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AN *IN VITRO* METHOD FOR DETERMINING THE BIOACCESSIBILITY OF PHARMACEUTICALS IN WILDLIFE

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ABSTRACT

Wildlife can be exposed to human pharmaceuticals via prey that have accumulated the compounds from wastewater, surface water, sediment and soil. One factor affecting internal absorption of pharmaceuticals is bioaccessibility, the proportion of the compound that enters solution in the gastrointestinal tract. Currently, the bioaccessibility of most pharmaceuticals in prey remains unknown for most wildlife species. Here, we evaluate the potential of a two-compartment in vitro gastrointestinal tract model to compare the bioaccessibility of the antidepressant fluoxetine from invertebrate prey for birds and mammals. Samples of gizzard (or stomach) and intestinal phase digestive juices were obtained from the in vitro models along with the residual solid material. HPLC analysis revealed that the bioaccessibility of fluoxetine in the avian in vitr*o* models (75.9% and 78.6%) was statistically significantly lower than in the mammalian models (88.2-89.6%) as a percentage of what was recovered; however there were no statistically or biologically significant inter-species difference in terms of the amount recovered per gram of 'food' inserted at the start of the simulation. Nevertheless, this in vitro model provides a useful method of comparing the bioaccessibility of pharmaceuticals in different prey for species with different gastrointestinal conditions. There may be merit for ecological risk assessments in further developing this in vitro approach to improve estimates of internal exposure for organics.

Key words: Bioaccessibility, Earthworms, Pharmaceuticals, Starlings, Fluoxetine

INTRODUCTION

Over the last 15 years there has been increased attention on the dispersal and effects of active pharmaceutical ingredients (APIs) into/in the natural environment [1], but the potential risks of pharmaceuticals in the environment to mammalian and avian species are only just starting to be studied (e.g.[2-6]). One approach that has been proposed for estimating the impacts of pharmaceuticals on organisms in the natural environment is to read-across from the wealth of data that are available on the safety and pharmacology of pharmaceuticals for humans and model mammals [7, 8]. The read-across approach has already shown utility for evaluating the effects of selected pharmaceuticals on fish [9, 10]. As extensive in vivo testing is usually done on pharmaceuticals using laboratory mammalian species (e.g. rodents), the read-across approach could be particularly helpful in understanding potential impacts on birds and mammals in the environment.

One factor that will likely need to be considered in applying the read-across approach is bioaccessibility [7, 11]. Bioaccessibility is defined as the percentage of the pharmaceutical that goes from the ingested matrix (e.g. tablet, food or soil) into the digestive juices. While bioaccessibility does not necessarily equate to bioavailability (the percentage of the dose that reaches systemic circulation), an understanding of how much of the ingested contaminant is available for uptake is an important stepping stone towards predicting internal concentrations in an organism. As digestive systems of different species vary in terms of temperature, enzymatic composition, gastrointestinal tract transit time and physical breakdown [12-14], the degree of bioaccessibility of a pharmaceutical will vary in different species types. To add further complexity, it has also been demonstrated bioaccessibility will vary across different ingested matrices (e.g. soil, plant, fish and meat) [15-17]. Given these many sources of diversity in bioaccessibility, in vivo measurements of bioaccessibility relevant to ecologically relevant exposure scenarios are impractical. However, the use of in vitro approaches may provide the solution.

In vitro test systems, such as the Physiologically based extraction test (PBET), that simulates gastro-intestinal conditions, have already been developed and validated for assessing the bioaccessibility of trace metals for several organisms including humans, [12, 14, 17-22], small mammals [23, 24] and birds

[13, 25, 26]. PBETs have been used in a variety of applications including testing the safety of children's toys containing metals [27], in the assessment of contaminated land [11, 28] and to assess the risks presented by lead shot to wild birds [13]. Assessments for organics have largely been limited to poly-aromatic hydrocarbons (PAHs) in humans [29, 30].

It has been suggested that PBETs could play a wider role in the assessments of risks of chemicals to wildlife by helping to adjust for differences in bioaccessibility [31, 32]. However, as yet the approach has not been used for environmental risk assessment of pharmaceuticals. In this paper we therefore describe the application of a PBET approach to develop an understanding of the differences between humans and birds. We illustrate the approach using earthworms spiked with the selective serotonin reuptake inhibitor fluoxetine. As a secondary amine, the uptake of fluoxetine across lipoidal membranes is strongly affected by pH close to its pKa of 10.06 [33]. At a basic pH, the Log Dow (Octanol water distribution coefficient) of fluoxetine reduces, fluoxetine becomes increasingly present in its ionised form (AH+ is more easily dissolved in water than the non-ionised A species). The ionised form is less soluble than the unionised form and cannot cross lipid cell membranes in the intestine to reach the blood as easily. Thus the accumulation and toxicity of amines such as fluoxetine varies considerably at pH values just below the pKa due to the reduction in hydrophobic non-ionised species with decreasing pH [34].

While the present study has only investigated one pharmaceutical, food type and wildlife species, in the future it could be applied to a wider range of active ingredients, food items and wildlife species. Fluoxetine is the study compound in a series of in vivo uptake and effects studies we have performed using starlings (*Sturnus vulgaris*) [Bean et al. submitted and 35]. In these studies, concentrations of fluoxetine in plasma and tissues of the birds were much lower than anticipated based on uptake into humans. The in vitro investigations conducted here provide us with an opportunity to explore whether the differences observed in vivo are due to differences in bioacessibility or whether other factors are at play.

MATERIALS AND METHODS

Test chemicals and soil

Fluoxetine (≥98%), pepsin, pancreatin, malate, bile extract and sodium bicarbonate extract were obtained from Sigma Aldrich (Dorset, UK). Lactic acid, citric acid, acetic acid and methanol (High Performance Liquid Chromatography [HPLC] grade 99.9%) were obtained from Fisher Scientific (Loughborough, UK).

A sandy loam (pH 6.47) soil was collected from an unpolluted site (N 53.957045, W -1.137880) for use in the earthworm exposures. Roots and stones were first removed by hand. The soil was air dried for 24 h before passing through a 2 mm sieve. Details of how moisture content and maximum water holding capacity were determined are given in Supplemental Data.

Exposure of earthworms to fluoxetine

Earthworm prey were first exposed to fluoxetine: A colony of *Eisenia fetida* were obtained from The Food and Environment Research Agency (FERA York, UK) and maintained at optimal conditions [36], for 3 wk until a sufficient number of individuals weighed 0.5 g ± 0.1 g. Then, individuals were removed from the colony, soil, which had adhered to the earthworm, was removed by holding the earthworm with a pair of blunt ended forceps and pipetting deionised water. Earthworms were dabbed dry on paper towels prior to inserting into their individual exposure jars.

To expose individual earthworms to fluoxetine, 50 g of moist soil was weighed out into individual glass jars (approximate volume of jars was 100 mL). Soil was either spiked with 1 mL of fluoxetine solution, (30 mg/mL fluoxetine dissolved in methanol) to give an expected fluoxetine concentration of 600 µg/g soil. A fluoxetine concentration of 0.37 µg/g is an environmentally relevant concentration in treated sludge [1], while 0.019 µg/g is predicted for soil [37, 38]. The spiking concentration for soil was based on previous work on uptake of fluoxetine into earthworms [37], and was selected to yield a concentration of fluoxetine in earthworms of at least 60 µg per earthworm. The concentration in earthworms after 21-d was required to be greater than an environmentally realistic level to ensure levels

in digestive juice and RSM samples were above the limits of quantification of the of the HPLC analysis method (see Chemical analyses and Supplemental Data). The soil for the control group was spiked with 1 mL of methanol. After spiking, the soil was left for 2 h before stirring with a spatula. Jars containing soil were left for 48 h prior to adding a single earthworm to each jar.

The soil in each jar was made up to 60% of Maximum Water Holding Capacity (MWHC) with deionised water on a balance (Sartorius LL4800P); the balance was then tared and the earthworm was added. The earthworm weight was recorded to 0.01 g. Earthworms were provided with a small amount of food, approximately 0.1 g of dried mashed potato powder, by sprinkling a thin layer on to the surface of the soil. To prevent earthworms from escaping, jars were covered with a square of garden fleece held in place by an elastic band. Earthworms were kept in a Controlled Environment room (20°C, 70% humidity, 16 h light and 8 h dark).

To control for the effect of soil moisture content on uptake into worms, the soil moisture content was maintained at 60% MWHC over the 21-day exposure period. This was achieved by adding the required mass of deionised water daily to return the jar to its starting mass [36, 37]. A small amount of food (<0.5 g) was also re-applied as necessary, approximately every other day. The start of the experiment was staggered so that the PBETs were carried out on nine separate days (see Supplemental data).

Physiologically based extraction tests (PBETs)

Relative bioaccessibilities for the mammalian and avian digestive systems were quantified by inserting earthworms (*Eisenia fetida*), along with soil which had adhered to the worm, into the human and avian PBETs. The earthworms were not cleaned first in order to mimic avian foraging behaviour in the wild.

In total 5 PBETs were conducted: 2 avian PBETs (siliceous (Si) grit gizzard and calcareous (Ca) grit gizzard) and 3 for mammals (recently fed = pH 4, average = pH 2.5 and fasted (pH1.3). These PBETs were designed to cover the broad range in digestive tract conditions for birds and mammals. To simulate uptake of fluoxetine by wild birds, we inserted earthworms, which had been exposed to fluoxetine in

the soil, directly into each PBET. The mean mass of adhered soil was 0.155 g ± 0.042 g (determined by rinsing the soil from 4 earthworms that did not go into a PBET). To humanely kill the earthworms, the tubes containing the earthworm were placed in a -20°C freezer and brought back up to room temperature prior to digestion in the simulated gastrointestinal tract.

In addition to the fluoxetine and control earth earthworms, we also ran blank PBETs containing no food. All simulations were performed in triplicate as is widely used in PBETs (e.g., [12-15] (see Supplemental Data for details of experimental structure).

From each PBET, samples of stomach (mammals) or gizzard (bird) and intestinal digestive juice were obtained. Digestive juice samples were centrifuged and extracted with solvent, passed through a 0.2 µm polytetrafluoroethylene (PTFE) syringe filter and analysed by HPLC with fluorescence detection (see High Performance Liquid Chromatography and Supplemental Data). The residual solid material (RSM) remaining at the bottom of the intestinal digestive juice sample was also extracted as in Martinez-Haro et al. [13]. The fluoxetine recovered at the end of the intestinal phase was the total bioaccessible amount and the total fluoxetine recovered was given by the sum of intestinal fluoxetine and RSM fluoxetine.

Mammalian models based on Human PBET

The mammalian models (were adapted from the human PBETs of Li and Zhang [12], Ruby et al. [14] and the mammalian adapted human PBETs of Moriarty et al. [24] and Kaufman et al.[23] (see Figure 1a and Supplemental Data for further details). Mammalian PBETs were carried out in 50 mL centrifuge tubes using stomach digestive juice at three different pHs, to account for the effect of time since the last meal on stomach pH. Stomach digestive juices were prepared in conical flasks by adding pepsin (1.25 g/L), malate, citrate (both 0.5 g/L), lactic acid (420 µL/L) and acetic acid (500 µL/L) to deionised water. Stomach digestive juice was adjusted to one of three pH values using concentrated (35%) HCl: pH 1.3 was used to represent fasted conditions, pH 2.5 represented average stomach conditions and pH 4 recently fed conditions and then warmed to 37°C in a water bath [14].

To begin the mammalian PBET simulations, whole earthworms and any adhered soil were added to tubes intact along with 20 mL of stomach digestive juice. The head space was purged with N_2 to create a low oxygen environment. Tubes were placed in the shaking incubator at 37°C but were not shaken for 10 min as in Ruby et al. [14], after which tubes were shaken at 250 rpm for 1 h in total. At three time points (every 20 min) during this hour, shaking was stopped and a 1.5 mL aliquot of the stomach digestive juice was taken from each tube and, and replaced with a fresh 1.5 mL of gastric solution. The stomach digestive juice samples were transferred to a 1.5 mL sealed microcentrifuge tube and immediately centrifuged (10 min at 11,000 × g). The supernatant was decanted into a fresh tube and stored at -20°C.

Once the stomach simulation was complete, the digestive juice in each simulation tube was adjusted to pH 7 with $NaHCO_3$ powder (intestinal pH) [21]. For the pH 1.3 simulation, the worm had completely disintegrated after 1 h, pH 2.5 had partially disintegrated but at pH 4 the worm could clearly still be seen intact prior to the intestinal simulation. Once pH had been adjusted, 52.5 mg of bile extract and 15 mg of pancreatin were added to each replicate [12] before returning to the shaker. The 50 mL centrifuge tubes were shaken on their sides at 100 rpm to mimic the slow intestinal passage of food. After 1 h (data not presented here) and after 3.5 h of intestinal incubation, 1.5 mL aliquots were taken from each centrifuge tube. Unlike the stomach phase, the intestinal 1.5 mL aliquots were not replaced after sampling. The intestinal samples were centrifuged, the supernatant aliquoted into a new tube and the RSM retained. All samples were stored at -20°C until extraction and analysis.

Avian PBETs

To capture the complexity of the avian digestive tract, a dynamic avian gizzard-intestine system containing either 2 g of Si grit or 2 g of Ca grit was simulated following the methods of Martinez-Haro et al. [13]. The method used for the avian PBET is summarised in Figure 1b and explained in full in the Supplemental Data. Five samples of gizzard digestive juice were collected every 36 min and aliquoted into two 1.5 mL microcentrifuge tubes during a 3 h incubation at 42°C on a mechanical shaker (full details in Supplemental Data). At each of the 5 sampling time points, one aliquot was centrifuged and the supernatant stored at -20°C prior to analysis. The other sample was adjusted to pH 6 with a

concentrated $NaHCO_3$ solution, one-tenth of the volume was then discarded and replaced with a 10 × concentrated intestinal digestive juice containing bile and pancreatin (see Supplemental data). Intestinal simulations were returned to the incubated mechanical shaker for 3 h, before being centrifuged. The supernatant was aliquoted into a new 1.5 mL tube, the RSM was retained, and stored at -20°C until extraction and analysis.

Chemical analyses

After the PBETs had been run, the samples of stomach (or gizzard) and intestinal digestive juice were thawed and vortex mixed. A 500 µL aliquot was taken from each sample and combined with 500 µL methanol using a further vortex mix. Samples of RSM were extracted into 1 mL of methanol using sonication for 3 min. Sample and methanol mixtures were centrifuged for 10 min at 11,000 × g and filtered using a 0.2 µm PTFE filter.

Extracts were analysed by HPLC with fluorescence detection (excitation = 230 nm, emission = 305 nm). Separation was achieved using a C-18 column (Kinetex 5 µm C18 150 × 4.6 mm, Phenomenex, Macclesfield UK) and a gradient mobile phase comprising a mixture of water containing 0.1% H_3PO_4 and methanol ranging in concentration from 90% water/ H_3PO_4 to 10% water/ H_3PO_4. The run time was 23 min with a retention time of 11.7 to 11.8 min.

To validate the extraction procedure from digestive juice samples and faeces for each of the 5 PBETs, blank digestive juice and faecal samples were generated by running the PBET simulations without the addition of an earthworm (see Supplemental Data).

Data analysis and statistics

Effect of fluoxetine on earthworm growth during 21 d exposure via soil: To meet the assumption of normality, earthworm body weights data were log-transformed and the effect of fluoxetine or control on growth was assessed using a repeated measures model in R package nlme. The repeated measures were body weight before and after the 21 d exposure period.

Effect of in vitro model type on percentage of fluoxetine in stomach or gizzard, intestine and faeces: An explanation of how the amounts of fluoxetine (µg) recovered in the different PBET compartments is presented in Figure 2. The relation between mass of solid material added and amount of fluoxetine recovered was not linear (Pearson's correlation: N=15, r=0.265, p=0.36). This was because there was a significant inverse relationship between mass of solid material added and the percent of the total fluoxetine that was bioaccessible (Pearson's correlation: N=15, r=0.519, $p<0.05$). Therefore, the bioaccessibility data were normalised in 2 ways for separate analyses: Data set 1) µg/g of solid material (worm + soil) inserted at the start of the PBET and Data set 2) Percentage recovery, assuming the sum of bioaccessible and RSM fluoxetine (µg) was the total fluoxetine added (Figure 2).

Normality was assessed from Q-Q plots for both data sets (µg/g solid material and in percentage terms) and no transformation was applied. The effect of PBET model on fluoxetine recovered in the different PBET compartments was initially assessed using a multivariate analysis of variance (MANOVA). The response variables used in the MANOVA were the µg/g or percentage of the total µg of fluoxetine recovered in the PBET compartments (1. stomach or gizzard, 2. the intestinal phase minus the stomach or gizzard, 3. at the end of the intestinal phase [total bioaccessibility]). For the µg/g analysis, the RSM remaining at the end of the intestinal phase and the total (intestinal fluoxetine + RSM) were also included in the MANOVA. They were not included in the MANOVA for the analysis of percentages as the total would always be 100% and %RSM is just 100% minus the intestinal %. As we obtained an overall effect of PBET model in the MANOVAs, individual ANOVAs were used to identify which of the response variables contained significant differences between models. Finally, for the PBET compartments where there were significant differences in the individual ANOVAs, Post-Hoc Tukey's HSD tests (all on 4 degrees of freedom) were used to identify which PBET models (i.e. Mammalian pH 1.3, 2.5 or 4 and Bird Si grit and Bird Ca grit) these were for. MANOVA, ANOVA and post-hoc analyses were performed in SPSS (Version 23) and significance was tested for at the 5% level.

RESULTS AND DISCUSSION

Earthworm uptake and growth

The mean concentration in earthworms (537.1 µg/g ±47.3 µg/g) was 0.94 times the mean concentration measured in soil 572.7 µg/g ±9.4 µg/g. The biota-sediment accumulation factor (BSAF) estimated by Carter et al. 2014 using radio-labelled fluoxetine and earthworms that had been given 24 h to depurate their gut was 0.3 [37]. Our value of 0.94 is the same order of magnitude as that of Carter et al. [37], and would be closer had we depurated the earthworms prior to extraction. Earthworms were not given a chance to depurate as the aim of the PBETs was to mimic how fluoxetine would be encountered by a wild bird which in reality would consume both the worm and associated soil.

The fluoxetine and control earthworms had similar masses before the 21 d exposure period (fluoxetine, N=20, mean ±SE = 0.53 g±0.008 g; control, N=20, mean ±SE = 0.51 g ±0.008g). After 21 days exposure, the earthworms in the fluoxetine treated soil had lost weight, with mean body weights ±SE of 0.50 g ± 0.024 g, while the control earthworms gained weight (0.70 g ± 0.030 g). There was a significant effect of treatment on body mass change from pre to post-exposure time ($F_{38,1} = 33.49$, $p<0.0001$). Effects on growth have been observed in invertebrates exposed to fluoxetine in sediments before with lowest observed effect concentrations (LOECs) of 1.3 and 5.6 mg/kg being reported for *Chironomus tentans* and *Hyalella Azteca* respectively [33]. Our fluoxetine concentration was measured at 572 mg/kg, two orders of magnitude higher than these LOECs. However we observed weight loss and not just growth inhibition, which is the endpoint typically investigated. The findings indicate that fluoxetine has the potential to affect invertebrate growth, the mechanism for which is uncertain [33]. However all concentrations at which fluoxetine has been found to affect growth are several orders of magnitude higher than predicted in the environment (0.019 mg/kg) based on Carter et al. [37, 38] so these effects may not occur in reality.

Differences between PBETs

Concentrations of fluoxetine in samples from the control earthworms and blank treatments were below the limit of detection (< 0.2 µg/mL) so no blank correction was applied. The masses of solid material (worm + soil) and the amount of fluoxetine (µg) recovered in each PBET compartment are reported for the avian and mammalian PBETs in Table 1. These data were used to compute the 2 versions of the bioaccessibility data analysed: Data set 1; µg/g of solid material added and Data set 2; percentage of fluoxetine recovered. The MANOVAs for both data set 1 and 2 indicated there were significant differences between PBETs (µg/g: Pillai's score = 2.41, $F = 3.79$, $p < 0.001$; for %: Pillai's score = 2.35, $F = 8.99$, $p < 0.001$).

Data set 1, Micrograms/gram of solid material added: Figure 3a shows the amount of fluoxetine recovered in each PBET compartment when the data were normalised in terms of the amount of solid material added. There were no significant differences between any PBETS in the individual ANOVAs for the amount of fluoxetine µg/g of solid material added at the end of the intestinal phase (df=4, F=0.29, p=0.88) nor in terms of the overall amount of fluoxetine recovered (sum of intestinal fluoxetine and RSM) (df=4, F= 1.41, p=0.30). Importantly, this means there were no inter- or intra species differences in bioaccessibility relative to the amount of solid material added (and thus approximately the amount of fluoxetine added due to differences in uptake into earthworms we cannot definitively give the amount of fluoxetine inserted). Therefore the significant differences were only in terms of whether fluoxetine entered solution in the first (stomach or gizzard: df=4, F= 11.66, P=0.001) or second compartment (intestine – stomach or gizzard: df=4, F =24.43, P<0.001) of the PBET and the amount remaining in the RSM (df=4, F=15.35, p<0.001). These differences are likely to be of less biological significance than total bioaccesibility. Table 2 summarises where the differences occurred (inter-or intra species) and the nature of the differences from post-hoc Tukey's HSD tests, the mean µg/g values can be found in Supplemental Data Table S2. In general terms, the PBETs with a more acidic stomach or gizzard (mammal pH 1.3 and Bird Si), regardless of species, recovered more fluoxetine per g of solid material added in the stomach or gizzard than the less acidic models (mammal pH 2.5, 4 and Bird Ca). The

reverse was true for the difference between the total bioaccessible fluoxetine recovered at the end of the intestine and that which had already entered solution in the stomach or gizzard.

A greater amount of fluoxetine per gram of solid material was recovered in the RSM from both avian models than in any of the mammalian models. It is believed this was due to sorption to the grit in the avian models (absent from mammalian models) (see Supplemental Data Figure S1) which seemed to produce RSM (Bird N= 30, mean ±SE = 0.074 ±0.002; mammal N=18, mean ±SE = 0.043±0.002; independent samples t-test: df =46, t= -10.64, $p<0.001$).

Data set 2, Percentage of fluoxetine recovered: Figure 3b shows the percentage of fluoxetine recovered in the different compartments of the PBETs. Individual ANOVAs found that there were significant differences between all PBET compartments. Importantly, this means looking in percentage terms, there were significant differences in bioaccessibility (df=4, F=36.21, $p<0.001$). Table 2 shows that all significant differences in terms of total bioaccessibility were inter-species rather than intra-species differences (the mean percentages can be found in Supplemental Data Table S2). Percentage bioaccessibility in mammalian models ranged from 88.1-89.6% but for the avian models they were 78.6% (Ca grit) and 75.1% (Si grit) respectively (Figure 3b, Table S2). For the avian models, the percentage of fluoxetine in RSM was 21.6% and 24.9% which was significantly higher than the mammalian models which ranged from 10.4-11.9% (Figure 3b Table S2). In terms of significant differences in the area of the gastrointestinal tract where fluoxetine was recovered, (Table 2), the only models with similarities were Bird Ca with mammalian pH 2.5 (intestine – stomach or gizzard) and 4 (stomach or gizzard) and Bird Si grit with mammalian pH 1.3 (intestine – stomach or gizzard). Therefore in percentage terms, there were inter-species differences for bioaccessibility and RSM and both intra and inter-species differences in terms of whether fluoxetine entered solution in the stomach or gizzard or intestine.

How could PBETs be used to support read-across for ERA in wildlife

The bioaccessibility of fluoxetine in the avian system was actually very similar to the bioaccessibility in the human system. The statistically significant lower bioaccessibility in the avian models (9.6 to

13.7%) is unlikely to be of significance for ecological risk assessment. When the data were normalised based on the amount of solid material added, there were no significant inter-species differences, in fact the avian models actually extracted 25.6 to 58.7 µg/g of solid material more than the mammalian PBETs. Our data highlight the challenges of conducting in vitro assessments of bioaccessibility when trying to introduce food items that have accumulated contaminants via an environmentally relevant exposure route.

Regardless of whether the data were examined in percentage terms or relative to the amount of solid material present, we found significant differences in the area of the gastrointestinal tract where fluoxetine was extracted from the earthworm. We observed that in the stomach or gizzard, the lowest pH models (human pH 1.3 and Bird Si grit) extracted both more fluoxetine/g-solid material and a greater percentage of that recovered. These acidic models were observed to cause the worm tissues to break up within 30 to 60 min. Worms in the less acidic models remained intact until the intestinal phase. This finding follows what is expected based on the optimum pH for pepsin (pH 2), the main enzyme in the stomach and gizzard [39].

It is possible that the region of the gastrointestinal tract where fluoxetine becomes bioaccessible would be significant for risk assessment. Generally, the majority of pharmaceuticals are absorbed from the intestine due to the presence of microvilli, with uptake from the stomach possible but less efficient [40]. In some mammals, fluoxetine is reasonably well absorbed from the gastrointestinal tract with 72% bioavailable in dogs but not in others with only 38% bioavailable in rodents [41]. Dogs excrete 10.9% of a fluoxetine dose as fluoxetine, no data could be found for small mammals [42] our mean percentage in vitro for mammalian RSM was 11.4%±0.45% which is interestingly close to that excreted by dogs even if RSM is not exactly equivalent to faeces in vitro [40, 41].

Very little is known about the bioavailability and excretion of pharmaceuticals in the avian system. There are a large number of in vitro gastro-intestinal tract simulations available to researchers, the majority of which have been developed for inorganics based on a human/ child model to simulate exposure via ingestion of lead from soil [18]. Relatively few models that have been developed for

organics (e.g. the FOREhST model used for PAHs [30]) or wildlife, and none that have been developed specifically for the assessment of organics in wildlife. We would encourage future researchers to integrate what is known about the gastrointestinal tract physiologies of wildlife species from the PBETs used here [13, 23, 24] with what is known about in vitro bioaccessibility of organics from the FOREhST model [30].

In terms of the suitability of the avian PBET used here (designed for uptake of lead by waterfowl), [13], we found a good match between the percentage recovered in the RSM in vitro and excretion by European starlings from a recent in vivo study (Bean et al. submitted). Starlings fed fluoxetine spiked invertebrates excreted 19.3%±15.9% of the dose as fluoxetine (n = 3) (methods in Supplemental Data). In the avian Ca grit PBET, 21.4% ± 0.9% of the fluoxetine inserted to the PBET was recovered in the RSM (the diet of the starlings contained a calcium supplement). The good match between avian in vitro and in vivo data was surprising but encouraging given the considerable number of parameters involved in designing and validating a species and contaminant species in vitro model. For example, there is a difference of approximately 5 h in gastrointestinal transit time between waterfowl and small passerines such as starlings [13, 43]. Although the mammalian and avian in vitro RSM and in vivo excretion data in vitro seem to match well, plasma and tissue residues of fluoxetine in starlings in vivo were found to be up to 2 orders of magnitude lower than would be predicted from human kinetic data (Bean et al. submitted). To properly validate the PBETs, samples of digestive juice from the bird would have to be collected after the drug has been administered. Not only would digestive juice be challenging to sample, there is limited information on how much digestive juice is secreted by different species (and the timing of secretions). Collecting blood plasma is another option for in vivo evaluation of organics, but this relies on 1) the assumption that the bioaccessible contaminant is 100% bioavailable and 2) species specific pharmacokinetic data, e.g. time to peak plasma concentration are similar [44].

Future research needs

Expanding the PBET approach from inorganic to organic chemicals is highly relevant to the needs of ecotoxicology to help quantify differences, both between species and food types, in internal exposure

[32, 45]. This would enable the accuracy of ecological risk assessments could be improved, the scope of inter-species read-across extended, and the number of animals used in routine ecotoxicology studies could be reduced. However, there remain several challenges both in terms of developing and validating PBETs for new species and contaminants. We have identified 5 main challenges to expanding the PBET approach. Firstly, there is a lack of complete data sets on gastrointestinal tract physiology and digestive processes for a range of wildlife species. Secondly, our understanding of how bioaccessibility relates to bioavailability, e.g. approaches for assessing the behaviour of bioaccessible fluoxetine in cell membranes e.g. [46] is limited. Thirdly, it is difficult to obtain licences for in vivo validations and even then, they are difficult to conduct and standardise. Fourthly, the impact of microbes in the gastrointestinal tract is uncertain, but may well be important for organics that are less persistent than fluoxetine [47]. Finally access to existing sensitive data from pre-clinical and clinical drug trials that could be used to evaluate new PBETs is hard to obtain. Nevertheless, in vitro gastrointestinal tract models such as PBETs are both economically feasible and ethically viable compared to the equivalent in vivo assessments. They provide the potential to improve estimates of internal concentrations of pharmaceuticals and other organic compounds when using read-across approaches to predict the sensitivity of non-model species to environmental contaminants.

Expanding the PBET approach in terms of numbers of species and compounds included could help to protect both wildlife, by setting safer threshold levels, and protect laboratory animals (from further tests). We believe the incentives for expanding the PBET approach outweigh the challenges faced. These challenges can be overcome through inter-disciplinary research across areas including, but not limited to, ecotoxicology, physiology and bioaccessibility.

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Table 1: Mass of solid material (g) and amount (µg) of fluoxetine recovered in avian and mammalian PBETs

Table 2: Inter- and Intra-species (B = bird, M =mammal) differences among PBETs and PBET compartments in post-hoc Tukey's tests at or below the 5% level

Figure 1: Schematics of (a) the mammalian *in-vitro* model adapted from a human PBET and (b) the avian PBET. Earthworms which had accumulated fluoxetine from soil (or control earthworms which were exposed to methanol spiked soil), plus any soil which adhered to earthworms, were inserted into the PBETS. Initially earthworms went into the stomach (a) or gizzard (b) along with digestive juice at a low pH. Simulations took place in 50 mL centrifuge tubes. Digestive juice samples were taken and replaced with fresh digestive juice three times for the mammalian simulation (a) and five times for the avian simulation (b) to simulate the dynamic nature of the digestive tract. The intestinal phase was simulated by taking the samples from the stomach or gizzard simulation, raising the pH to 7 (a) and 6.2 (b) and adding bile extract and pancreatin. Digestive juice samples were centrifuged and the residual solid material (RSM) at the bottom of the intestinal sample was also collected for extraction.

Figure 2: Simplified schematic illustrating how the fluoxetine concentrations (µg/mL) in samples of digestive juice and solvent extracted residual solid material (RSM) collected throughout the avian and mammalian PBETs were used to determine the amounts of fluoxetine (µg) recovered in the avian gizzard or mammalian stomach, the intestine and RSM.

Figure 3: Mean ±SE of fluoxetine extracted in the avian gizzard or mammalian stomach (gizz or stom), intestine (int) and Residual solid material (RSM) for a) µg/g of worm + soil added to the PBETs and b) as a percentage of the total mass of fluoxetine recovered at the end of the PBETs.

Table 1: Mass of solid material (g) and amount (µg) of fluoxetine recovered in avian and mammalian PBETs

PBET	Solid material added to PBET[a] (g)		Gizzard or stomach[b] (µg)		Intestine[c] (µg)		Intestine-gizz or stom[d] (µg)		RSM[e] (µg)		Total[f] (µg)	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Bird Ca	0.64	0.04	105.82	15.85	255.70	38.82	132.94	20.96	69.72	11.35	325.43	49.61
Bird Si	0.50	0.04	165.67	3.45	201.28	4.84	22.60	7.81	63.84	3.68	265.12	6.42
Mammal pH 1.3	0.70	0.03	245.07	31.38	246.10	28.86	1.03	6.75	28.75	3.63	274.85	32.45
Mammal pH 2.5	0.72	0.09	159.34	18.95	267.40	24.05	108.07	5.15	36.27	6.48	303.67	29.20
Mammal pH 4	0.61	0.02	71.42	3.42	226.89	11.51	155.47	13.43	30.25	2.87	257.14	10.74

[a] The mass of solid material added included earthworm and an average of 0.155 g of soil which had adhered to the earthworm, the mean±SE concentration of fluoxetine in worm tissue was 537.1 µg/g ±47.3 µg/g, in soil 572.7 µg/g ±9.4 µg/g.

[b] Gizzard for birds, stomach for mammals

[c] Total bioaccessible amount of fluoxetine

[d] The difference between the amount of fluoxetine in the intestine and that which had already been extracted in the gizzard or stomach

[e] The residual solid material (RSM) remaining at the end of the intestinal phase,

[f] The total amount of fluoxetine recovered was given by the sum of fluoxetine in the intestine and RSM.

Table 2: Inter- and Intra-species (B = bird, M =mammal) differences among PBETs and PBET compartments in post-hoc Tukey's tests at or below the 5% level

PBET compartment	**Difference**		**µg/g solid material[a]**	**% of total fluoxetine recovered[b]**
Gizzard or stomach[c]	Inter-species	B&M	1.3>Ca*, Si>4†	1.3>Ca‡, 2.5>Ca‡ 1.3>Si‡, Si>2.5*, Si>4‡
	Intra-species	B&B	Si>Ca*	Si>Ca‡
		M&M	1.3>4†	1.3>2.5‡, 1.3>4‡, 2.5>4‡
Intestine (total bioaccessibility)[c]	Inter-species	B&M	None	1.3> Ca ‡, 2.5>Ca†, 4>Ca† 1.3> Si ‡, 2.5> Si ‡, 4> Si ‡,
	Intra-species	B&B	None	None
		M&M	None	None
Intestinal minus (gizzard or stomach)[c]	Inter-species	B&M	Ca>1.3‡, 2.5>Si* 4>Si ‡	Ca>1.3‡, Ca>4†, 2.5>Si‡ 4>Si‡
	Intra-species	B&B	Ca>Si†	Ca>Si‡
		M&M	1.3>2.5, 1.3>4	2.5>1.3‡ 4>1.3‡, 4>2.5‡
RSM[c]	Inter-species	B&M	Ca>1.3†, Ca>2.5* and Ca>4*; Si >1.3†, Si>2.5† and Si>4†	Ca> 1.3 ‡, Ca>2.5†, Ca>4†; Si> 1.3 ‡, Si> 2.5‡, Si>4‡
	Intra-species	B&B	None	None
		M&M	None	None
Total[c]	Inter-species	B&M	None	NA
	Intra-species	B&B	None	NA
		M&M	None	NA

Data were normalised in 2 ways:

[a]µg of fluoxetine recovered relative to the mass (g) of solid material inserted to the PBET as worm and soil

[b] as a percentage of the total amount of fluoxetine recovered (intestine + RSM)

PBETs:Ca= Bird Ca grit, Si = Bird Si grit, 1.3 = Mammal pH 1.3, 2.5 = Mammal pH 2.5 and 4 = Mammal pH 4

[c]PBET compartments: Gizzard or stomach, Intestine (total bioaccessible fluoxetine), Intestine minus gizzard or stomach, Residual solid material (RSM) and Total (Intestine + RSM)

P<0.05 *,P<0.01 †, P<0.001‡, NA = Not applicable, None = No significant differences

A)

B)

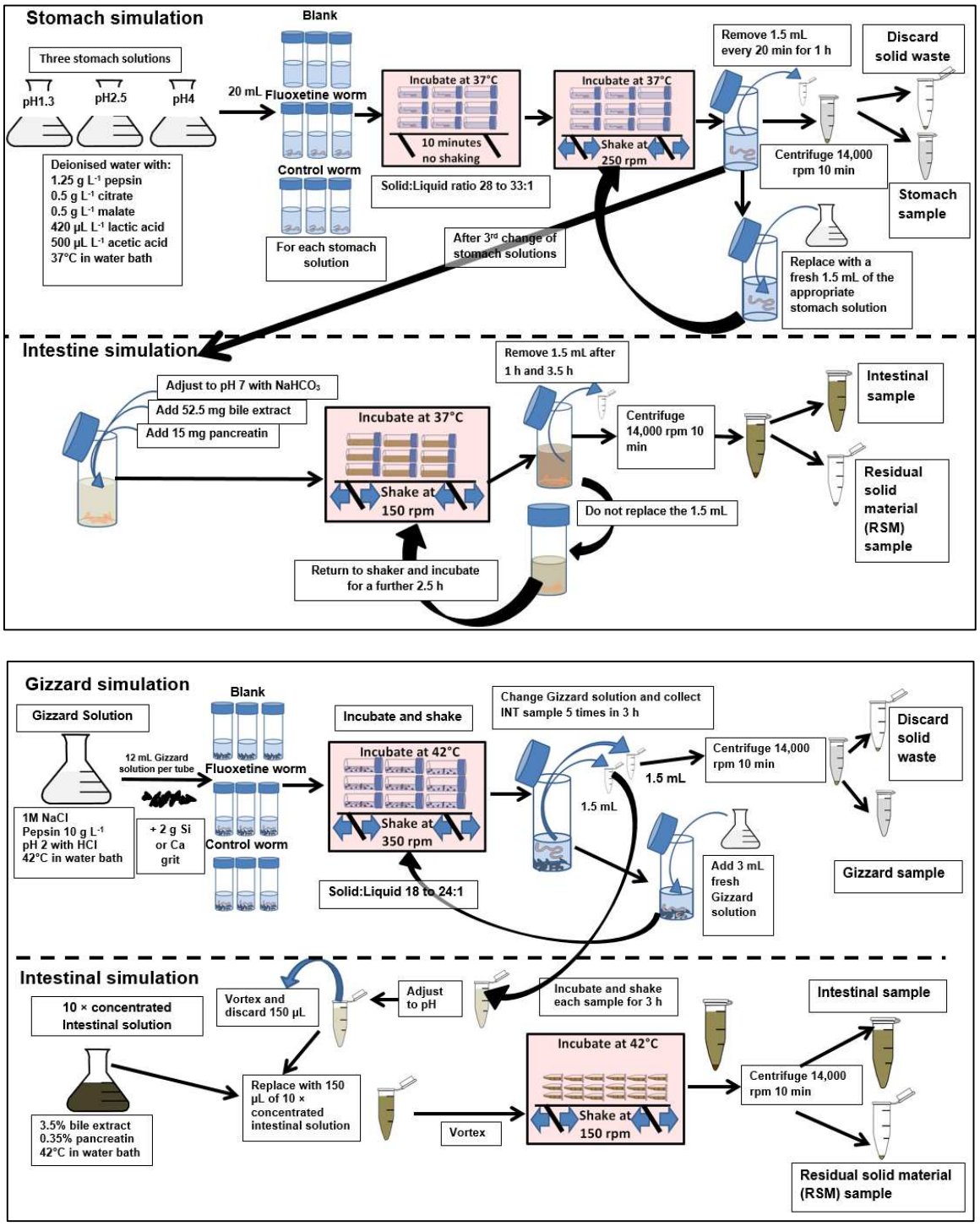


Figure 1: Schematics of (a) the mammalian in vitro model adapted from a human PBET and (b) the avian PBET. Earthworms which had accumulated fluoxetine from soil (or control earthworms which were exposed to methanol spiked soil), plus any soil which adhered to earthworms, were inserted into the PBETS. Initially earthworms went into the stomach (a) or gizzard (b) along with digestive juice at a low pH. Simulations took place in 50 mL centrifuge tubes. Digestive juice samples were taken and replaced with fresh digestive juice three times for the mammalian simulation (a) and five times for the avian simulation (b) to simulate the dynamic nature of the digestive tract. The intestinal phase was simulated by taking the samples from the stomach or gizzard simulation, raising the pH to 7 (a) and 6.2 (b) and adding bile extract and pancreatin. Digestive juice samples were centrifuged and the residual solid material (RSM) at the bottom of the intestinal sample was also collected for extraction.

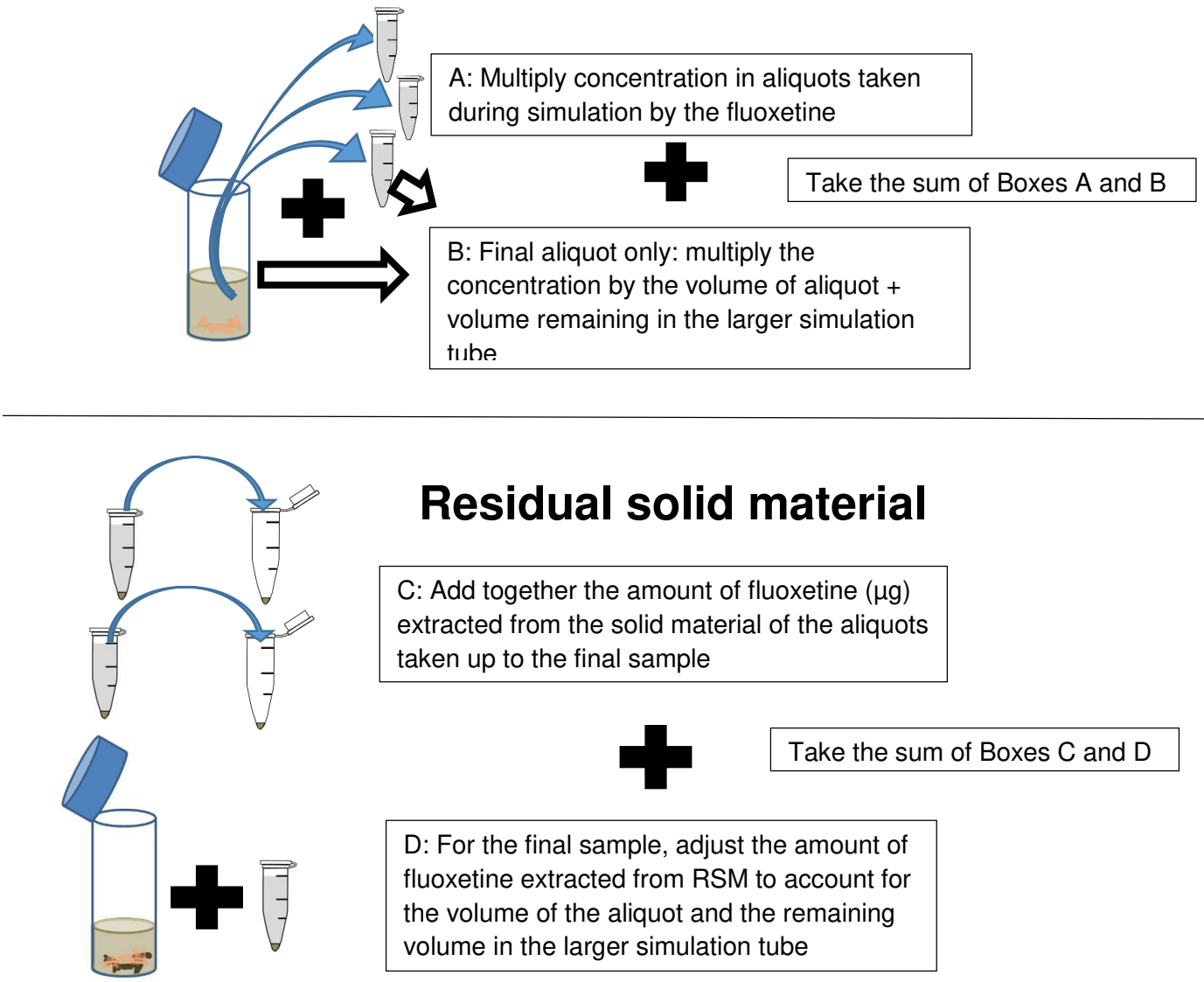
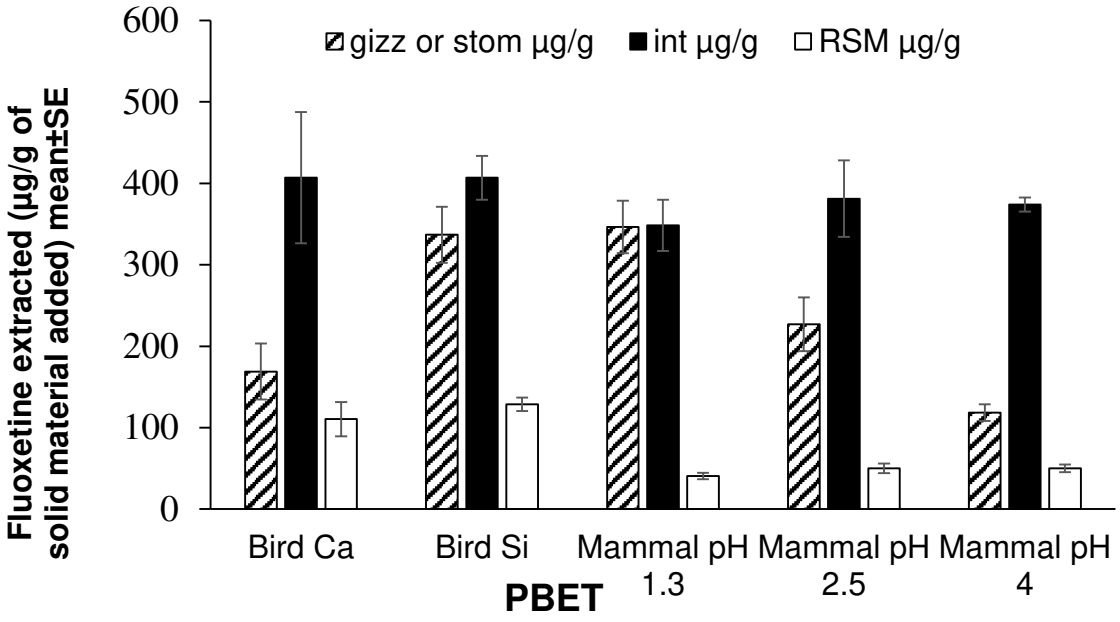


Figure 2: Simplified schematic illustrating how the fluoxetine concentrations (µg/mL) in samples of digestive juice and solvent extracted residual solid material (RSM) collected throughout the avian and mammalian PBETs were used to determine the amounts of fluoxetine (µg) recovered in the avian gizzard or mammalian stomach, the intestine and RSM.

A)



B)

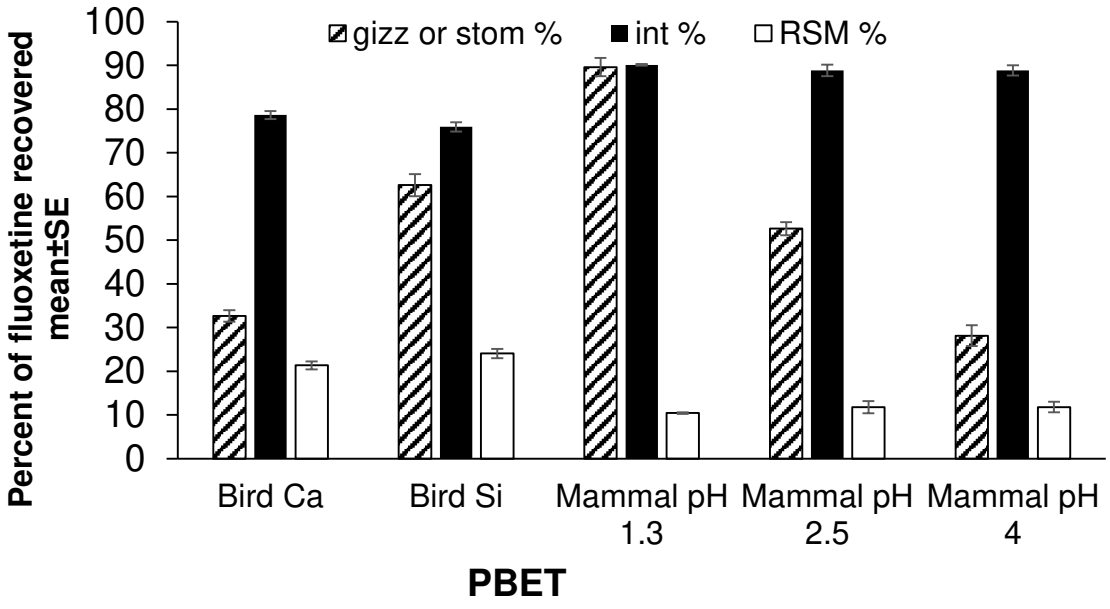


Figure 3: Mean ±SE of fluoxetine extracted in the avian gizzard or mammalian stomach (gizz or stom), intestine (int) and Residual solid material (RSM) for a) µg/g of worm + soil added to the PBETs and b) as a percentage of the total mass of fluoxetine recovered at the end of the PBETs.

SUPPLEMENTAL DATA

These supplemental data provide additional information on the exposures of earthworms to fluoxetine via soil, the experimental structure, QA/QC and the methods of the mammalian and avian PBETs.

1 Preparation of food for PBETs: Earthworm exposures

The uptake of fluoxetine into earthworms followed OECD Guideline 317 "Bioaccumulation in Terrestrial Oligochaetes" [1].

1.1 Determination of Soil moisture content

Twenty grams of soil was weighed out and placed in the oven at 105°C for 24 h. The percentage moisture content was determined from the mean loss in mass from four replicates.

Equation 1

$$MC = 100 / (Dry - Cont)*(Wet-dry)$$

Where: ***MC***= moisture content of matrix sub sample (%)

Dry= The mass of container and dry matrix sub sample (g)

Wet= The mass of container and wet matrix sub sample (g)

Cont= mass of empty container (g)

1.2 Determination of Soil Maximum Water Holding Capacity

A square of muslin was fixed to the bottom of each of five separate plastic rings (volume = 40.707 cm^3) with elastic bands. The weight of rings, muslin and elastic band were recorded for each of the 5 replicates. Soil was then packed into each of the 5 rings, tapping the rings carefully during the process to ensure that the packing density fell in the range of 0.76-1 g cm^{-3} (Equations 2 and 3).

Equation 2

$$Dsoil=[(RS-R)/(100+MC)]*100$$

Where: ***Dsoil***= Dry mass of soil in ring (g)

RS= mass of ring and moist soil (g)

R= mass of empty ring (g)

Equation 3 ***Den = Dsoil /RingVol***

Where: ***Den***= Packing density of soil in the ring (g cm^{-3})

RingVol=Volume of ring (cm^3). The ring used had external diameter of 5 cm and height of 2.3 cm giving a volume of 40.707 cm^3.

The rings containing the soil were weighed and if the packing density (Equation 3) fell in the range 0.76-1 g cm^{-3} then it was acceptable, if the packing density was outside these limits then it was necessary to re-pack and re-weigh until the packing density fell within the acceptable range. The soil rings were then placed into a glass tray which was then slowly filled to a depth of approximately 3 mm with deionised water. The soil rings were left for three hours to saturate with the water level checked every hour and topped up as necessary.

Prior to preparation of the rings, a 4 cm deep quartz sand bed with average grain size 0.60 mm was saturated in a bath of deionised water filled to just below the top of the surface of the sand for one hour. The sand bed was prepared in a plant growth tray with a sheet of garden fleece in the bottom and holes in the bottom of the tray that are partially closed with tape so that water can drain but the sand will not fall out. After one hour in the water bath, the sand bed was removed from the water and left to drain in a second tray for 2 h.

After three hours saturation time, the soil rings were placed muslin side down on the sand bath for 24 h to drain. After which the soil rings were removed and the soil was scraped out from the ring into individual pre-weighed foil dishes and re-weighed. The foil dishes containing saturated soil were oven dried at 105°C for 24 h and reweighed. The maximum water holding capacity is the moisture content of the saturated soil (Equation 3).

1.3 Determination of mass of moist soil for a certain dry mass content

We needed 50 g of dry soil for the earthworm exposures and this was maintained at 60% of MWHC throughout the earthworm exposures.

Firstly, the mass of water in 50 g of moist soil was determined using Equation 4.

Equation 4

Water50*= (50*MC*)/(100+*MC*)**

Where: ***Water50*=** Mass of water in 50 g of moist soil (g)

***MC*=** Moisture content (dry weight basis) of 50 g of soil (%)

Secondly, we determined the mass of water required in order for the soil to be at MWHC using Equation 5.

Equation 5

WCR*= [(50-*Water50*)/100]*MWHC***

Where: ***WCR*=** Mass of water required for moist soil to be at MWHC (g)

***MWHC*=** Maximum water holding capacity of soil (%)

Finally, the mass of water to be added to soil to make up to 60% of MWHC was calculated using Equation 6.

Equation 6

RW*= [(WCR/100)*60]-*Water50

Where: ***RW*=** Mass of Water to be added to 50 g of moist soil to make it up to 60% of MWHC (g)

For the soil used here, 4.75 g of water was added to the 50 g of moist soil to make it up to 60% of MWHC. Earthworms were then exposed for 21 d, maintaining the moisture content at 60% MWHC each day by adding deionised water, full details of the 21 d exposure are given in the main paper 'Preparation of earthworms'.

2 Structure of the experiment

The earthworm exposures were staggered so that one set of triplicates (maximum number of replicates that could be dealt with by a single worker) plus an additional replicate to track pH change were entered into the PBET on any one day; therefore the PBET was completed over six days with blanks (no earthworm), controls and fluoxetine earthworms for 2 grit types. For the mammalian PBET, the experiment was structured so that all simulations for blanks, control and fluoxetine earthworms took place on separate days to avoid contamination from the pH probe.

3 Quality Assurance / Quality Control

3.1 Limits of quantification (LOQ) detection of analytical methods

The LOQof the high performance liquid chromatography method (µg/mL) and the percentage recovery of the methanol extraction used to extract fluoxetine from the digestive juice samples are given in Table S1. The LOQ was determined from a pilot study where sample obtained from PBETs were spiked with fluoxetine at 1%, 10%, 50%, 100% and 200% of the levels we were expecting to see in the PBETs. The lowest standard detected with recovery >85% (relative to solvent standard) and %RSD <10% between triplicates was selected as LOQ.

Table S1: Limits of Quantification (LOQ) of the High Performance Liquid Chromatography (HPLC) method (µg/mL), mean percentage recoveries and percentage Relative Standard Deviations using methanol to extract fluoxetine spiked into blank digestive juice and faecal samples.

	Avian						Mammalian								
	Si grit			**Ca Grit**			**pH 1.3**			**pH 2.5**			**pH 4**		
	LOQ	% rec.	% RSD	LOQ	% rec.	% RSD	LOQ	% rec.	%RSD	LOQ	% rec.	%RSD	LOQ	% rec.	%RSD
Gizzard/ Stomach	0.76	94.3	7.7	4.8	105.9	7.5	0.55	121.9	3.5	0.58	114.2	2.5	0.14	117.7	9.5
Intestine	0.48	112.3	7.0	0.57	108.1	2.8	0.045	116.1	6.0	0.35	113.2	3.4	0.095	118.2	3.1
Faeces	0.75	92.6	1.7	1.42	85.9	2.4	1.5	106.0	2.3	2.1	91.5	2.8	5.1	91.9	1.9

3.2 Extraction of fluoxetine from earthworms as a QA/QC

To determine the amount of fluoxetine inserted to each PBET replicate, we used the mean concentration per gram of earthworm as determined from the QA/QC and corrected for the mass of the earthworm. The four replicates of fluoxetine and control worms that were exposed under the same conditions as the earthworm samples were rinsed of soil with deionised water. Earthworms were then dabbed dry on paper towels. The weight was recorded (± 0.1g) and earthworms were extracted by homogenisation (Turrax) with 6 mL of methanol. Extracts were ultrasonicated in an ultrasonication water bath for 10 minutes, centrifuged for 10 minutes at 4500 × g before analysing by HPLC. The mean concentration in earthworm tissue was 537.1 µg/g (RSD = 17.6 %).

3.3 Extraction efficiency of fluoxetine from earthworms

To validate the earthworm extraction, we extracted earthworms spiked with 0, 0.6, 6, 30, 60, 120 µg of fluoxetine. Earthworms were left for 2 h to absorb the spiked fluoxetine prior to extraction. Earthworms were homogenised (Turrax, UK) in 6 mL methanol. Samples were ultrasonicated for 10 min, centrifuged for 10 min (4500 × g, 20°C) before passing through a 0.2 µm PTFE syringe filter into an amber glass vial with a crimp cap. Analysis was performed by HPLC with fluorescence (Recovery = 75.3%, LOQ = 0.6 µg/mL, RSD between triplicates = 10.0%).

3.4 Soil extractions

To determine the amount of fluoxetine contained in soil that adhered to earthworms, we determined the concentration in the soil that earthworms were exposed in. Five grams of soil (at 60% maximum water holding capacity) was taken from each jar of the QA/QC earthworms and placed into a 50 mL centrifuge tube. Soil was extracted twice using 10 mL of 70:30 acetonitrile:water (HPLC fluorescence grade). Samples were shaken on their side at 420 rpm, they were then ultrasonicated for 10 min before centrifuging for 10 min (4500 × g, 20°C). The supernatant was decanted into a second 50 mL centrifuge tube before adding a fresh 10 mL of the 70:30 acetonitrile:water mixture and repeating the extraction process. After centrifuging for the second time, the two 10 mL aliquots were combined in the second falcon tube, vortex mixed before taking an aliquot and passing through a 0.2 µm PTFE filter into a vial and analysing by HPLC (See main paper). The mean fluoxetine concentration in soil was 572.7 µg/g (RSD = 3.3 %). The mean mass of adhered soil was 0.155 g, i.e. this soil contained on average 0.88 µg of fluoxetine.

3.5 Extraction efficiency of fluoxetine from soil

We validated the extraction method for soil by spiking at 0, 1, 10, 50, 100 and 200% of the expected concentration. Five grams of soil was extracted on a wet weight basis. Mean percentage recovery was 77.2% (RSD = 5.2%).

3.6. Validation of chemical analyses

To validate the extraction procedure from digestive juice samples and faeces for each of the five PBETs, blank digestive juice and faecal samples were generated by running the PBET simulations without the addition of an earthworm. The samples collected were spiked with 0, 1, 10, 50, 100 and 200% of the expected fluoxetine concentration (based on a pilot study) using stock solutions of fluoxetine in methanol. To extract fluoxetine from samples, 500 µL aliquot was taken, spiked with 100 µL of the stock and extracted with 400 µL methanol. Faecal samples were spiked with 100 µL methanol, vortex mixed before adding 900 µL methanol. Samples were then vortex mixed for 5 secs, centrifuged for 10 minutes at 11,000 × g before passing through a 0.2 µm PTFE filter and analysing by HPLC (see Chemical analyses). The percentage recoveries of the methanol extraction are presented in Table S1 along with the limits of quantification. The limits of detection were below the levels detected in all of the real samples. PBET

3.7 Fluoxetine inserted into PBETs

To maintain environmental relevance, the earthworms inserted to PBETs accumulated fluoxetine from soil, rather than being spiked with a known amount. As a result, the exact concentration of fluoxetine in each earthworm and adhered soil was unknown and so we normalised data in terms of mass of solid material added (worm + adhered soil). Nevertheless, as a quality assurance/control, we exposed eight additional earthworms for 21-days, four fluoxetine earthworms and four control earthworms. Exposure conditions exactly replicated that of the earthworms that were inserted to the PBET. Prior to extraction, earthworms were weighed, rinsed of soil with deionised water using a pipette and paper towels and then re-weighed. The weight difference before and after rinsing was used to quantify the mass of soil adhered to the earthworms. We extracted the earthworms using the same method as above and analysed the extract using HPLC to quantify the mass of fluoxetine per gram of earthworm on a wet weight basis. The concentration in the earthworm extract was corrected for a 75.3% recovery (as found in the earthworm validation) and the mean concentration was found to be 537.1 µg/g, RSD = 17.6%. No fluoxetine was detected in the control earthworms.

Additional information on the mammalian PBET

The mammalian PBETs were adapted from the human PBETs of Li and Zhang [2] and Ruby et al. [3]. Mammalian PBETs were run using digestive juice at three different timings since the consumption of food. In conical flasks, gastric digestive juice was prepared by adding pepsin (1.25 g/L), malate, citrate (both 0.5 g/L), lactic acid (420 µL/L) and acetic acid (500 µL/L) to deionised water. Gastric juice was adjusted to the desired pH with concentrated HCl (pH 1.3 was used to represent fasted conditions, pH 2.5 represented average gastric conditions and pH 4 recently fed conditions [3]) and then warmed to 37°C in a water bath.

To begin the PBET simulation, earthworms and any adhered soil were added to tubes along with 20 mL of gastric digestive juice. The head space was purged with N_2 to create a low oxygen environment. Tubes were placed in the shaking incubator at 37°C but were not shaken for 10 minutes as in [3], after which tubes were shaken at 250 rpm for one hour in total. At three time points (every 20 minutes) during this hour, shaking was stopped and a 1.5 mL aliquot of gastric juice was taken from each tube and, and replaced with a fresh 1.5 mL of gastric solution. The gastric juice samples were transferred to a 1.5 mL sealed microcentrifuge tube and immediately centrifuged (10 min at 11,000 × g). The supernatant decanted into a fresh tube and stored at -20°C.

Once the intestinal simulation was complete, the digestive juice in each simulation tube was adjusted to pH 7 with $NaHCO_3$ powder (intestinal pH) [4]. Once pH had been adjusted, 52.5 mg of bile extract and 15 mg of pancreatin were added to each replicate [2] before returning to the shaker. Tubes were shaken on their sides at 100 rpm to mimic the slow intestinal passage. A 1.5 mL aliquot was taken from each sample and not replaced after 1 hour intestinal incubation with a final aliquot of 1.5 mL taken after a total of 3.5 hours of the intestinal incubation. The intestinal samples were centrifuged and stored as the gastric phase samples, the only exception being that the remaining solid material was considered to be faeces.

5. Additional information on the avian PBET

The avian gizzard digestive juice was prepared in a 1 N NaCl solution, as in [5], and contained either 2 g of siliceous (Si grit) or 2 g of calcareous grit (Ca grit) (obtained from a local pet shop), as in [5], to account for the distinct geo-chemistries and the influence on gastrointestinal tract pH experienced by wild birds [6]. The intestinal digestive juice was made up in deionised water containing bile extract (3.5%) and pancreatin (0.35%), as in [5]. To begin the simulation, 12 mL of gizzard digestive juice [5] was added to a 50 mL centrifuge tube containing grit and an earthworm. Tubes were placed in the shaking incubator on their sides for a total of three hours (350 rpm, 42°C) following a PBET validated for waterfowl [5]. Gizzard digestive juice changes took place as in the mammalian PBETs, but at 36 minute intervals to give a total of five changes in the three hour gizzard simulation. At each change of digestive juice two 1.5 mL aliquots were removed and replaced with 3 mL of fresh digestive juice. The first aliquot (the gizzard sample) was centrifuged; the supernatant was transferred to a fresh tube and stored at -20°C.

The second aliquot (which became the intestinal simulation) went into a separate 1.5 mL centrifuge tube. The pH was adjusted to 6.2 using a $NaHCO_3$ solution (9 g/100 mL deionised water) as outlined in [5], however we encountered a number of issues with their approach. Firstly, when adjusting the pH of the gizzard digestive juice samples to intestinal pH (6.2), the initial pH of the siliceous grit samples was approximately pH 3.6 for the first gizzard digestive juice change, and had fallen to pH 2.8 by the fifth change, meaning that a stronger base was required to raise the pH to 6.2. We added 10 µL of a 5%

NaOH solution to each, and made the remaining adjustments to pH with the $NaHCO_3$ solution (approximately 15-25 µL). For the Calcareous grit, the initial pH was actually above 6.2 (approximately 6.7); we still added 10 µL of $NaHCO_3$ and made the residual change with 0.2 M HCl. An issue we experienced was that the method of Martinez-Haro et al. [5] used triplicates for each grit simulation and a fourth replicate was used to monitor pH change (to speed up the procedure). Martinez-Haro et al. [5] noted down the amount of $NaHCO_3$ added to the fourth replicate and then added the same amount to the other three samples so that pH only had to be measured once for each digestive juice change time point (instead of three times). However when we was validating the methods, we found that using the approach of Martnez-Haro [5] to speed up digestive juice changes led to variable pHs in the triplicates, which we suggest could be due to differences in earthworm weight added to each PBET. Subsequently, the pH of each replicate was changed individually.

Once the pH had been adjusted to 6.2, one 10^{th} of the volume in the tube was removed and replaced by the 10 × concentrated intestine solution. The intestinal simulations were shaken on their side (150 rpm, 42°C), as an end over end shaker as used in Martinez-Haro et al. [5] was not available to us. After three hours of the intestinal simulation, samples were removed from the shaking incubator and processed as in the mammalian PBETs.

6 Differences between PBETs

Table S2: Mean and SE of fluoxetine extracted in stomach or gizzard, intestine, from RSM and total in terms µg/g of solid material added and percentage of the total (Intestinal µg + RSM µg). The mass of fluoxetine added to each PBET and recovered at the various phases are given in Table 1 in the main paper.

PBET	Data set	Stomach or gizzard		Intestinal		RSM		Total	
		mean	SE	mean	SE	mean	SE	mean	SE
Bird Ca	µg/g	168.8	34.4	407.1	80.7	110.5	21.0	517.6	101.0
Bird Si	µg/g	336.9	34.5	406.8	27.0	128.7	8.4	535.6	32.9
Mammal pH 1.3	µg/g	346.3	32.3	348.4	31.4	40.6	3.8	389.0	35.1
Mammal pH 2.5	µg/g	226.9	32.9	381.2	47.0	50.2	5.9	431.4	50.2
Mammal pH 4	µg/g	118.5	10.3	373.8	8.6	50.0	4.8	423.8	3.9
Bird Ca	%	32.7	1.3	78.6	0.9	21.4	0.9	100.0	0.0
Bird Si	%	62.6	2.6	75.9	1.1	24.1	1.1	100.0	0.0
Mammal pH 1.3	%	89.6	2.1	89.6	0.2	10.4	0.2	100.0	0.0
Mammal pH 2.5	%	52.6	1.5	88.2	1.3	11.8	1.4	100.0	0.0
Mammal pH 4	%	28.2	2.4	88.2	1.2	11.8	1.2	100.0	0.0

7 Sorption of fluoxetine to grit

For each grit type (Ca and Si), we weighed out 2 g of pre-rinsed (deionised water) and dried grit into six sets of triplicate 50 mL centrifuge tubes (50 mL, BD UK). For each set of triplicates, 1 mL of fluoxetine solution (1:7 methanol:water) was added along with 11 mL of deionised water so that total volumes were 12 mL as they were for the PBET. Samples were shaken at 200 rpm on their side for 48 hours, after which they were centrifuged for 10 minutes (4500 × g, 20°C). An aliquot from each sample was filtered (0.2 µm PTFE) and analysed by HPLC.

We found sorption of fluoxetine to be particularly high with approximately 85% of fluoxetine adsorbing to Ca grit and 45% to Si grit at the concentration used in the experiment (Figure S1). Therefore sorption could potentially explain why a greater percentage of fluoxetine was recovered in the faeces for birds than mammalians.

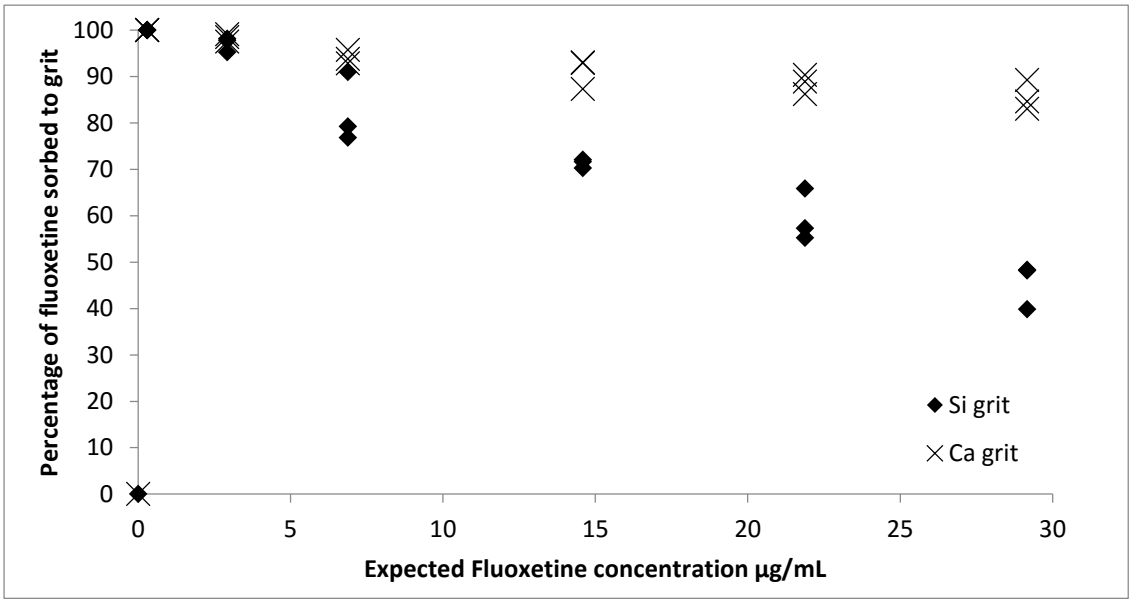


Figure S1: Percentage of fluoxetine adsorbed to 2 g of Si grit (diamonds) and Ca grit (Crosses) between 0 and 30 µg/mL fluoxetine after 48 hours shaking at 250 rpm.

8 In vivo evaluation: Excretion of fluoxetine by starlings

The avian PBET we used was designed and validated for waterfowl [5]. Here, the PBET was evaluated for passerines, specifically the European starling; a species that forages on invertebrates at wastewater treatment works and on fields fertilised with sewage sludge throughout the year [7-11]. Wild caught starlings were dosed in captivity [12] with lesser wax worms spiked with fluoxetine (1300 ng/worm 5-

d/wk, i.e. average dose of 928 ng/day) or carrier medium (n = 12 per treatment group). After 22 weeks of dosing, three birds from each treatment group were placed in an individual test cage for 2 hours after the final dose. Immediately prior to euthanasia, a sample of faeces was collected and placed in a 1.5 mL microcentrifuge tube using a swab and placed on dry ice while post-mortem dissections took place. Approximately three hours later, samples were placed in the oven at 40°C until dry. Drying faecal samples were weighed daily until there was no further change in mass. The mean dry weight of faeces was (0.169 g ± 0.022). Dried samples were then ground and stored in small glass vials at -20°C until analysis.

To extract fluoxetine from dried faeces, each sample was first spiked with 100 µL of 25 µg/mL of internal standard (fluoxetine-d5 in methanol, >99.4% purity purchased from TLC Pharmachem, Vaughan, Canada) before adding 1 mL of methanol. Faeces and solvent were shaken on their sides at 420 rpm for 30 min, ultrasonicated for 5 minutes before centrifuging for 10 min in a microcentrifuge at 11,000 × g. The supernatant was transferred to a glass tube containing 100 µL of 9:1 Methanol:ethylacetate to prevent analytes from sticking to the glass during the concentration phase. Glass tubes were transferred to a Turbovap set at 45°C and blown until dry under a steady flow of nitrogen (typically 5-10 psi).

Samples were reconstituted first with 0.5 mL of methanol and vortex mixed for 10 seconds before adding 0.5 mL of deionised water and vortex mixing again. Samples were passed through a PTFE filter into a clear glass total recovery vial. Vials were sealed using a crimp cap containing a PTFE/Silica septa. Samples were analysed following the method outlined in [12].

The percentage of the dose excreted unchanged was determined using LC-MS/MS. The percentage of the dose excreted unchanged was compared between in vivo and in vitro data to provide an indication as to how well the avian PBET predicted the percentage excretion of fluoxetine by passerines. We compared the in vivo data with the Ca grit model as the in vivo starling diet included a calcium supplement.

8.1 Ethics statement

In vivo evaluations were carried out under Home Office Licence (PPL 60/4213) and was approved by the ethics committees of FERA and the University of York. Birds were captured under licences from the British Trust for Ornithology and Natural England

8.2 LC-MS/MS

The concentrations of fluoxetine in faecal samples were determined using liquid chromatography coupled to a triple quadrupole mass spectrometer. Liquid Chromatography was performed using a Dionex Acclaim® RSLC C18 Polar Advantage II column (2.2 µm 120A 2.1 × 100 mm). The triple

quadrupole mass spectrometer was an Applied Biosystems/MDS Sciex API 3000 triple quadrupole in positive ion mode for LC-MS/MS analyses. Multiple reaction monitoring (MRM) transitions using the Applied Biosystems/MDS Sciex API 3000 triple quadrupole in positive ion mode were Fluoxetine: 310.2>147.9 (time = 200 msec); CV/CE = 13 and Fluoxetine-d5: 315.2>153.2 (time = 100 msec), CV/CE = 13.

For the Liquid Chromatography a Dionex Acclaim® RSLC C18 Polar Advantage II column (2.2 µm 120A 2.1 × 100 mm) was used. A ramp gradient method was used consisting of A: H2O 0.1% formic acid, B:Acetonitrile 0.1% formic acid was used at a flow rate of 200 µL/min with a total run time of 9 min. The gradient was as follows 1 min 15% B, 1.5 min, 40% B, 5.5 min 45% B, 5.6 min 95% B, 7 min 95% B, 7.2 mins 15% B, 9 mins 15% B. Retention times were 5.5 min for both analytes and internal standard. The LOD was 1.78 ng/g of dry faeces, the LOQ was 5.92 ng/g of dry faeces and the percentage recovery was 135.7±3.2%.

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