pesticide exposure.

Human Pesticide Exposure				
Compound:	**CAS #:**	**Matrix**	**Concentration (unit)**	**Author (Year)**
Milbemectin A4	51596-11-3	R	0.044 (mg/kg)	Cabrera and Pastor (2021)
Milbemectin A3	51596-10-2	R	0.044 (mg/kg)	Cabrera and Pastor (2021)
Chlorpyrifos	2921-88-2	B	0.4 (ng/mL)	Huen *et al.* (2012)
		I	2.376 (ng/kg/day)	Winter (2015)
Prosulfocarb	52888-80-9	H	0.62 (pg/mg)	Béranger *et al.* (2018)
		H	0.26 (pg/mg)	Peng *et al.* (2020)
		H	0.31 (pg/mg)	Peng *et al.* (2020)
		U	3.03×10^5 (pg/mL)	Hardy *et al.* (2021)
Fipronil Sulfone	120068-36-2	B	1.41 (ng/mL)	Shi *et al.* (2021)
		H	10.51 (pg/mg)	Béranger *et al.* (2018)
		H	3.05 (pg/mg)	Peng *et al.* (2020)
		H	1.75 (pg/mg)	Peng *et al.* (2020)
		H	48.7 (pg/mg)	Hardy *et al.* (2021)
		U	5.06 (pg/mL)	Hardy *et al.* (2021)
Trifluralin	1582-09-8	H	0.17 (pg/mg)	Béranger *et al.* (2018)
		H	0.11 (pg/mg)	Peng *et al.* (2020)
		H	0.04 (pg/mg)	Peng *et al.* (2020)
		H	1.34 (pg/mg)	Hardy *et al.* (2021)
		U	7.47 (pg/mL)	Hardy *et al.* (2021)
Endosulfan*	115-29-7	H	0.4 (pg/mg)	Béranger *et al.* (2018)
		H	0.15 (pg/mg)	Peng *et al.* (2020)
		H	<LOD	Peng *et al.* (2020)
		H	7.81 (pg/mg)	Hardy *et al.* (2021)
		U	5.5 (pg/mL)	Hardy *et al.* (2021)
		I	9.3737 (ng/kg/day)	Winter (2015)
Hexachlorophene	70-30-4	B	0.24 (ng/mL)	Ulsamer *et al.* (1973)
Metofluthrin	240494-70-6			
Imazalil	35554-44-0	H	1.63 (pg/mg)	Béranger *et al.* (2018)
		H	<LOD	Peng *et al.* (2020)
		H	0.56 (pg/mg)	Peng *et al.* (2020)
		I	0.5742 (ng/kg/day)	Winter (2015)
Pyraclostrobin	175013-18-0	H	0.04 (pg/mg)	Béranger *et al.* (2018)
		H	0.06 (pg/mg)	Peng *et al.* (2020)
		H	0.02 (pg/mg)	Peng *et al.* (2020)
Fenitrothion	122-14-5	I	0.0132 (ng/kg/day)	Winter (2015)
Oxadiazon	19666-30-9	H	0.29 (pg/mg)	Béranger *et al.* (2018)
		H	0.01 (pg/mg)	Peng *et al.* (2020)
		H	0.06 (pg/mg)	Peng *et al.* (2020)
		H	1.96 (pg/mg)	Hardy *et al.* (2021)
		U	<LOD	Hardy *et al.* (2021)
Lindane	58-89-9	H	2.2 (pg/mg)	Béranger *et al.* (2018)
		H	0.36 (pg/mg)	Peng *et al.* (2020)
		H	0.59 (pg/mg)	Peng *et al.* (2020)
		H	90.5 (pg/mg)	Hardy *et al.* (2021)
		U	1.35 (pg/mL)	Hardy *et al.* (2021)
		I	0.0693 (ng/kg/day)	Winter (2015)

Pentachlorophenol	87-86-5	H	28.47 (pg/mg)	Béranger *et al*. (2018)
		H	0.6 (pg/mg)	Peng *et al*. (2020)
		H	0.11 (pg/mg)	Peng *et al*. (2020)
		H	1041 (pg/mg)	Hardy *et al.* (2021)
Prochloraz	67747-09-5	H	0.028 (pg/mg)	Béranger *et al*. (2018)
		H	0.25 (pg/mg)	Peng *et al*. (2020)
		H	0.39 (pg/mg)	Peng *et al*. (2020)
Cypermethrin	52315-07-8	H	2.86 (pg/mg)	Béranger *et al*. (2018)
		H	3.89 (pg/mg)	Peng *et al*. (2020)
		H	3.21 (pg/mg)	Peng *et al*. (2020)
		H	15.3 (pg/mg)	Hardy *et al.* (2021)
		U	0.75 (pg/mL)	Hardy *et al.* (2021)
		I	8.9699 (ng/kg/day)	Winter (2015)
Propiconazole	60207-90-1	H	1.44 (pg/mg)	Béranger *et al*. (2018)
		H	0.36 (pg/mg)	Peng *et al*. (2020)
		H	0.73 (pg/mg)	Peng *et al*. (2020)
		I	0.0002 (ng/kg/day)	Winter (2015)
Chlorothalonil	1897-45-6	I	0.1205 (ng/kg/day)	Winter (2015)
Permethrin	52645-53-1	H	91.61 (pg/mg)	Béranger *et al*. (2018)
		H	25.1 (pg/mg)	Peng *et al*. (2020)
		H	15.7 (pg/mg)	Peng *et al*. (2020)
		H	1241 (pg/mg)	Hardy *et al.* (2021)
		U	<LOD	Hardy *et al.* (2021)
		I	73.4122 (ng/kg/day)	Winter (2015)
Deltamethrin	52918-63-5	H	0.651 (pg/mg)	Béranger *et al*. (2018)
		H	0 (pg/mg)	Peng *et al*. (2020)
		H	0 (pg/mg)	Peng *et al*. (2020)
		H	44.1 (pg/mg)	Hardy *et al.* (2021)
		U	<LOD	Hardy *et al.* (2021)
Tebuconazole	107534-96-3	H	0.62 (pg/mg)	Béranger *et al*. (2018)
		H	0.26 (pg/mg)	Peng *et al*. (2020)
		H	1.19 (pg/mg)	Peng *et al*. (2020)
Desthioprothioconazole	120983-64-4			
Boscalid	188425-85-6	H	1.41 (pg/mg)	Béranger *et al*. (2018)
		H	<LOD	Peng *et al*. (2020)
		H	<LOD	Peng *et al*. (2020)
Triticonazole	131983-72-7			
3-Phenoxybenzoic acid	3739-38-6	H	3.76 (pg/mg)	Béranger *et al*. (2018)
		H	1.28 (pg/mg)	Peng *et al*. (2020)
		H	0.79 (pg/mg)	Peng *et al*. (2020)
		H	35.3 (pg/mg)	Hardy *et al.* (2021)
		U	5189 (pg/mL)	Hardy *et al.* (2021)
		U	1.88×10^4 (pg/mL)	Norén *et al.* (2020)
		U	393 (pg/mL)	Wielgomas *et al*. (2013)
		U	13.29×10^3 (pg/mL)	Wielgomas and Piskunowicz (2013)
Cyprodinil	121552-61-2	H	0.175 (pg/mg)	Béranger *et al*. (2018)
		H	0 (pg/mg)	Peng *et al*. (2020)
		H	0 (pg/mg)	Peng *et al*. (2020)
		I	12.9971 (ng/kg/day)	Winter (2015)
Prothioconazole	178928-70-6	R	0.079 (mg/kg)	Cabrera and Pastor (2021)

R= pesticide residue on food products, H= hair, U= urine, B=blood plasma, I=estimated intake. *β-endosulfan.

Appendix 1.5 - Figure F1

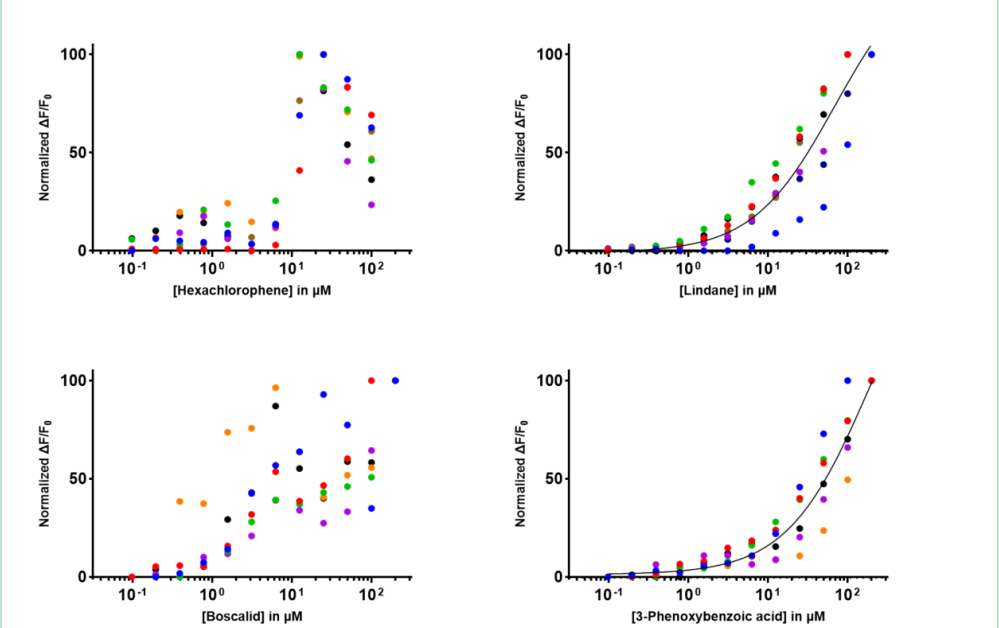
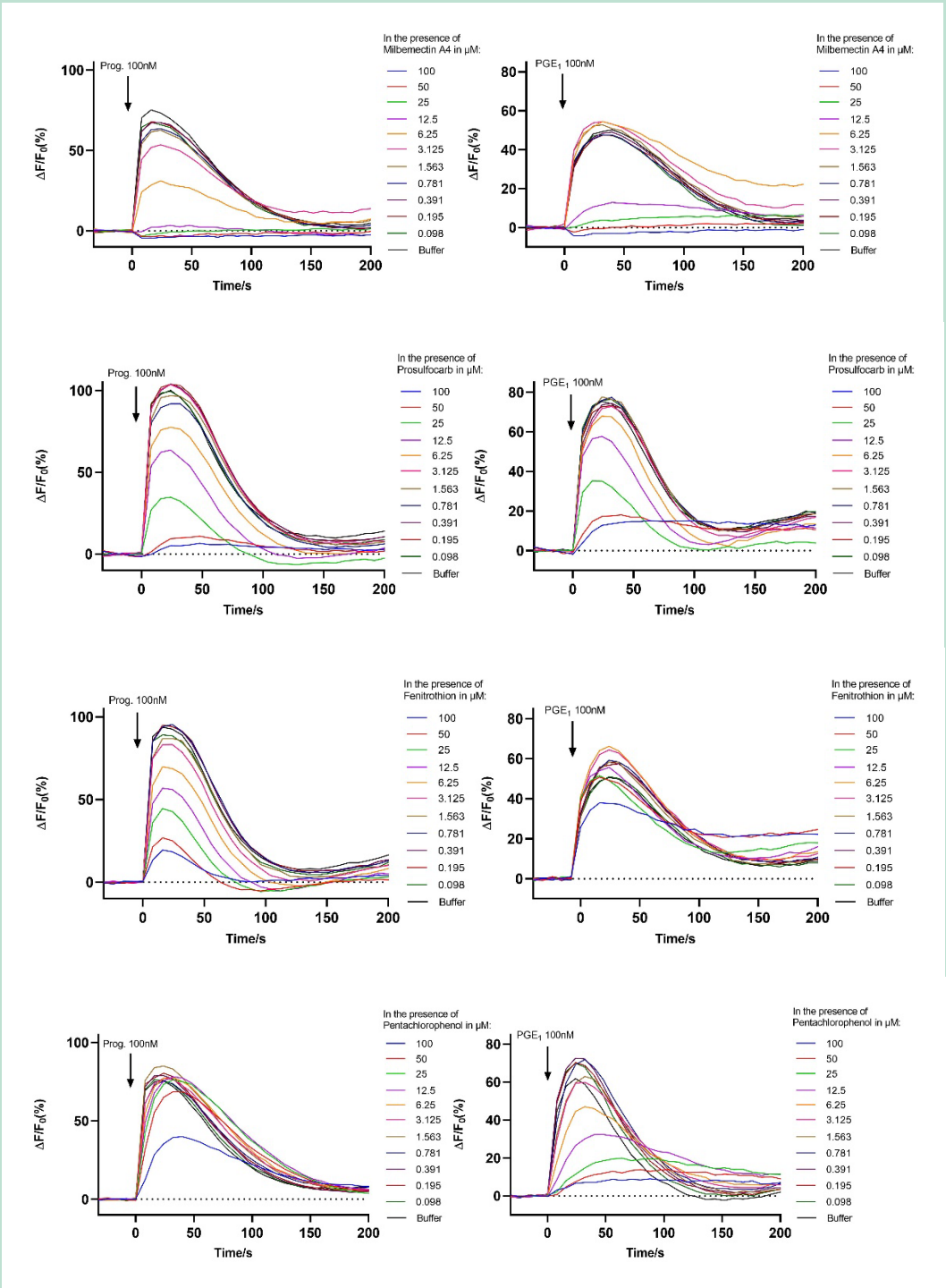
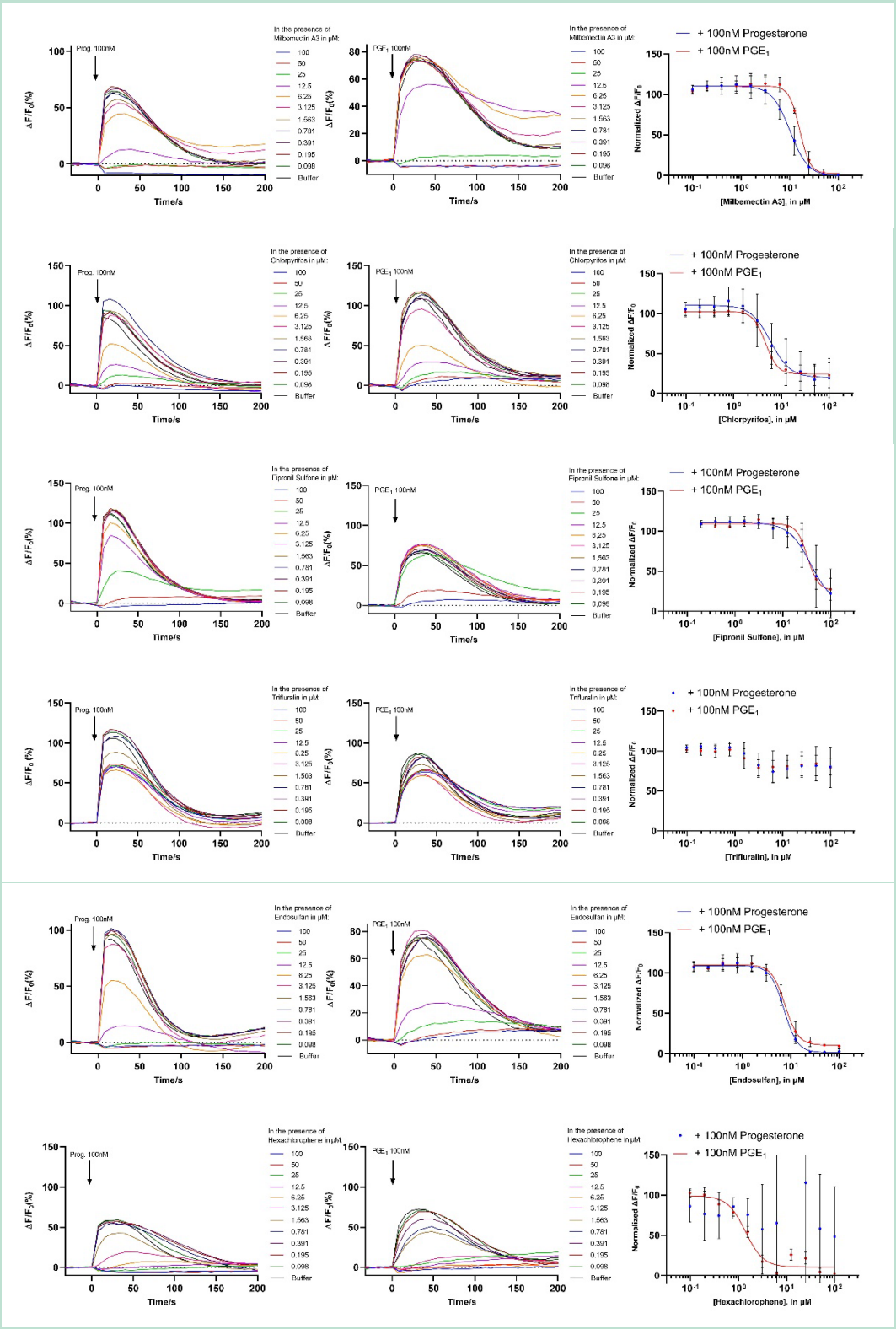
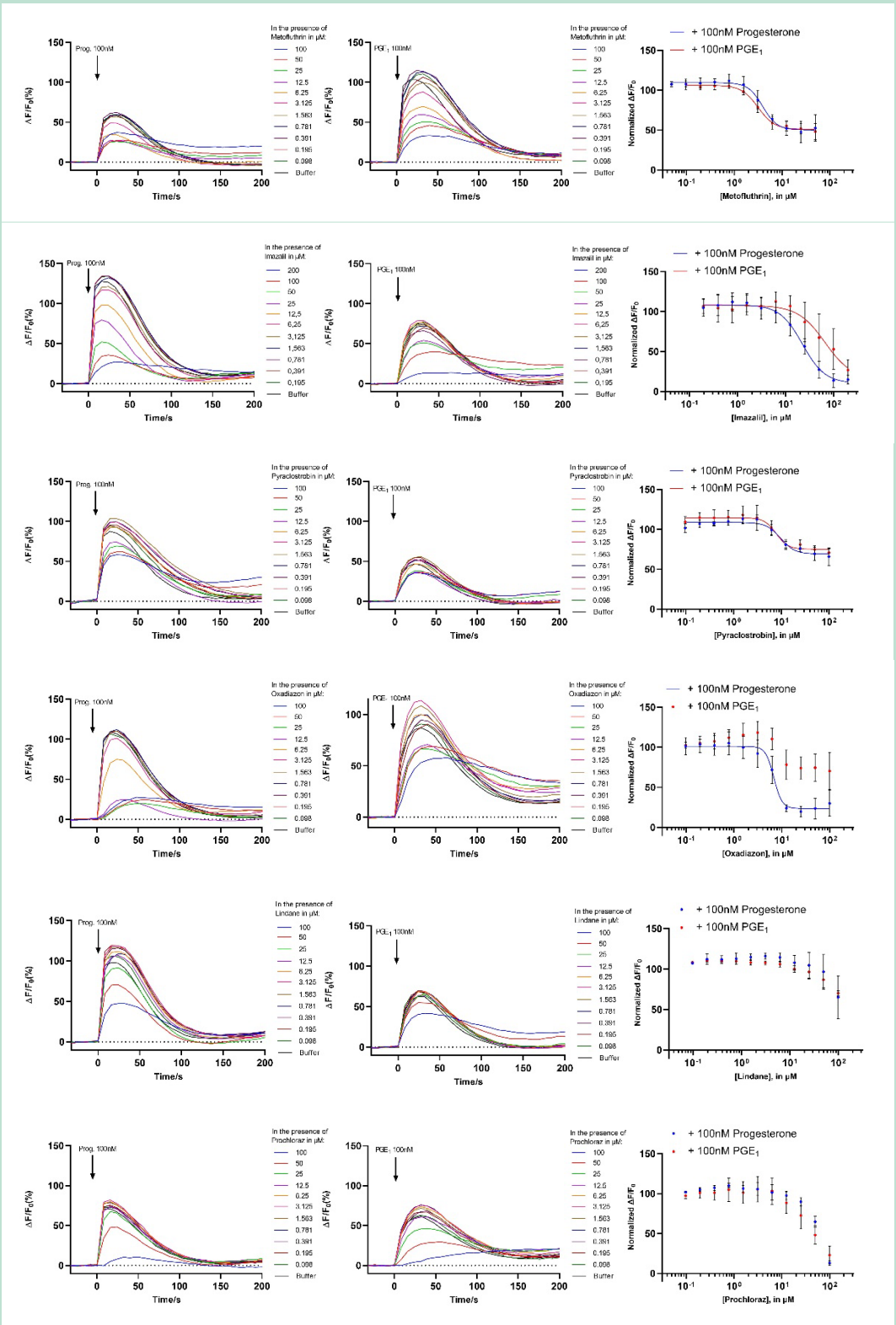


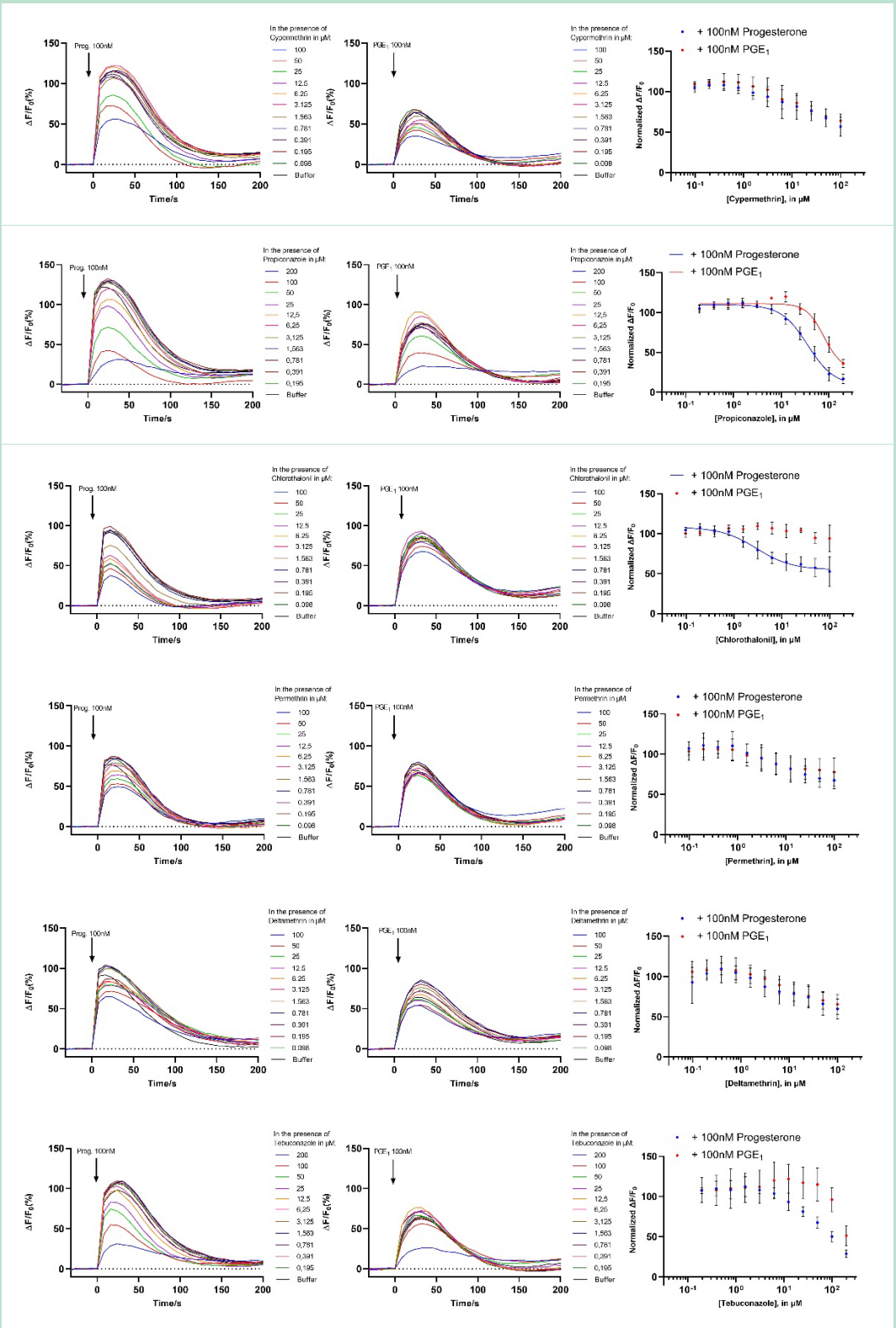
FIGURE S1. Left, Normalized dose-response data for hexachlorophene and boscalid where no dose-response rela-tion is present. Right, Normalized dose-response data for lindane and 3-phenoxybenzoic acid that did not yield saturated dose-response curves.

Appendix 1.6 - Figure F2




Prog. 100nM
PGE1 100nM
In the presence of Milbemectin A3 in µM:
In the presence of Chlorpyrifos in µM:
In the presence of Fipronil Sulfone in µM:
In the presence of Trifluralin in µM:
In the presence of Endosulfan in µM:
In the presence of Hexachlorophene in µM:
100
50
25
12.5
6.25
3.125
1.563
0.781
0.391
0.195
0.098
Buffer
ΔF/F0(%)
Time/s
+ 100nM Progesterone
+ 100nM PGE1
Normalized ΔF/F0
[Milbemectin A3], in µM
[Chlorpyrifos], in µM
[Fipronil Sulfone], in µM
[Trifluralin], in µM
[Endosulfan], in µM
[Hexachlorophene], in µM


Prog. 100nM
PGE1 100nM
In the presence of Metofluthrin in µM:
In the presence of Imazalil in µM:
In the presence of Pyraclostrobin in µM:
In the presence of Oxadiazon in µM:
In the presence of Lindane in µM:
In the presence of Prochloraz in µM:
100
50
25
12,5
6,25
3,125
1,563
0,781
0,391
0,195
0,098
Buffer
ΔF/F0(%)
Time/s
+ 100nM Progesterone
+ 100nM PGE1
Normalized ΔF/F0
[Metofluthrin], in µM
[Imazalil], in µM
[Pyraclostrobin], in µM
[Oxadiazon], in µM
[Lindane], in µM
[Prochloraz], in µM


Prog. 100nM
PGE1 100nM
In the presence of Cypermethrin in µM:
In the presence of Propiconazole in µM:
In the presence of Chlorothalonil in µM:
In the presence of Permethrin in µM:
In the presence of Deltamethrin in µM:
In the presence of Tebuconazole in µM:
100
50
25
12.5
6.25
3.125
1.563
0.781
0.391
0.195
0.098
Buffer
200
100
50
25
12,5
6,25
3,125
1,563
0,781
0,391
0,195
Buffer
ΔF/F0(%)
Time/s
+ 100nM Progesterone
+ 100nM PGE1
Normalized ΔF/F0
[Cypermethrin], in µM
[Propiconazole], in µM
[Chlorothalonil], in µM
[Permethrin], in µM
[Deltamethrin], in µM
[Tebuconazole], in µM

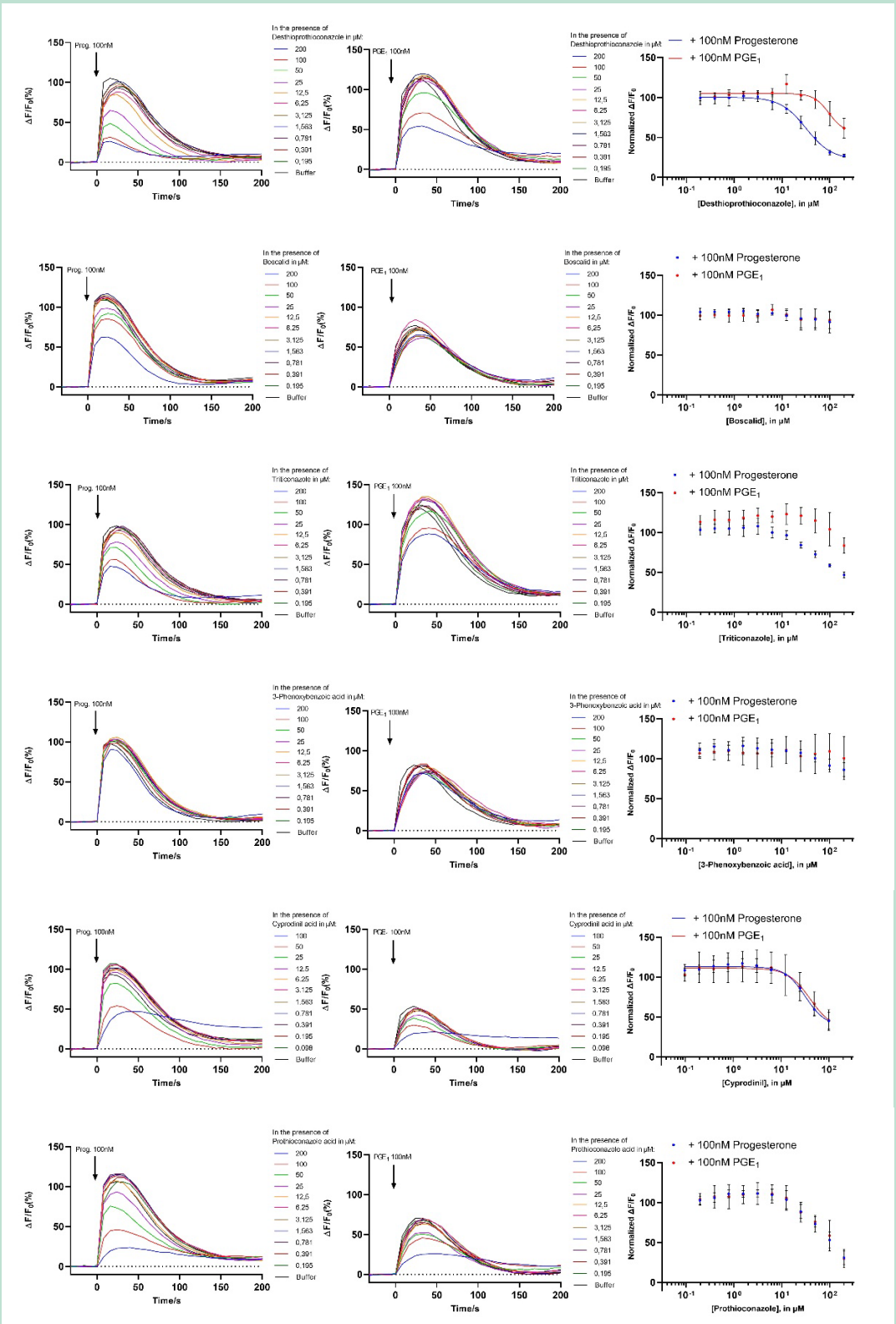


FIGURE S2. Dose-inhibition data for the remaining 22 active pesticides. Right column, inhibition of Ca2+-signals induced by 100 nM progesterone after 5 minutes of preincubation with the negative buffer control and different concentrations of the pesticides. Middle column, inhibition

of Ca2+-signals induced by 100 nM PGE1 after 5 minutes of preincubation with the negative buffer control and different concentrations of the pesticides: Normalized dose-response relation (n ≥ 3, with ≥ 3 different donors) of the Ca2+-signals in-duced by 100 nM progesterone and PGE1 after 5 minutes of preincubation with different concentrations of the pesticides.

Direct effects of pesticides and pesticide metabolites on the CatSper Ca^{2+}-channel in human sperm

Background: Ca^{2+}-signaling controls sperm cell functions necessary for successful fertilization. Multiple endocrine disrupting chemicals have been found to interfere with normal Ca^{2+}-signaling in human sperm cells through an activation of the sperm-specific CatSper Ca^{2+}-channel, which is vital for normal male fertility.

Objectives: We investigated 53 pesticides for their ability to interfere with CatSper mediated Ca^{2+}-signaling and function in human sperm cells.

Methods: Effects of the pesticides on Ca^{2+}-signaling in human sperm cells were evaluated using a Ca^{2+}-fluorometric assay. Effects via CatSper were assessed using the specific CatSper inhibitor RU1968. Effects on human sperm function and viability were assessed using an image cytometry-based acrosome reaction assay and the modified Kremer's sperm–mucus penetration assay.

Results: 28 of 53 pesticides were found to induce Ca^{2+}-signals in human sperm cells at 10 µM. The majority of these 28 active pesticides induced Ca^{2+}-signals through CatSper and interfered with subsequent Ca^{2+}-signals induced by the two endogenous CatSper ligands progesterone and prostaglandin E1. Multiple active pesticides were found to affect Ca^{2+}-mediated sperm functions and viability at 10 µM. Low nM dose mixtures of the active pesticides alone or in combination with other environmental chemicals were found to significantly induce Ca^{2+}-signals and inhibit Ca^{2+}-signals induced subsequently by progesterone and prostaglandin E1.

Conclusions: Our results show that pesticides, both alone and in low nM dose mixtures, interfere with normal Ca^{2+}-signaling in human sperm cells *in vitro* at physiologically relevant concentrations. Biomonitoring of pesticides in relevant matrices such as blood and reproductive fluids is very limited and the effects of real time human pesticide exposure on human sperm cells and fertility thus remains largely unknown. To which extent human pesticide exposure affects the chances of a successful fertilization in humans *in vivo* needs further research.

The Danish Environmental
Protection Agency
Tolderlundsvej 5
DK - 5000 Odense C

www.mst.dk