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Fipronil and fipronil sulfone in chicken: From *in vitro* experiments to *in vivo* PBK model predictions

L.S. Lautz *, G. Stoopen, A.J. Ginting, R.L.A.P. Hoogenboom, A. Punt

Wageningen Food Safety Research, Akkermaalsbos 2, 6708, WB Wageningen, the Netherlands

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ABSTRACT

In 2017 a large-scale fipronil contamination in eggs occurred in several European countries. Fipronil and its metabolites have the potential to be transferred into the eggs of laying hens, thereby entering the human food chain. Here, first the metabolism of fipronil was measured *in vitro* using chicken liver S9. The results show that fipronil is mainly metabolised into fipronil sulfone and the clearance obtained *in vitro* was extrapolated to *in vivo* liver clearance. In a second step a physiologically based kinetic model was developed with a focus on fipronil and its major sulfone metabolite and the model outcome was compared to available *in vivo* data in eggs from the literature. The experimentally obtained clearance was used as model input to evaluate whether such an *in vitro*-based model can be used in an early phase of a contamination incident to predict the time-concentration curves. Overall, all model predictions were within a 10-fold difference and the estimated elimination half-life for fipronil equivalents was 14 days. *In vitro* experiments are definitely recommended compared to *in vivo* studies, since they provide a fast first insight into the behaviour of a chemical in an organism.

1. Introduction

In 2017 a large-scale incident with fipronil in eggs occurred in the Netherlands and several other European countries (EFSA, 2018). The cause of this incident was the illegal use of fipronil as a disinfection product against poultry lice, resulting in exposure of the chickens, which led to absorption, accumulation in tissues and excretion via eggs and excreta (EFSA, 2006; Gerletti et al., 2020). Fipronil belongs to the phenylpyrazole-class insecticides and was released to the market in 1993 (Gerletti et al., 2020). Although fipronil can be used in anti-flea products in for example pet collars (Dryden et al., 2000), it is not approved for use in food-producing animals, as the transfer of fipronil and its metabolites into the human food chain poses a human health risk.

The biological activity and transfer of fipronil not only relates to fipronil itself, but also to its major metabolite fipronil sulfone. Fipronil sulfone is suggested to have similar toxic potential compared to fipronil, and is more metabolically stable (EFSA, 2006). Acute biological effects of fipronil by poisoning include headache, dizziness, sweating, nausea, vomiting and seizures (Mohamed et al., 2004). The WHO classified fipronil as a moderately hazardous pesticide. EFSA established an acute reference dose (ARfD) of 0.009 mg/kg BW, based on neurobehavioral effects in rats, and an acceptable daily intake (ADI) of 0.0002 mg/kg BW/day, based on reduced growth in rats (EFSA, 2006; Safety, 2005). The fipronil levels found in eggs in the Netherlands during the 2017 incident could not rule out an exceedance of the ADI, but considering the short duration of the incident a potential health risk was considered to be small (RIVM, 2018). Given the risks for human health, incidents like the fipronil incident require early stage risk-management decisions on the potential re-call of food, in this case eggs and egg-derived products. In addition, there is generally a need for insights on how long the contamination maintains after ending the use of the compound and applying follow-up actions, like the cleaning of the stables, in order to judge whether animals need to be destructed or not.

Kinetic models can be useful in the assessment of the transfer of chemicals, such as fipronil, since they determine the internal concentration in various tissues and can be used to predict the excretion in e.g. eggs. Especially kinetic models based on *in vitro* and *in silico* data could be helpful, since they do not require time-consuming animal studies and may be extrapolated to other chemicals. With regards to chicken, there are three kinetic models available which include fipronil as a chemical; one feed-to-egg kinetic transfer model and two physiologically based kinetic (PBK) models (Gerletti et al., 2020; Lautz et al., 2020; MacLachlan, 2010). These models focus on fipronil as parent compound only and do not take into account metabolites. While all model input

* Corresponding author. Wageningen Food Safety Research, Wageningen University and Research, Akkermaalsbos 2, 6708, WB Wageningen, the Netherlands.
E-mail address: leonie.lautz@wur.nl (L.S. Lautz).

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parameters still rely on *in vivo* chemical-specific data, opportunities to predict internal concentrations of chemicals using *in vitro* kinetic data (Vmax, Km, intrinsic clearance) and *in silico* calculated partition coefficients as model input are increasingly highlighted in the literature and have been explored-for the predictions of monensin residues in chicken (Dorne and Fink-Gremmels, 2013; Henri et al., 2009; Lautz et al., 2019, 2020).

The present paper describes the implementation of *in vitro* metabolism data and *in silico* calculated partition coefficients in a PBK model for chicken (*Gallus gallus domesticus*) to evaluate whether such an *in vitro-in silico* based model can be used in an early phase of a contamination incident to predict the time-concentration curves. A model was developed with a focus on fipronil and its major metabolite fipronil sulfone and the model outcome was compared to available *in vivo* data in eggs from the literature.

2. Material and methods

2.1. Chemicals and reagents

Fipronil was purchased from HPC (Cunnersdorf, Germany) and fipronil sulfone from Sigma-Adrich (Zwijndrecht, The Netherlands). Potassium chloride was purchased from Merck chemicals (Amsterdam, The Netherlands), Tris (Tris(hydroxymethyl)aminomethane) from Fisher Scientific (Landsmeer, The Netherlands), acetonitrile ULC-MS, Methanol Ultra LC-MS, water ultra LC-MS and formic acid LC-MS from Actu-All Chemicals (Oss, The Netherlands). NADPH and Regensys A were purchased from Trinova Biochem (Giessen, Germany), Germany.

2.2. Preparation of chicken liver S9

Livers of mother hens were collected at the slaughterhouse approximately 8 min after killing the animals. Livers were washed with ice cold DPBS (Fisher Scientific, Landsmeer, The Netherlands), cut into smaller chunks, snap frozen in liquid nitrogen and stored at −80 °C. To prepare S9 homogenate, livers were homogenized in ice cold Tris/KCl buffer (1.15% KCl in 50 mM Tris/HCl pH 7.4) in a precooled stainless steel blender at a ratio of 1 g tissue to 2 mL of buffer. The crude homogenate was centrifuged in a precooled rotor for 25 min at 8960 rcf and 4 °C. The supernatant was collected and 500 μL aliquots were snap frozen in liquid nitrogen and stored at −80 °C. The protein concentration was determined using the Biorad DC Protein Assay (Biorad, Lunteren, The Netherlands) according to the manufacturers protocol with BSA as a calibration standard.

2.3. In vitro metabolism assay

The *in vitro* metabolism studies were performed in 1.5 mL low-bind protein Eppendorf tubes at 37 °C. The incubation mixture contained (final concentrations) 5 mM NADPH, 0.5 mg chicken S9 protein per mL and 1 μM fipronil or fipronil sulfone in Regensys A (100 mM sodium phosphate buffer pH 7.4, 33 mM KCl, 8 mM $MgCl_2$ and 5 mM glucose-6-phosphate). A fipronil stock solution of 1 mM was prepared in DMSO and after dilution in the assay mixture the final DMSO concentration was 0.1%. Reactions were started after a pre-incubation period of 5 min at 37 °C by addition of the S9. After 10, 20, 40, 80 and 120 min 100 μL of the samples was transferred to a tube containing 100 μL of ice-cold methanol to stop the reaction. The samples were vortex mixed thoroughly and put on ice. T = 0 samples were prepared by mixing the incubation mixture with methanol prior to the addition of S9. The samples were stored at −80 °C.

2.4. UPLC-MS analysis

Samples were defrosted, mixed thoroughly and centrifuged 5 min at 14,000 rpm at ambient temperature. The supernatants were diluted 5 times in NADPH reagensys and 20 μL was analysed on a Waters Acquity UPLC Class system equipped with a sample manager, photodiode array detector (PDA), Quadrapole Dalton (QDa) detector and a C18 UPLC column (Acquity UPLC BEH C18 1.7 μm column, 2.1 × 100 mm, Waters, Etten Leur, The Netherlands). The mass spectrophotometer was equipped with an electrospray ionization interface (ESI). The ESI was operated in negative mode (ESI^-) with a capillary voltage of 0.8 kV, a desolvation gas temperature of 600 °C and source temperature 150 °C. The column and autosampler were set at a temperature of 50 °C and 20 °C respectively. The eluents consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The injection was set for 12 min and the flow rate was set at 0.4 mL/min. MassLynx software (version 4.1) was used to record and analyse the peaks. A full scan analysis was performed (m/z 100–600). Within the full scan, different potential metabolites were identified by associating the detected mass in this study with the mass as provided in PubChem, and in case of fipronil and fipronil sulfone by comparison of the retention time and m/z with the reference standards. Quantification of the metabolic rate constants focused on fipronil and on fipronil sulfone as the predominant metabolite (±85% of metabolic conversion). Quantification of the peak areas of fipronil and fipronil sulfone was achieved by comparison of the peak areas to the calibration curves of the reference standards at an m/z (-H) of 435 for fipronil and 451 for fipronil sulfone.

2.5. PBK model

The model structure for fipronil and fipronil sulfonate was based on the generic model for chicken (Lautz et al., 2020). This includes two-sub-models for the parent compound and the major metabolite fipronil sulfonate, with each sub-model consisting of 11 compartments, including gut tissue, liver, adipose tissue, brain, bone, heart, kidney, lung, muscles and egg (Fig. 1). All organs and tissues are modelled as well-mixed compartments with a blood-flow limited distribution. For the parent compound the compartment gut lumen was included since fipronil is simulated as oral uptake and therefore the absorption from the gut lumen into the gut tissue is modelled as a first order process. Distribution throughout the body is modelled by systemic circulation.

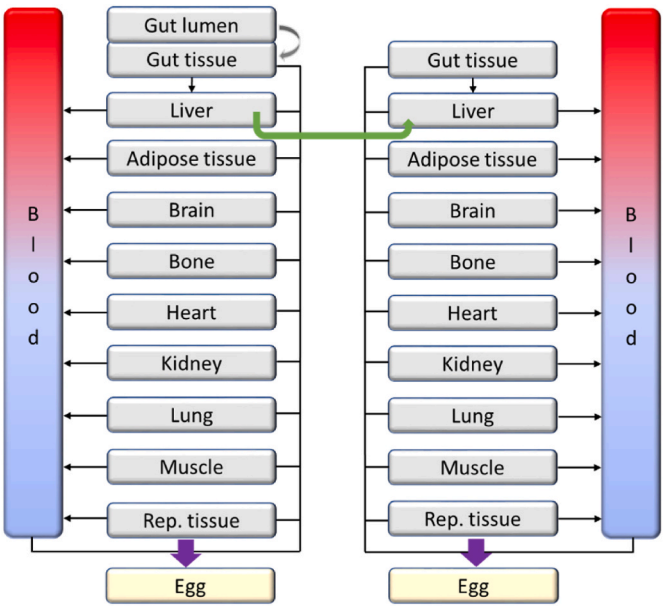

Fig. 1. Structure of the PBK models for fipronil (left) and its metabolite fipronil sulfone (right) for chicken. Fipronil is taken up orally and enters the body via the gut lumen and via the liver enters the blood. In the liver fipronil is metabolised to fipronil sulfone and excreted via eggs. Rep. Tissue: reproductive tissue.

Elimination of fipronil and fipronil sulfone was modelled by implementing hepatic metabolism and transfer to eggs. The transfer to eggs was calculated by inclusion of the reproductive tissue (Lautz et al., 2020). Computer implementation of all differential equations was performed in the R software (version 3.6.3) to provide model codes and syntax (R Core Development Team, 2014). The model code is available in the supporting information (SI). Physiological parameters for chicken were taken from another paper as well as the absorption rate constant of fipronil which was previously estimated to be 0.01 h^{-1} (Lautz et al., 2020). The fraction absorbed was set on 1 to simulate the default value (FAO, 1998; Pendse et al., 2020), and the value of 0.5 as reported in literature (Gerletti et al., 2020; Kitulagodage, 2011). The fipronil intrinsic clearance was based on the *in vitro* result (section 3.2) and was calculated by determining the slope of the linear part of the ln-transformed substrate depletion curve representing the elimination rate constant. After calculation of the half-life and volume of incubation, the intrinsic clearance can be calculated as followed (Equation (1)):

$$Clint\ (\mu L / min / mg\ S9\ protein) = \frac{\ln(2)}{t_{\frac{1}{2}}}\ x\ Volume\ (\mu L / mg\ S9\ protein)$$

The intrinsic clearance was scaled to total liver by using a liver weight and estimated S9 protein per gram liver (60 mg/g liver). The estimated S9 yield used for chicken is based on the ratio liver microsomal protein yield and S9 yield in humans. The clearance of fipronil sulfone was set to 0 in the model given no further conversion of the metabolite occurs. Partition coefficients for both compounds in the various tissues were estimated using an available QSAR (Rodgers and Rowland, 2006) using a log Kow of 4.0 for fipronil (Kim et al., 2020) and 4.42 for fipronil sulfone estimated with EPI Suite™ (US EPA, 2021). The QSAR allowed the calculation of the chemical affinity for all tissues based on the octanol/water partition coefficient and tissue composition through considering the tissues constituents' lipids (both neutral and phospholipids) and water. The partition coefficients for fipronil and fipronil sulfone are depicted in Table 1.

3. Results

3.1. Identification of fipronil and metabolites

After the incubation with chicken S9 for 2 h, fipronil was metabolised to fipronil sulfone as the main metabolite. Besides fipronil sulfone, another compound with similar mass molecule with fipronil amide (m/z = 453.014(-H)) (Kim et al., 2016) was also detected. This compound and fipronil sulfone showed the same retention time (5.76 min). However, after the mass range was set smaller (i.e. m/z±1 rather than m/z±2), the two compounds could be observed separately. An unknown compound (m/z = 284.93(-H)) was also detected and was not identified, but it was suspected to be the pyrazole degradation product from fipronil (Cravedi et al., 2013). The integrated chromatograms of fipronil and the metabolites in the MS analysis are presented in Fig. 2.

Table 1
Partition coefficients for fipronil and fipronil sulfone.

Tissue	Partition coefficients fipronil	Partition coefficients fipronil sulfone
Adipose tissue	104	117
Bone	14.16	14.54
Brain	12.98	13.3
Heart	3.21	3.29
Intestine	10.25	10.5
Kidney	4.94	5.1
Liver	8.12	8.34
Lung	1.17	1.19
Muscle	5.01	5.14
Reproductive tissue	8.12	8.34

3.2. Kinetics in vitro experiment

The time related degradation of fipronil and formation of the sulfone are shown in Fig. 3. From this graph the inherent half-life, elimination rate constant and R^2 values can be estimated. Fipronil intrinsic clearance was 29.6 μL/min/mg S9 protein. The results are presented as the average and the standard deviation from three different experiments. In these experiments fipronil sulfone was the major metabolite formed (±85%) compared to the others measured in the MS analysis. An incubation with fipronil sulfone as substrate revealed a loss of the chemical due to non-specific binding as 0.3 μM of the added 1 μM could be found back in the incubation (Fig. 3B). No metabolic clearance of fipronil sulfone occurred as the concentration of fipronil sulfone remained stable throughout the incubation.

3.3. PBK model results

Fig. 4 shows the comparisons between measured fipronil equivalents in egg (MacLachlan, 2010; Stewart, 1994) and the PBK model predictions based on *in vitro* clearance data and *in silico* calculated partition coefficients. In this case the fraction absorbed was set on a default value of 1 (Pendse et al., 2020). The predicted egg concentration shows a systematic overprediction of the concentration of fipronil equivalents, especially for lower time points, compared to measured egg concentrations (Fig. 4). However, as shown in Fig. 5, 88% of the PBK model predictions were within a 3-fold difference for fipronil equivalents and only 12% of the predicted egg concentrations were overpredicted by more than 3-fold. The estimated elimination half-life for fipronil equivalents was 14 days.

Fig. 6 shows the comparisons between measured fipronil equivalents in egg (MacLachlan, 2010; Stewart, 1994) and the PBK model predictions for the scenario that additional information about fipronil from literature is taken into account on the fraction absorbed. In this case the fraction absorbed was set on 0.5 (Gerletti et al., 2020; Kitulagodage, 2011). The predicted egg concentration shows a better fit in Fig. 6 compared to Fig. 4. As shown in Fig. 7 all PBK model predictions were within a 3-fold difference for fipronil equivalents. The estimated elimination half-life for fipronil equivalents was 14 days.

4. Discussion and conclusions

This study describes the implementation of *in vitro* metabolism data and *in silico* partition coefficients in PBK models in laying hens, based on the example for fipronil, using an R-based algorithm. The performance of the model was illustrated and evaluated for fipronil equivalents by comparing measured egg concentrations and predicted results. Overall, all of the model predictions were within a 10-fold difference, showing an overprediction compared to the measured data in the first scenario, while all model predictions were within a 3-fold difference when additional information about the fraction absorbed was taken into account.

The *in vitro* incubations of the present study were performed with chicken liver S9 and NADPH as cofactor. A clear conversion of fipronil to fipronil sulfone as major metabolite (±85%) was observed, which was also observed in *in vitro* experiments with rat microsomes (Tavares et al., 2015). Other metabolites such as fipronil-desulfinyl and fipronil-sulfide were not detected, whereas fipronil-amide and a pyrazole were detected in low quantities as about 85% of fipronil was converted into fipronil sulfone. This confirms that fipronil sulfone is the main relevant metabolite in chicken, which was also indicated previously for chicken as well as other species. Fipronil sulfone also contributes to the toxicological effects (EFSA, 2006; JMPR, 2001; Kitulagodage et al., 2011). Further conversion of fipronil sulfone was not detected in our incubations. It is possible that the *in vitro* experiments did not include all necessary co-factors which are needed for the further metabolism of fipronil sulfone. However, based on the available evidence it is suggested that fipronil sulfone is very stable in chicken (EFSA, 2006; Gerletti et al.,

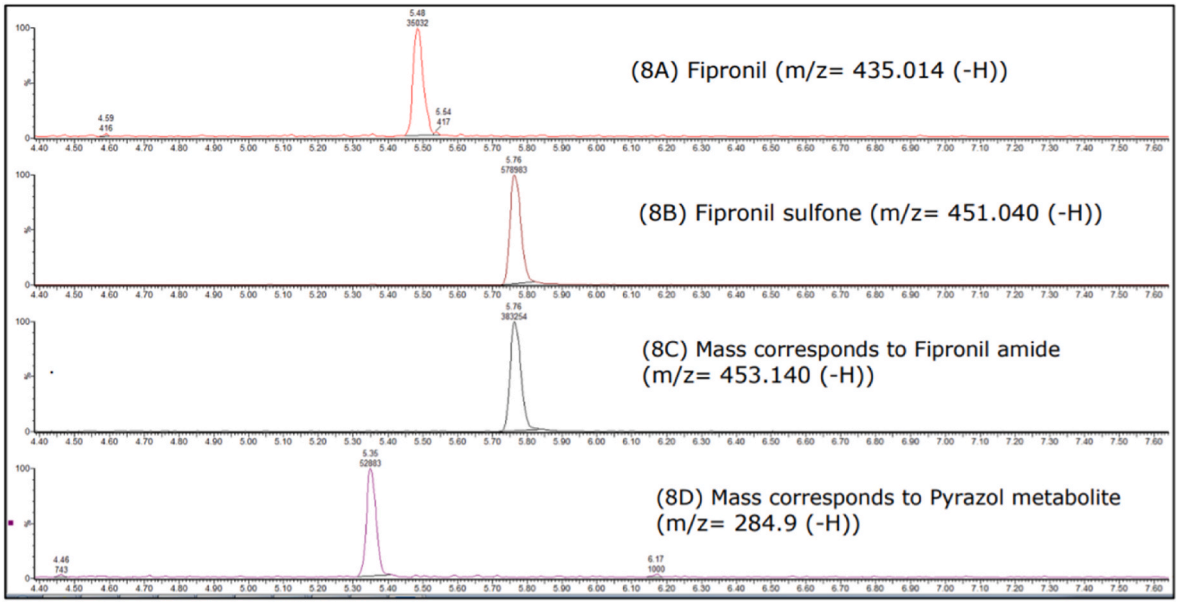

Fig. 2. Integrated chromatograms of fipronil and the metabolites in the MS analysis.

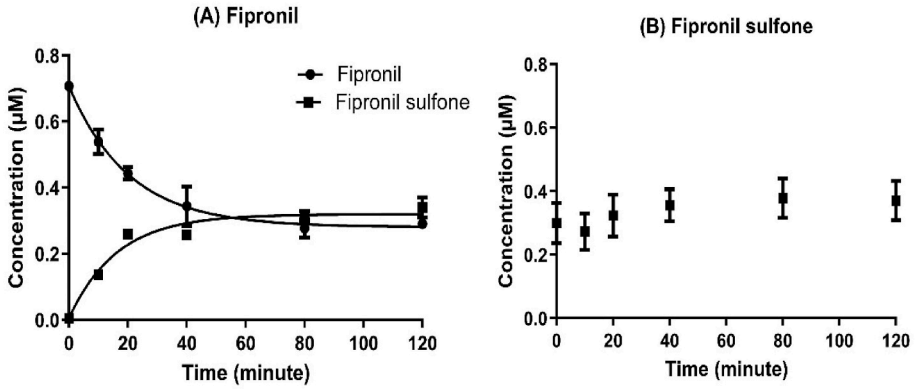

Fig. 3. Profile of the concentration of fipronil and fipronil sulfone during the *in vitro* incubation of fipronil (A) and fipronil sulfone (B) with chicken S9 for up to 2 h. Black dots represent measured values with standard deviation. Fipronil: half-life ($t_{1/2}$) = 14.2 min; elimination rate constant (min^{-1}) = 0.05; R^2 = 0.92.

2020; Kitulagodage, 2011). In this study, existing literature information about fipronil and its major metabolite fipronil sulfone were used. For other chemicals without pre-knowledge of their behaviour and metabolism, a two-step approach is advised. For example, incubations can first be analysed with high-resolution mass-spectrometry in combination with specialised software to identify possible metabolites such as MetabolitePilot Software 2.0 (AB Sciex, USA) or Compound Discoverer Software (Thermo Scientific, USA) (Meijer et al., 2022; Yang et al., 2021). In the second step *in vitro* studies can be performed, where it is advised to include a co-factor mixture to not only account for cytochrome P450 enzyme-mediated metabolism, but also glucuronidation.

Compared to other available models for fipronil in chicken, this model performed slightly less (Gerletti et al., 2020; Lautz et al., 2020; MacLachlan, 2010). But unlike the available models, the model parameter for clearance was extrapolated from *in vitro* data and is not fitted based on *in vivo* data, which makes it also suitable for other chemicals. Furthermore, the fraction absorbed for fipronil was assumed to be 1 (FAO, 1998), whereas a fraction absorbed between 0.44 and 0.72 was reported for chicken (Gerletti et al., 2020; Kitulagodage, 2011). Differences in the fraction absorbed greatly influence the model outcome, since they determine the dose taken up and thus also the transfer to eggs. However, for chemicals with no information available about the absorbed fraction the use of a default factor of 1 is common for PBK models (Pendse et al., 2020). This model calculated an elimination half-life of 14 days, which is similar to the half-life of 11.32 days found by Gerletti et al. (2020). MacLachlan (2010) and Corrias et al. (2021) reported a half-life of 8 days. This shows that *in vitro* data can give insight into the elimination time of a chemical, which is especially useful for chemicals with little or no information available in literature. Overall, the PBK predicted egg concentrations and half-life predictions were validated results from *in vivo* animal studies and were shown to be appropriately and reasonably more conservative than the actual data (*references*). This study illustrates how a combination of *in vitro/in silico* methods can reduce animal use while at the same time providing rapid and valuable data useful for risk management decisions associated with unanticipated contamination scenarios. This approach is in line with the recommendations of EFSA regarding *in silico* and *in vitro* methods (EFSA, 2021).

PBK models for farm animals are currently fitted on *in vivo* animal experiments, while so far a few PBK models implemented *in vitro* metabolism results (Henri et al., 2017; Lautz et al., 2019, 2020). This PBK model also implemented *in vitro* metabolism data, indicating the possibility and giving future opportunities to predict internal concentrations of chemicals using *in vitro* kinetic data as model inputs. There is

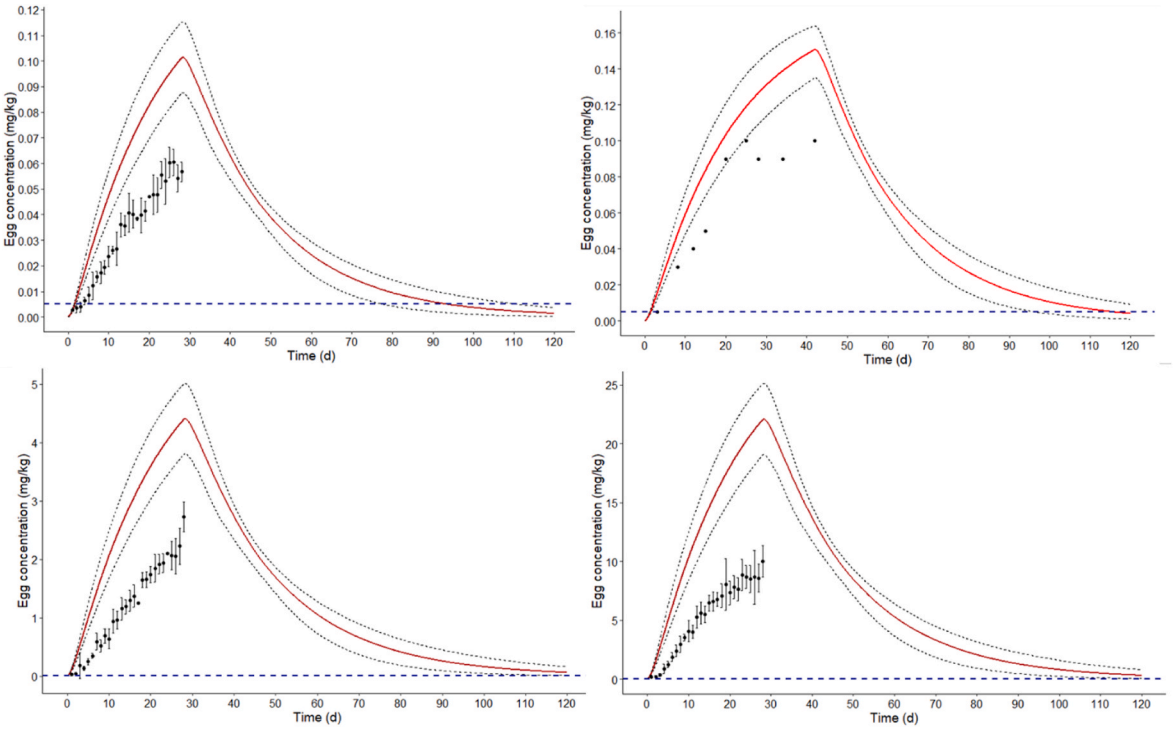

Fig. 4. Comparison of model prediction (solid red line and 95th confidence interval) and observed mean data±standard deviation (black dots) (MacLachlan, 2010; Stewart, 1994) are shown for different concentrations of fipronil equivalents in chicken eggs. The blue line represents the maximum residue limit of 0.005 mg/kg (European Commission, 2018). A) 0.004 mg/kg BW fipronil; B) 0.005 mg/kg BW fipronil; C) 0.174 mg/kg BW fipronil; D) 0.872 mg/kg BW fipronil. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

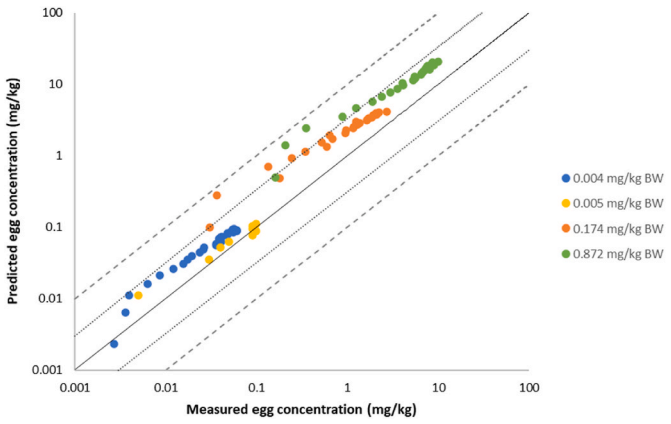

Fig. 5. Comparison between mean quantities measured in chicken and PBK model predictions of fipronil equivalents in egg. The dots for each dose represent the time course. Dotted lines represent 3-fold and 10-fold differences, respectively.

an increasing demand to reduce animal testing in chemical risk assessment and to shift towards the use of mechanistic data based on *in silico* models or *in vitro* toxicity tests (Dorne and Fink-Gremmels, 2013; Lautz et al., 2020). Overall, the challenge for the PBK modelling community is to base the model parameterisation increasingly or entirely on ADME properties derived from non-animal studies, with limited or no availability of *in vivo* kinetic data (EFSA, 2020; Leonard and Tan, 2019; Madden et al., 2019; Punt et al., 2017). However, the case study about fipronil shows, how available background information about the chemical in literature can be used to fine-tune the outcome of the model predictions.

In the case of fipronil sufficient information about the chemical was available based on reports and scientific literature to conduct and compare the model outcome, but for many chemicals, especially environmental chemicals or natural toxins, this is not often the case. *In vitro* experiments are therefore recommended compared to *in vivo* studies to provide fast first insights into the behaviour of a chemical in an organism. Overall, *in vitro* kinetic data are still anecdotic in the literature for chicken and other avian test species (e.g., goose, quail), but increasing availability and generation of *in vitro* kinetic data and its use in PBK models can help to increase the confidence in the model outcome. Harmonisation of protocols for *in vitro* experiments are necessary to reduce further uncertainties (Louisse et al., 2020; Punt et al., 2018). Overall, the combination of *in silico/in vitro* methods is a promising approach for transfer predictions and to reduce animal testing for transfer studies.

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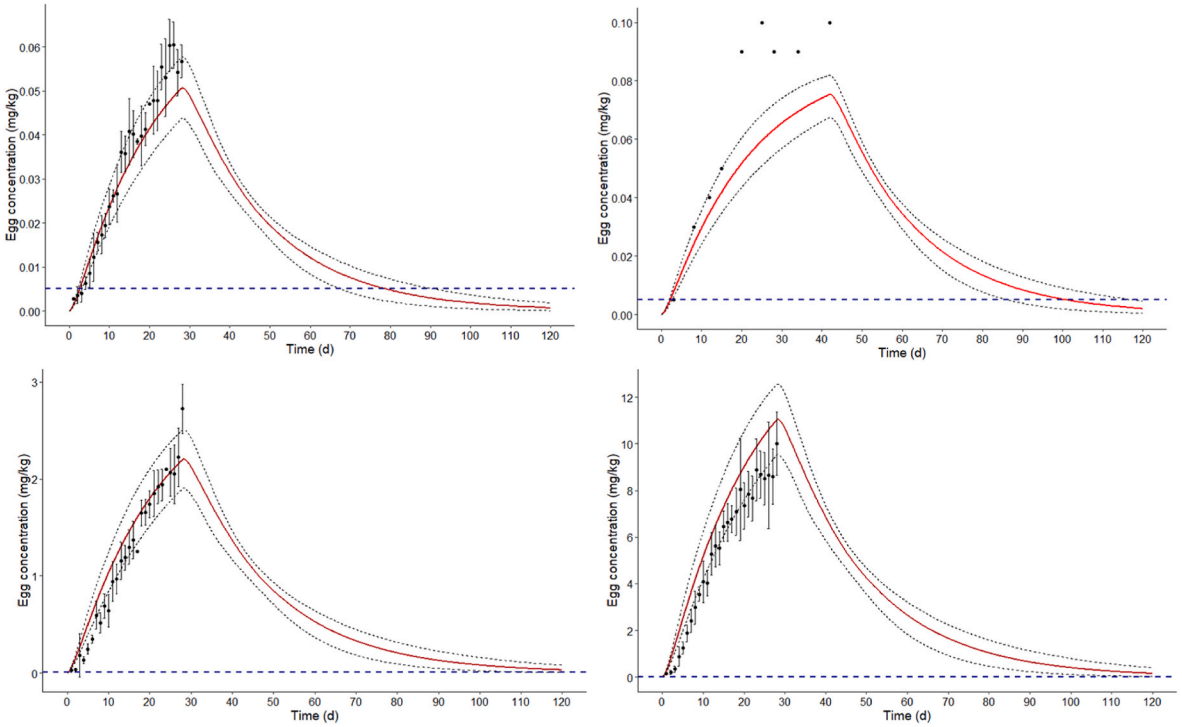
CRediT authorship contribution statement

L.S. Lautz: Methodology, Conceptualization, Writing – original draft, Writing – review & editing. **G. Stoopen:** Data curation, Formal analysis, Writing – review & editing. **A.J. Ginting:** Data curation, Writing – review & editing. **R.L.A.P. Hoogenboom:** Writing – review & editing. **A. Punt:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

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



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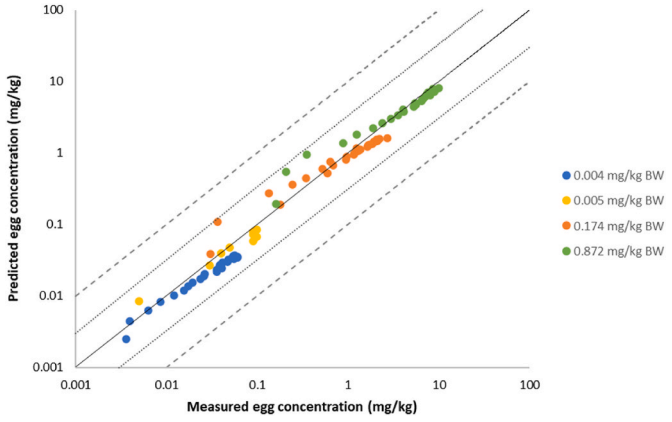

Fig. 7. Comparison between mean quantities measured in chicken and PBK model predictions of fipronil equivalents in egg. The dots for each dose represent the time course. Dotted lines represent 3-fold and 10-fold differences, respectively.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.113086.

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