Environment International 126 (2019) 193–201

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Detecting fluoxetine and norfluoxetine in wild bird tissues and feathers

Sophia E. Whitlock[a,*], M. Glória Pereira[b], Julie Lane[c], Darren Sleep[b], Richard F. Shore[b], Kathryn E. Arnold[a]

[a] Environment Department, University of York, Heslington, York YO10 5NG, UK
[b] Centre for Ecology & Hydrology, Lancaster Environment Centre, Bailrigg, Lancaster LA1 4AP, UK
[c] Animal and Plant Health Agency, National Agri-Food Innovation Campus, Sand Hutton YO41 1LZ, UK

ARTICLE INFO

Handling Editor: Olga-Ioanna Kalantzi

Keywords:
Pharmaceuticals
Fluoxetine
Biomonitoring
Wild birds
Bioaccumulation
Feathers

ABSTRACT

The contamination of the environment with human pharmaceuticals is widespread and demand for such products is mounting globally. Wild vertebrates may be at particular risk from any effects from pharmaceuticals, because of the evolutionary conservation of drug targets. However, exposure of wildlife to pharmaceuticals is poorly characterised, partly due to challenges associated with detecting rapidly metabolised compounds. As part of a wider study on the behavioural effects of fluoxetine (Prozac) on Eurasian starlings (*Sturnus vulgaris*), we investigated which avian samples are best suited for detecting exposure to fluoxetine in free-living birds. We analysed plasma, various tissues and tail feathers (grown both in the wild and in captivity during the dosing period) from fluoxetine-treated birds (dosed daily with 0.035 mg kg^{-1} bodyweight for 28 weeks), and liver tissue and tail feathers from sham-dosed birds. We detected fluoxetine in only two of twelve plasma samples from dosed birds. In dosed birds, median concentrations of free fluoxetine/norfluoxetine in tissues (two hour post-final dose) were: 111.2/67.6 ng g^{-1} in liver, 29.6/5.7 ng g^{-1} in kidney, 14.2/4.0 ng g^{-1} in lung, 15.1/1.6 ng g^{-1} in brain. We estimated that fluoxetine would remain detectable in liver and kidney approximately 4.5 times longer (90 h) than in brain (20h). In dosed birds, fluoxetine was detected in feathers regrown during the dosing period (median concentration = 11.4 ng g^{-1}) at concentrations significantly higher than in regrown feathers from control birds. Fluoxetine residues were detected in wild-grown feathers (grown before the birds were brought into captivity) at concentrations up to 27.0 ng g^{-1}, providing some evidence of likely exposure in the wild. Our results show liver and kidney can be used for detecting fluoxetine in avian carcasses and provide a first indication that feathers may be useful for assessing exposure to fluoxetine, and possibly other pharmaceuticals.

1. Introduction

Over the past decades, the widespread occurrence of human pharmaceuticals in the environment has increasingly come to light (Daughton and Ternes, 1999). The amounts discharged to the environment are likely to increase further over the coming years (QuintilesIMS Institute, 2016). Human pharmaceuticals have been detected in both aquatic and terrestrial ecosystems and biota (Hughes et al., 2013; Monteiro and Boxall, 2010) and, although concentrations are typically low relative to therapeutic doses, they may yet pose a threat to biota as they are designed to be biologically active at low concentrations (Boxall, 2004). Free-living vertebrates are predicted to be particularly at risk from human pharmaceutical exposure due to the degree of orthology between humans and other vertebrates (Gunnarsson et al., 2008). However, there is relatively little understanding of the extent to which higher vertebrates, such as birds, are exposed to human pharmaceuticals. Potential exposure routes include ingestion of contaminated invertebrates at wastewater treatment plants, consumption of contaminated fish and scavenging on contaminated carcasses (Shore et al., 2014). The concentrations of pharmaceuticals associated with these exposure routes can be sufficient to exert sub-lethal effects on avian fitness (Bean et al., 2014; Whitlock et al., 2018) or, under rare circumstances, can cause mortality (Oaks et al., 2004).

The selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac), prescribed as an antidepressant in people, has received considerable attention regarding its effects on wildlife. Its active demethylated metabolite, norfluoxetine, is also a SSRI and similarly considered to pose an environmental risk, albeit to a lesser extent (Kumar and Xagoraraki, 2010). Fluoxetine and norfluoxetine have both

* Corresponding author at: Department of Environment and Geography, University of York, Heslington, York YO10 5NG, UK.
E-mail address: sew553@york.ac.uk (S.E. Whitlock).

https://doi.org/10.1016/j.envint.2019.01.083
Received 3 November 2018; Received in revised form 30 January 2019; Accepted 31 January 2019
Available online 22 February 2019

been detected in freshwater fish tissues and plasma, generally at micro to nanogram per gram concentrations (Brooks et al., 2005; Chu and Metcalfe, 2007; Schultz et al., 2010), but relatively little is known about subsequent trophic transfers. A recent study on an estuarine/riverine food web detected trace amounts of fluoxetine in all water and some fish plasma samples but not in osprey (*Pandion haliaetus*) plasma (Bean et al., 2018). However, detecting exposure is likely to be more challenging for higher trophic terrestrial wildlife than for fish. This is because fish in effluent-dominated freshwaters may be continually living in dilute concentrations of fluoxetine/norfluoxetine (Christensen et al., 2009) and such ongoing, chronic exposure may result in a persistence presence of residues in plasma. In contrast, non-aquatic higher vertebrates are likely to be exposed to pharmaceuticals more sporadically when they consume contaminated prey, water or vegetation. Ability to detect exposure to compounds that have short plasma and tissue half-lives, such as fluoxetine, will be highly dependent on the magnitude and timing of exposure relative to sampling. False negatives (non-detection of residues because individuals have metabolised the compound before it can be measured) are likely to be common.

Characterising contaminant exposure specifically in wild birds can involve measuring residues in various types of sample (Espin et al., 2016). Plasma is probably the most frequently taken non-destructive sample, generally collected to determine circulating concentrations and/or provide evidence of recent exposure. However, it is clear from the multitude of human studies that the plasma half-life of many pharmaceuticals can be short. Elimination in birds may occur even more quickly than in humans; for example, studies with another SSRI, paroxetine, found that the plasma half-life in grey parrots (*Psittacus erithacus*) given therapeutic doses was 5.4 h compared to 21 h in humans (van Zeeland et al., 2013). Environmental concentrations of pharmaceuticals to which wild birds are likely to be exposed are also typically orders of magnitude lower than therapeutic doses. This combination of low exposure and fast metabolism suggests that the reduction of compound to concentrations too small to be detected is likely to be rapid in birds (Caccia et al., 1990). Consistent with this, a recent study found that neither fluoxetine nor norfluoxetine were detected in the plasma of any of 12 Eurasian starlings (*Sturnus vulgaris*) 2 h after they were given a sub-therapeutic dose (1.58 µg per bird) of fluoxetine (Bean et al., 2017). Although faecal sampling is another non-destructive sample that can be taken, faecal elimination of pharmaceuticals can likewise be rapid (Bean et al., 2017) and obtaining suitable faecal samples from wild-caught birds can be problematic.

Exposure to contaminants in wild birds is also characterised by analysing tissues from individuals found dead (Espin et al., 2016; Gómez-Ramírez et al., 2014). As with plasma, the suitability of such measurements for pharmaceuticals depends on dose and tissue half-life; the latter is still likely to be rapid and shorter for pharmaceuticals than for more persistent contaminants that are more commonly monitored (Gómez-Ramírez et al., 2014). Elimination half-lives for fluoxetine may be longer in tissues than in plasma (Bean et al., 2017) but avian tissue distribution and persistence is not well characterised. Feathers are also used for detecting contaminant exposure. Residues can be the result of past exposure that occurred at the time of feather growth (Espin et al., 2016), as circulating plasma contaminant concentrations are stored within the feather as it grows and remain stable therein over long periods of time (Garcia-Fernandez et al., 2013). Feather analysis has been used to demonstrate exposure to a range of contaminants (Garcia-Fernandez et al., 2013; Abdullah et al., 2015; Espin et al., 2012), although, in some cases, contamination may be on the feather surface where lipophilic contaminants have been expressed in preen oil (Jaspers et al., 2008). Feathers can be collected from carcasses and non-destructively from live birds through plucking, whilst moulted feathers can be collected from nests (Garcia-Fernandez et al., 2013). We are unaware of any published data on the transport of fluoxetine and norfluoxetine into feathers but it is known that fluoxetine is deposited in mammalian hair (Lefebvre et al., 1999; Fisichella et al., 2014), and is found in poultry feather meal, presumably a result of treating poultry with veterinary feed additives (Love et al., 2012).

Detection of exposure is key for understanding whether pharmaceuticals could pose a significant direct risk to wildlife. The main aim of this paper was to characterise which avian tissue samples are best suited for detecting fluoxetine exposure in free-living birds. We quantified and compared free concentrations of fluoxetine and norfluoxetine in a range of tissues (plasma, brain, kidney, liver and lung) taken from wild Eurasian starlings held untreated in captivity (during which time they would have metabolised any existing plasma and internal tissue residues) and subsequently dosed chronically with an environmentally-relevant dose of fluoxetine. We used starlings as they are known to feed on invertebrates in trickling filter beds at waste water treatment plants (WWTPs) (Fuller and Glue, 1978) and so are at high risk of exposure to pharmaceuticals present in sewage, such as fluoxetine (Lajeunesse et al., 2012). We coupled our measured concentrations to fluoxetine tissue decay curves developed in a previous study (Bean et al., 2017) to determine the time period over which tissue residues were likely to be analytically detectable; such information is crucial for informing the design of biomonitoring programmes. We also quantified the levels of free fluoxetine and norfluoxetine in feathers from the same birds. These samples comprised: (i) feathers regrown during the period in which birds were chronically dosed, thereby providing a test of whether feathers accumulate fluoxetine in birds known to have been exposed; (ii) feathers grown by the birds before they were captured - detection of fluoxetine in these feathers would provide some evidence that starlings are currently exposed to fluoxetine in the wild in Britain.

2. Methods

2.1. Ethics statement

The birds used in the study were captured under licences from the British Trust for Ornithology and Natural England. The aviary study and all experimental protocols conducted therein were assessed and approved by ethics committees at the University of York (UK) and the Animal and Plant Health Agency. The work was carried out under Home Office licence, number PPL 60/4213.

2.2. Aviary study

The samples analysed were from birds that underwent an in vivo chronic effects experiment. These were 28 wild-caught Eurasian starling (*Sturnus vulgaris*), captured under licence in October 2015 from a site in North Yorkshire (UK). The captive birds were housed in five outdoor aviaries in single sex groups (three aviaries containing 7, 6 and 6 females; two aviaries with 6 and 3 males). Within each aviary, individuals were allocated randomly to either fluoxetine treatment or control treatment ($n = 14$ per group), within the constraint that numbers in each treatment group were balanced as closely as possible. We housed treated and control birds together to avoid biases in behavioural experiments that were carried out during the chronic dosing period as part of a wider study.

Fluoxetine-treated starlings were exposed chronically to a nominal daily dose of 2.7 µg bird^{-1} fluoxetine. This was equivalent to a mean (± SD) dose of 34.3 (± 1.44) µg kg^{-1} bodyweight (BW) daily based on a mean (± SD) starling weight of 78.9 (± 3.37) g across the duration of the dosing period. The dose was calculated to be representative of an environmentally relevant exposure, based on: i) the feeding rates for starlings, ii) fluoxetine concentrations detected in invertebrate prey at a waste water treatment plant (Bean et al., 2017; Feare, 1984). We administered a five-day corrected dose of 3.8 µg bird^{-1} each weekday (as birds were not dosed at weekends) in the early afternoon, by hand-feeding individuals with a spiked waxworm (*Galleria mellonella*) (Bean et al., 2014) containing 3.8 µg fluoxetine dissolved in 2.5 µl deionised water. Control birds were given a waxworm

injected with 2.5 µl deionised water only. To confirm the dose administered, we quantified the amount of free fluoxetine in a subset of spiked waxworms and the mean (± SE) concentration was 3.71 ± 0.18 µg worm^{-1} (n = 10; see (Whitlock et al., 2018)). Dosing of birds commenced in late November 2015 and continued for 28 weeks, at which point 24 individuals remained alive (n = 12 per group). Details of the dose calculation, animal capture and husbandry are given in the SI.

Tail feathers (rectrices R4 and R9) were plucked (and retained as pooled samples for each bird) from all individuals one week after dosing commenced, except for two fluoxetine-treated birds and one control group bird that were missing R4 and/or R9. Plucking was undertaken at this time, rather than before dosing was started, due to ethical concerns associated with plucking feathers simultaneously with other stressful procedures, such as baseline behavioural assays, blood sampling and acclimation to captivity. The feathers, grown when the birds were free-living in the wild (hereafter termed wild-grown feathers), were plucked so that they would regrow during the dosing period. This would allow us to determine whether fluoxetine residues could be detected in feathers that were regrown (hereafter termed regrown feathers) during a period when birds were exposed to an environmentally relevant concentration of fluoxetine. We restricted our feather analyses to two specific rectrices because contaminant concentrations can vary significantly between feathers (Furness et al., 1986; Gochfeld, 1980; Braune, 1987).

After 28 weeks of dosing, the birds were euthanised by cervical dislocation to allow harvesting of various tissues for the determination of free concentrations of fluoxetine and norfluoxetine. Euthanasia of each bird occurred 2 h after administration of the final fluoxetine/control dose. Brain, kidney, liver and lung were immediately excised, frozen within 30 min using dry ice and subsequently stored at −80 °C until analysis. In between administration of the final dose and euthanasia, a blood sample was taken from each fluoxetine-treated bird for analysis of free fluoxetine and norfluoxetine plasma residues. Guided by findings in (Bean et al., 2017), both to enhance the chance of detecting residues and to allow decay curves to be constructed, we blood sampled six individuals 30 min after dose administration, whilst the remaining six individuals were sampled after 1 h. Assignment of birds to the 30 or 60 min bleed was randomised, within the constraint that sex and treatment were balanced between the timings. Blood samples (500 µl) were collected by jugular venepuncture (23 G needle AN-2316R, Terumo UK, Bagshot, UK, and 1 ml syringe, BD, Wokingham, UK), transferred to a heparinised 3.5 ml Microtainer (BD, Wokingham, UK), and centrifuged within 30 min of collection at 16,000g for 10 min. The plasma was then collected, transferred to a 1.5 ml polypropylene Eppendorf tube (Eppendorf UK, Stevenage, UK) and stored at −20 °C until analysed.

The regrown R4 and R9 rectrices were also removed from each bird, pooled (per individual) for analysis and stored in brown paper envelopes at room temperature. Regrown feathers were not collected from three (two fluoxetine-treated, one control) birds as their rectrices had not regrown (SI, Table S5). Samples for analysis contained both rectrices from each bird except for four control samples, which contained only one feather of the pair; in two of these four individuals, the regrown feather was lost or damaged before it could be harvested and in the other two, the feather never regrew (SI, Table S5). We excluded these birds from any statistical comparison of residue magnitude as concentrations in a single feather may not be directly comparable to those in a pooled pair (Bortolotti, 2010).

2.3. Analysis of samples for fluoxetine residues

We screened the livers from all the control birds to determine whether it was worthwhile analysing other tissues from individuals in this group. Limited previous results (Bean et al., 2017) have indicated that residues occur at higher concentrations in liver compared to other tissues, therefore an absence of detectable liver residues was assumed to be indicative of an absence of residues in other tissues. We could not detect fluoxetine residues in the livers of eleven of the twelve control birds, whilst the remaining bird had a concentration close to the limit of detection (free concentration in sample: 0.34 ng g^{-1}, liver limit of detection (LOD): 0.27 ng g^{-1}). Free norfluoxetine was not detected in any control liver sample. Consequently, we restricted further analysis of residues in other body tissues (kidney, lung, brain) to samples taken only from fluoxetine-treated birds. We likewise only analysed plasma samples from fluoxetine-treated birds, as the aim was solely to examine the timeframe of disappearance of residues from this labile pool.

In contrast, we analysed residues in regrown and wild-grown feathers from both fluoxetine-treated and control birds. This was to assess: i) whether there was fluoxetine in the regrown feathers of control birds, which may have arisen from cross-contamination given that the birds were housed in mixed-treatment groups; ii) whether there were residues in wild-grown feathers that were indicative of past exposure.

Tissue samples were thawed, cut in half, homogenised and a subsample (nominal mass of 300 mg for brain, kidney and liver, 100 mg for lung) was taken for analysis. Each subsample was weighed accurately to the nearest 0.1 mg. Samples were each ground with sand in a glass pestle and mortar before being transferred to a Pyrex test tube. Feather samples were washed with diluted soap (1% solution of Decon 90 in HPLC grade water; Decon Laboratories, Hove, UK). There is evidence that surfactants can remove some proportion of lipophilic contaminant from the feather surface (Jaspers et al., 2008). The feathers were then washed with HPLC grade water and allowed to dry for 24 h before being cut into small pieces and ground in ceramic pestle and mortars with liquid N_2. After grinding, the entirety of each feather sample was transferred to a Pyrex test tube. Plasma samples were thawed to room temperature and an 80 µl aliquot was taken from each for analysis. Four of the twelve plasma samples had insufficient volume to provide an 80 µl aliquot, so smaller volumes were taken instead (25, 60, 60 and 70 µl aliquots).

HPLC grade solvents (including HPLC grade water) were used throughout the sample preparation and analysis process. Prior to extraction, all sample types were spiked with fluoxetine-d5 (98% purity, Sigma Aldrich, Gillingham, UK) internal standard. The extraction method was based on (Bean et al., 2017). All samples were extracted by adding 2 × 2 ml portions (i.e. 4 ml total) of 0.2% formic acid in methanol/water (50:50 v/v), with vortex mixing for 20–30 s after each addition of solvent. The samples were placed in a sonication bath at 20 °C for 15 min and subsequently centrifuged for 10 min at 20 °C and 4696g. The supernatant was transferred to a new test tube and 8 ml water (HPLC Grade) was added, before clean-up by SPE. Following (Grabic et al., 2012), Oasis HLB cartridges (6 cm^3, 200 mg sorbent, 30 µm particle size; Waters, Elstree, UK) were first conditioned with methanol, then water, before the sample extracts were run through them at a flow rate of 1 ml min^{-1}. After drying at full vacuum for 30 min, the samples were eluted into fresh test tubes with methanol followed by acetonitrile, before being concentrated to dryness under a N_2 stream, in a water bath at 40 °C. The residues were then reconstituted in 1 ml of 0.2% formic acid in water/acetonitrile (80:20 v/v), vortex mixed and transferred to LC vials for LC-MS/MS analysis.

2.4. LC-MS/MS analysis

The residues of free fluoxetine and norfluoxetine (i.e. the free parent compounds only) were quantified by reverse phase LC-MS/MS. The analytes were separated on a Thermo Scientific UltiMate 3000 HPLC with a C18 column (Thermo Scientific Hypersil GOLD; particle size 1.9 µm, length 100 mm, internal diameter 2.1 mm). The column temperature was held at 30 °C and the analytes were eluted using a binary gradient of mobile phases A (water containing 0.2% formic acid) and B (acetonitrile containing 0.2% formic acid). The volume of injection of

the sample extracts was 25 µl and the mobile phase flow rate was as follows: 0.35 ml min^{-1} from 0 to 7.5 min, 0.25 ml min^{-1} from 7.5 to 12 min and 0.30 ml min^{-1} after 12 min. The gradient was performed as follows: 18% mobile phase B from 0 to 7.5 min, 60% B from 7.5 to 12 min, 100% B from 12 to 15 min, 18% B from 15 to 20 min.

The HPLC system was coupled to a TSQ Quantum Ultra triple quadrupole mass spectrometer. The sample extracts were ionised by electrospray ionisation, in positive ion mode. Detection was performed by monitoring two MS/MS transitions per analyte in selected reaction monitoring (SRM) mode. See Table S1 in the SI for fragmentation parameters.

For quality control in each batch we ran a reagent blank, matrix blank and spiked control. We constructed calibration curves of the target compounds (Sigma Aldrich, Gillingham, UK), to cover the expected analyte concentration range in the samples. Concentrations were recovery corrected using the internal standard method (see Table S2 in the SI for method recoveries and detection limits for fluoxetine and norfluoxetine).

2.5. Statistical analyses

All statistical analyses were carried out in the software package R (R Core Team, 2016). Kruskal-Wallis non parametric tests (with Dunn's *post-hoc* tests using R package "dunn.test" (Dinno, 2017)) were used to compare the four tissue types (brain, kidney, liver, lung) in treated individuals for free concentrations of: (i) fluoxetine; (ii) norfluoxetine; (iii) fluoxetine:norfluoxetine ratio. We examined the significance of associations between fluoxetine concentrations in different tissues and between fluoxetine and norfluoxetine concentrations within tissues using Spearman's Rank correlation coefficients. Comparison of the free fluoxetine concentrations in regrown feathers between the treatment and control groups was made by Mann-Whitney non-parametric test; non-detected fluoxetine concentrations in feathers were assigned a value of 0.5*LOD (0.42 ng g^{-1}). Non-parametric tests were selected due to non-normal distribution of datasets. Total tissue burdens (sum of parent compound and metabolite) were estimated by summing the mass of parent fluoxetine present (concentration in the tissue multiplied by the tissue mass) and the mass of fluoxetine that had been metabolised. The latter was back-calculated using the number of moles of norfluoxetine in the tissue (assuming a 1:1 molar ratio with fluoxetine), itself derived using the mass of norfluoxetine in the tissue (concentration in the tissue multiplied by the tissue mass). We expressed the calculated burden in each tissue as a percentage of the measured administered oral daily dose (3.71 µg). Tissue burdens and their percentage of administered dose are reported as medians with interquartile ranges.

2.6. Estimation of time for concentrations to decay to the limit of detection (LOD) in brain, kidney, and liver

We used concentration decay curves developed in a recent study (Bean et al., 2017) to estimate how long the dose of fluoxetine used in the present study would remain detectable as free fluoxetine and norfluoxetine in brain, kidney and liver. The curves were derived from in vivo experimental data from Eurasian starlings given 1.58 µg fluoxetine $bird^{-1}$, i.e. 42.6% of the dose administered in our study (Bean et al., 2017). We assumed that the decay of residues in tissues follows a first order relationship ($y = Ce^{-kx}$) and applied the rate constants (k) from (Bean et al., 2017) to the mean concentration of fluoxetine (y) at 2 h (x) measured in the present study (in brain, kidney and liver), allowing the estimation of C for each tissue. We then plotted the decay curves and derived the time (x) when C would be equal to the limit of detection (LOD) for each tissue type.

3. Results and discussion

3.1. Free concentrations and distribution of fluoxetine and norfluoxetine within the body of fluoxetine-treated birds

Fluoxetine was detected in two of the six plasma samples taken from fluoxetine treated birds 30 min after administration of the final oral dose (free concentration 0.20 and 0.42 ng ml^{-1} respectively) but was not detected any of the plasma samples ($n = 6$) taken 60 minute post-dose. Norfluoxetine was not detected in any of the plasma samples at either time point. Because of this very rapid elimination relative to our sampling times, we were unable to construct a simple plasma decay curve, as we had originally planned. However, our results indicate that plasma elimination of fluoxetine is faster in starlings than previously thought. A previous study using a lower dose (1.58 µg per bird) found no detectable fluoxetine or norfluoxetine in plasma after 2 h (Bean et al., 2017) but our data indicate that a (> 2 fold) higher dose is completely eliminated from plasma within 1 h. Clearance may also be faster than might be predicted from mammalian studies (which form the bulk of available pharmacokinetic data for pharmaceuticals), as the metabolic rates of certain passerines and psittaciformes can exceed those of comparably sized mammals (i.e. rodents) (McNab, 1988) and of humans (van Zeeland et al., 2013). Given that only small volumes of plasma can be collected non-destructively from many birds due to their low body mass and given the apparently short window for successful detection of fluoxetine post-exposure, we conclude that avian plasma is of severely limited use for determining exposure of wild birds to fluoxetine.

Concentrations of free fluoxetine and norfluoxetine were both detected in the brain, kidney, liver and lung of each of the fluoxetine-treated birds. Fluoxetine is relatively lipophilic and has a large volume of distribution in mammalian tissues (Caccia et al., 1990; Hiemke and Hartter, 2000) and this also appears to be true in birds. In the present study, concentrations of parent compound and metabolite varied significantly between tissue types ($H_{(3)} \geq 32.5$, $p < 0.001$ for fluoxetine and norfluoxetine). *Post-hoc* tests indicated that free fluoxetine concentrations decreased in the order liver > kidney ≥ lung ≥ brain (Fig. 1a and SI, Table S3) and free norfluoxetine concentrations in the order liver > kidney ≥ lung > brain (Fig. 1b and SI, Table S3). These results clearly indicate that fluoxetine and its metabolite distribute unevenly between tissues and that the highest concentrations of free compound are found in the liver. A limited previous study in which birds were dosed at half the rate to the present study had also suggested that free residues may be highest in the liver, but residues had been quantified in only three birds (Caccia et al., 1990). When tissue fluoxetine burdens were calculated for the present study, the magnitude of distribution to each tissue followed a similar pattern to the observed concentrations of fluoxetine (Table 1). On average, the equivalent of 8.59% of the final dose (given 2 h earlier) was accounted for by the total burden of free compound in the four tissues, with most (7.40%) in the liver (Table 1). We also examined the relationships between fluoxetine concentrations in different organs to determine whether residues in one tissue could be used to predict concentrations in others. There were positive relationships in all cases but only the correlation between brain and kidney fluoxetine was statistically significant (Fig. 2 and SI, Table S4). Thus, it might be possible to use information on fluoxetine levels in the kidney to predict fluoxetine concentrations in the brain, the target organ for this compound, although such estimates would be approximate.

Based on our concentration data and because of the relatively large mass that can be harvested for analysis, liver and kidney would appear to be the most appropriate tissues for biomonitoring fluoxetine exposure in starlings and potentially other wild birds. Measuring kidney concentrations may also provide a means for predicting brain concentrations, whilst liver concentrations are likely to be higher than in kidney, lung or brain tissue. However, liver, and particularly kidney,

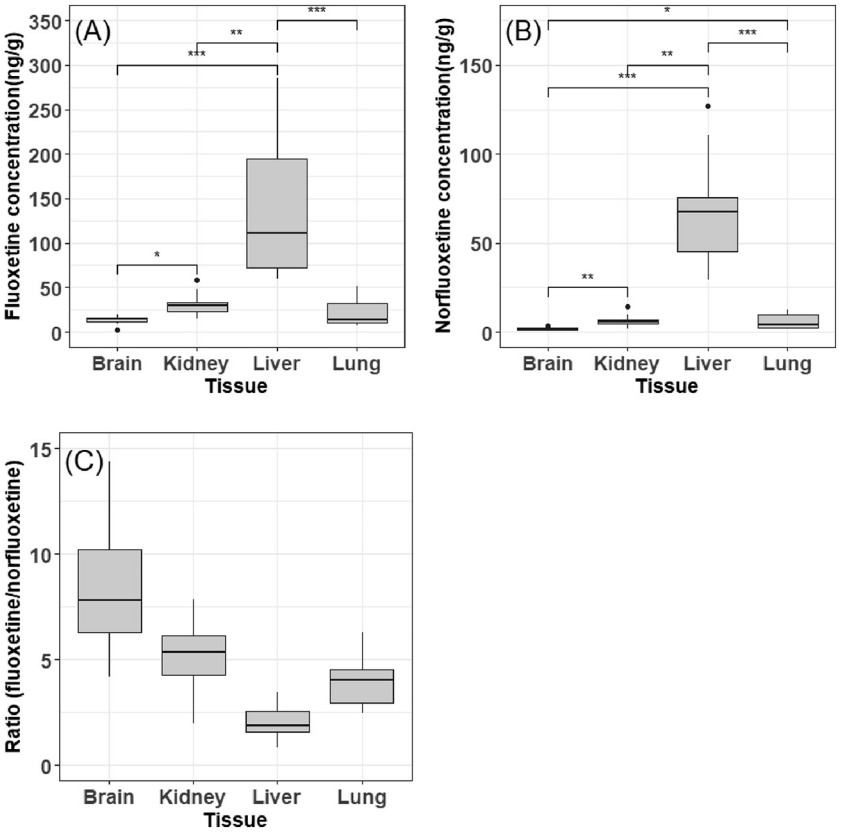

Fig. 1. Median free concentrations of (a) fluoxetine and (b) norfluoxetine in brain, kidney, liver and lung ($n = 12$ per organ). In (a) and (b), significant differences in concentrations between pairs of tissues are starred (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). See SI, Table S3 for full statistical test results, based on Dunn's test. Plot (c) shows the median ratio of fluoxetine to norfluoxetine (i.e. concentration of fluoxetine divided by concentration of norfluoxetine) in each tissue. Each plot corresponds to concentrations of residues 2 h after the final dose (3.71 μg bird^{-1}) of a 28-week dosing period. Boxes correspond to upper and lower quartiles and points shown outside of whiskers are outliers (1.5*IQR, as defined by Tukey (1977)).

Table 1
Median free fluoxetine tissue burdens (in ng) estimated for brain, kidney, liver and lung; shown with interquartile range (IQR). $n = 12$ per organ. Table also expresses the burdens as median percentages of the total oral dose (3.71 μg), with corresponding IQRs shown.

Tissue	Median burden/IQR (ng)	Median percentage of dose/IQR (%)
Brain	14.5/11.8–15.3	0.390/0.318–0.414
Kidney	18.9/12.5–28.4	0.509/0.338–0.766
Liver	274/215–396	7.40/5.80–10.7
Lung	10.9/9.48–19.8	0.293/0.255–0.534

may not be the best choice of organs for monitoring exposure in other vertebrate wildlife, such as wild mammals. Mammalian studies have shown that fluoxetine is distributed extensively in liver, lung and brain tissues (Caccia et al., 1990; Pohland and Bernhard, 1997; Johnson et al., 2007), primarily to the lysosome-rich lung and liver tissues (Daniel and Wojcikowski, 1997), but with less distribution to the kidney. For example, a study that analysed concentrations of fluoxetine and norfluoxetine in deceased human pilots that had taken antidepressant medication found that distribution coefficients in tissues decreased in the following order for both compounds: lung > liver > brain > kidney (Johnson et al., 2007). Further studies are needed to determine which may be the best organs for biomonitoring fluoxetine exposure across the range of wildlife taxa.

We also examined the ratio of free fluoxetine to norfluoxetine in the dosed starlings and found that this also differed significantly with tissue type (Kruskal-Wallis: $H = 32.23$, 3 *d.f.*, $p < 0.001$; Fig. 1c). However, within kidney, liver and lung tissue, fluoxetine and norfluoxetine concentrations were significantly correlated with each other (Fig. 3 and SI, Table S4). A similar relationship was apparent in brain but did not achieve statistical significance (Fig. 3 and SI, Table S4). The median fluoxetine:norfluoxetine ratio in starling brain tissue was 7.81. This ratio appears to be very different to that recorded in an earlier mammal study, in which rats were given a single oral dose of 5 mg kg^{-1} fluoxetine (two orders of magnitude larger than the dose used in the present study), leading to a mean fluoxetine:norfluoxetine ratio in rat brain of 0.26 (here calculated as the inverse of a stated norfluoxetine:fluoxetine ratio of 3.8 (Caccia et al., 1990)). Although the ratio in rat brain increased with dose, with fluoxetine:norfluoxetine ratios of 1 and 1.4 (inverse of stated norfluoxetine:fluoxetine ratios of 1 and 0.7) in rats given fluoxetine doses of 10 and 20 mg kg^{-1} respectively (Caccia et al., 1990), these were still much lower than the median brain tissue ratio of 7.81 for starlings in the present study. Even though we dosed the starlings chronically rather than administering a single dose, we estimated that fluoxetine would likely be cleared from the brain after approximately 20 h (see below), limiting the potential for fluoxetine to accumulate in brain tissue as a result of successive, chronic dosing. Our results suggest that the ratio of fluoxetine:norfluoxetine in the brain may be higher in birds compared to mammals following low doses of fluoxetine. This may indicate that birds are more susceptible to adverse effects on behaviour than mammals following exposure in the environment, since the R-form of fluoxetine is a more potent inhibitor of serotonin reuptake than R-norfluoxetine, although this difference in activity between parent and active metabolite is not observed in the respective S-enantiomers (Fuller et al., 1992).

3.2. Decay of fluoxetine in avian tissues following oral exposure

Based on decay curves derived from those given in (Bean et al., 2017), the times taken for organ concentrations to decay to the detection limit in the present study were estimated to be 20 h for brain, 90 h for kidney and 93.5 h for liver (Fig. 4). This calculation confirms that liver or kidney are likely to be preferred organs for biomonitoring exposure because residues persist for longer than in the brain, in which fluoxetine parent compound arising from environmentally-relevant exposures would be eliminated in less than a day. These estimates of time to reach detection limits are however only approximate, as our

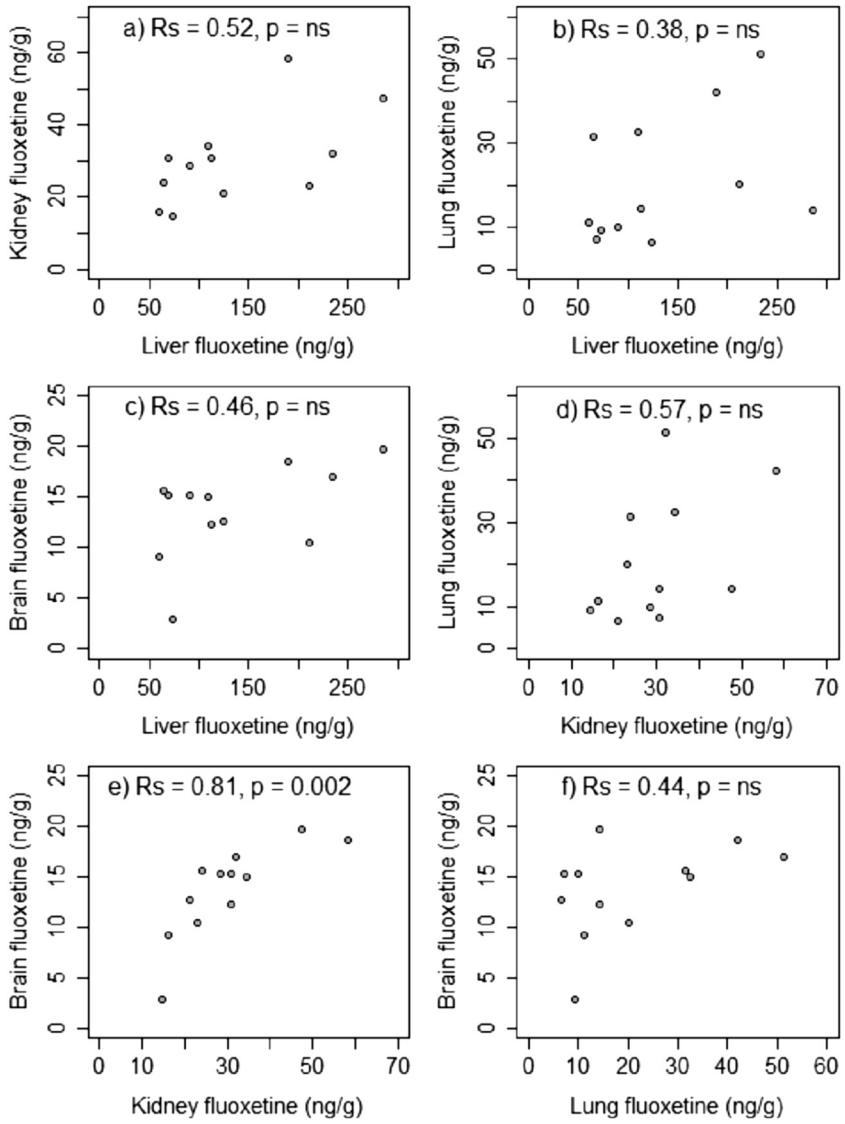

Fig. 2. Scatter plots of free fluoxetine concentrations between pairs of different tissues. Spearman correlation coefficients are shown, as are corresponding *p*-values for statistically significant correlations, whilst non-significant correlations are ascribed a *p*-value of "ns" ($\alpha = 0.05$). Specifically, plots show correlations between: a) liver and kidney fluoxetine, b) liver and lung fluoxetine, c) liver and brain fluoxetine, d) kidney and lung fluoxetine, e) kidney and brain fluoxetine, f) lung and brain fluoxetine. Note that full results from Spearman's Rank-Order correlations are reported in the SI, Table S4.

calculations suggest that fluoxetine persists in liver and kidney for > 24 h and therefore would have been bioaccumulated to some extent over the course of the dosing period that we used. Thus, time to reach detection limit will in part be a function of dosing duration. Nevertheless, our data provide evidence that analysis of kidney and especially liver tissue may allow fluoxetine residues to be detected in the organs of dead wild birds. As the brain is the intended site of therapeutic action for fluoxetine, presence in wild bird brain tissue might be expected to indicate a degree of risk from adverse effects, such as behaviour alteration (Bean et al., 2014; Whitlock et al., 2018). Yet according to our decay curve estimates, residues in avian brain drop to undetectable concentrations rapidly compared to those in liver and kidney tissue.

3.3. Free concentrations in regrown and wild-grown feathers

Fluoxetine was detected in all the regrown rectrice feathers analysed from fluoxetine-treated individuals (11/11) and in all but two of the regrown feather samples from control individuals (6/8). Such frequent detection of fluoxetine in the regrown feathers of control birds was unexpected. It suggested that contact with excreta and possibly preen oil from dosed birds may have led to surface contamination of feathers in control birds. Although all feathers were thoroughly washed with mild soap, previous studies have found that washing is not always able to fully remove contaminant residues from feather surfaces (Garcia-Fernandez et al., 2013; Jaspers et al., 2008; Cardiel et al., 2011). However, it is noteworthy that an earlier study found that washing feathers with a surfactant removed at least some external residues of lipophilic persistent organic pollutants, likely those derived from preen oil (Jaspers et al., 2008). The surfactant we used may have likewise reduced external feather fluoxetine contamination from preen oil, although this may have been less effective than for the persistent organic pollutants in (Jaspers et al., 2008), as fluoxetine is less lipophilic. It is also possible that contamination arose from control birds ingesting food and water containing fluoxetine-contaminated faeces (or even consuming contaminated faces directly), resulting in circulating fluoxetine residues in the plasma and subsequent deposition into growing feathers. Such contamination was possible as the experimental design of our wider behavioural studies necessitated housing starlings in mixed treatment groups.

Whilst we were unable to determine whether residues in control

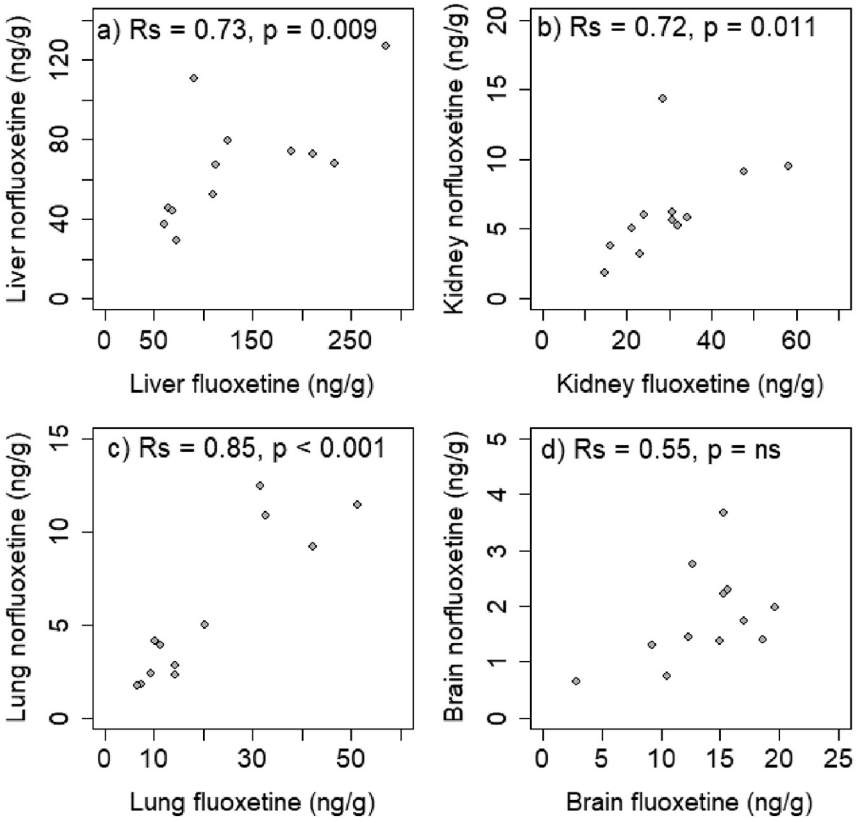

Fig. 3. Scatter plots of fluoxetine and norfluoxetine free concentrations (ng g^{-1}) within each tissue: a) liver, b) kidney, c) lung and d) brain. Spearman correlation coefficients are also displayed.

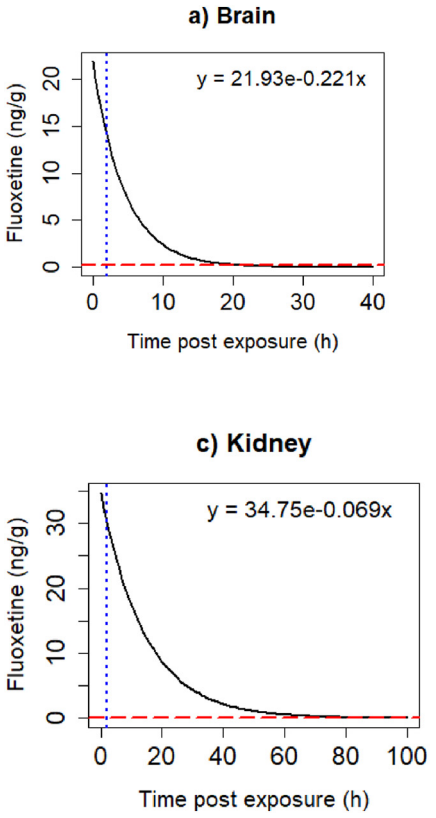

Fig. 4. Decay curves that estimate the concentration of free fluoxetine in various tissues (brain, liver, kidney) over time (hours) post exposure ($n = 12$ birds per tissue). Curves generated using rate constants derived in another study, during an in vivo experiment (Bean et al., 2017). The black line is the decay curve, the blue dotted vertical line marks $t = 2$ h post-dose (corresponding to concentration measurements from organs) and the red dashed line indicates method limit of detection (LOD). The equation of each decay curve is shown on each plot. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

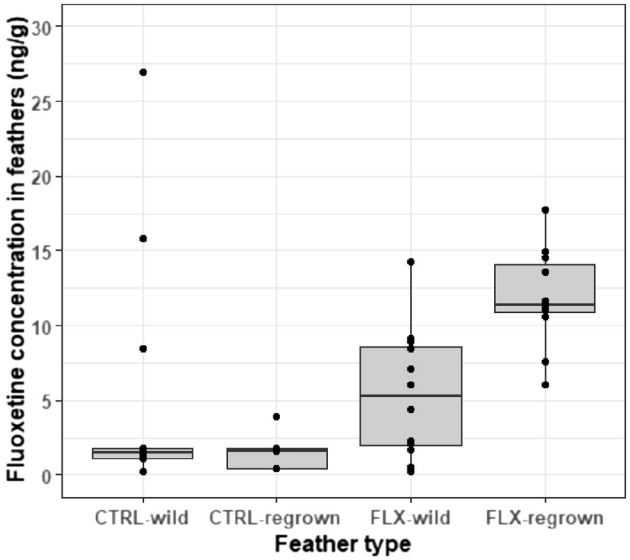

Fig. 5. Concentrations of free fluoxetine in rectrice feathers (R4 and R9). Data shown correspond to regrown control (CTRL) group feathers ($n = 5$), regrown fluoxetine-treated (FLX) group feathers ($n = 11$) and wild-grown feathers ($n = 25$). Only samples with pooled R4 and R9 feathers are included. The boxes represent the upper and lower quartiles, whilst outliers are defined as 1.5*IQR (Tukey, 1977). The regrown feathers grew during the dosing period, whereas the wild-grown feathers were fully grown before the onset of dosing and were harvested the week after dosing commenced.

feathers reflected external and/or internal contamination, it is noteworthy that we only detected fluoxetine in the liver of one control bird (and then only at a concentration close to the detection limit). This suggests that control birds were not routinely ingesting fluoxetine. Furthermore, the fluoxetine concentration was around fourfold higher in regrown feathers from dosed than from control birds (Mann Whitney U test, $U = 0$, $p = 0.002$; $n = 11$ fluoxetine-treated and $n = 5$ control birds; Fig. 5). Some of the higher fluoxetine residues in fluoxetine-treated bird regrown feathers are likely due to fluoxetine deposition into feathers from systemic circulation, analogous to deposition into hair following oral administration in mammals (Lefebvre et al., 1999; Fisichella et al., 2014). However, preen gland secretion is a suggested excretory route for persistent organic pollutants (Solheim et al., 2016) and preen oil is a major source of external feather contamination for highly lipophilic contaminants (Jaspers et al., 2008; Solheim et al., 2016). Therefore, although fluoxetine is less lipophilic than many persistent organic pollutants, we cannot rule out the possibility that surface contamination was reinforced in dosed birds by residues in preen oil.

There were no significant correlations between feather and organ fluoxetine concentrations (see SI, Table S6). Norfluoxetine was not detected in any regrown-feathers from control birds but was found in four of the fluoxetine-treated birds (concentration range of 0.74–1.25 ng g^{-1}). This may possibly be an additional indicator of deposition of the compound from the plasma into feathers in treated birds, although it is unclear why norfluoxetine was not found in regrown feathers from all the chronically dosed birds. Overall, our results suggest that feathers could be used to assess the occurrence of exposure to fluoxetine in wild birds, although currently the mechanism of contamination (whether via systemic circulation or preen oil) is unclear. Further work is needed to elucidate the dynamics between exposure and deposition of residues into and onto feathers.

Fluoxetine was also detected in wild-grown feathers from most (21 of 25) of the starlings brought into captivity, whilst norfluoxetine was not detected in any wild-grown feathers. The frequent detection of fluoxetine in wild-grown feathers may in part have resulted from surface contamination during the week-long dosing of the experimental birds that occurred before the wild-grown feathers were plucked or, in fluoxetine-treated birds, from fluoxetine in preen oil applied to the feathers. Indeed, based on the higher rates of fluoxetine observed in fluoxetine-treated compared to control bird regrown feathers (see Fig. 5), this seems plausible. However, it seems unlikely that external (surface) contamination would account for all the observed residues in wild-grown control bird feathers, especially since two of the three highest concentrations were in birds from the wild control group, and one of these was the highest concentration in any feather (wild-grown or regrown) that we analysed. The data suggest that some individuals may have been exposed to fluoxetine in the wild, likely between late May and early September in the summer preceding the experiment, as all feathers are moulted and regrown over approximately 100 days in Eurasian starlings (Rothery et al., 2001). Further analyses of feathers from free-living birds are needed to confirm such exposure and assess its prevalence.

4. Conclusions

Our results suggest that fluoxetine exposure in wild birds can be monitored by analysing residues in liver and kidney tissues harvested from dead birds, and possibly in feathers from live or dead birds. Any such monitoring presents challenges. Residues in the liver and kidney are relatively labile, becoming undetectable several days post-exposure. Measured concentrations are also likely to vary markedly with both magnitude of exposure and the period between the last exposure event and death. Internal feather residues (those incorporated into the feather structure) provide an indication of exposure during the period when feathers are regrown (Daniel and Wojcikowski, 1997) and the processes by which deposition into feathers occurs can be complex (Bortolotti, 2010). There is also the potential to confuse external and internal contamination (Jaspers et al., 2007), leading to overestimation of internal residue concentrations. However, even if the contribution of preen oil and deposition by systemic circulation to feather fluoxetine concentrations cannot be differentiated, valuable data regarding exposure on a presence/absence basis can still be collected. If internal and external concentrations could be differentiated, it might then be possible to collect additional information on the variability of exposure over time within the feather growth period, by analysing sequential sections from the same feather. Further work is needed to determine the extent to which feathers can be used to characterise the scale of wild bird exposure to fluoxetine, and indeed other pharmaceuticals, especially those species whose foraging strategies put them at particular risk of exposure. Finally, our analysis of wild feathers suggests that exposure of free-living starlings to fluoxetine does occur. This is of concern, as fluoxetine has been found to alter Eurasian starling behaviours at a dose less than half of the environmentally relevant dose used in the present study (Bean et al., 2014). Although the likelihood and significance of effects in free-living birds resulting from pharmaceutical exposures remains to be determined, the implementation of pharmaceutical-appropriate biomonitoring campaigns could significantly further our understanding in this area.

Competing interests

I/we have no competing interests. Declarations of interest: none.

Acknowledgements

We thank the ASIST team and M. Brash (FERA Science Ltd.), J. Warwick and the East Dales Ringing Group, S. Warwick and C. Pennock (Tarmac Ltd.).

Funding statement

This work was supported by a Natural Environment Research Council (NERC) grant within the Adapting to the Challenges of a Changing Environment DTP [grant number NE/L002450/1], and by funding from the University of York (UK) and the Animal and Plant Health Agency (UK).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.01.083.

References

Abdullah, M., Fasola, M., Muhammad, A., Malik, S.A., Bostan, N., Bokhari, H., Kamran, M.A., Shafqat, M.N., Alamdar, A., Khan, M., et al., 2015. Avian feathers as a non-destructive bio-monitoring tool of trace metals signatures: a case study from severely contaminated areas. Chemosphere 119, 553–561. https://doi.org/10.1016/j.chemosphere.2014.06.068.

Bean, T.G., Boxall, A.B.A., Lane, J., Herborn, K.A., Pietravalle, S., Arnold, K.E., 2014. Behavioural and physiological responses of birds to environmentally relevant concentrations of an antidepressant. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369.

Bean, T.G., Arnold, K.E., Lane, J.M., Bergström, E., Thomas-Oates, J., Rattner, B.A., Boxall, A.B.A., 2017. Predictive framework for estimating exposure of birds to pharmaceuticals. Environ. Toxicol. Chem. 36, 2335–2344. https://doi.org/10.1002/etc.3771.

Bean, T.G., Rattner, B.A., Lazarus, R.S., Day, D.D., Burket, S.R., Brooks, B.W., Haddad, S.P., Bowerman, W.W., 2018. Pharmaceuticals in water, fish and osprey nestlings in Delaware River and Bay. Environ. Pollut. 232, 533–545. https://doi.org/10.1016/j.envpol.2017.09.083.

Bortolotti, G.R., 2010. Flaws and pitfalls in the chemical analysis of feathers: bad news-good news for avian chemoecology and toxicology. Ecol. Appl. 20, 1766–1774. https://doi.org/10.1890/09-1473.1.

Boxall, A.B.A., 2004. The environmental side effects of medication. EMBO Rep. 5, 1110–1116.

Braune, B.M., 1987. Comparison of total mercury levels in relation to diet and molt for nine species of marine birds. Arch. Environ. Contam. Toxicol. 16, 217–224. https://doi.org/10.1007/bf01055802.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24, 464–469. https://doi.org/10.1897/04-081r.1.

Caccia, S., Cappi, M., Fracasso, C., Garattini, S., 1990. Influence of dose and route of administration on the kinetics of fluoxetine and its metabolite norfluoxetine in the rat. Psychopharmacology 100, 509–514. https://doi.org/10.1007/BF02244004.

Cardiel, I.E., Taggart, M.A., Mateo, R., 2011. Using Pb-Al ratios to discriminate between internal and external deposition of Pb in feathers. Ecotoxicol. Environ. Saf. 74, 911–917.

Christensen, A.M., Markussen, B., Baun, A., Halling-Sørensen, B., 2009. Probabilistic environmental risk characterization of pharmaceuticals in sewage treatment plant discharges. Chemosphere 77, 351–358. https://doi.org/10.1016/j.chemosphere.2009.07.018.

Chu, S., Metcalfe, C.D., 2007. Analysis of paroxetine, fluoxetine and norfluoxetine in fish tissues using pressurized liquid extraction, mixed mode solid phase extraction cleanup and liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1163, 112–118. https://doi.org/10.1016/j.chroma.2007.06.014.

Daniel, W.A., Wojcikowski, J., 1997. Contribution of lysosomal trapping to the total tissue uptake of psychotropic drugs. Pharmacol. Toxicol. 80, 62–68.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107, 907–938.

Dinno, A., 2017. dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums. R Package Version 1.3.5.

Espin, S., Martinez-Lopez, E., Maria-Mojica, P., Garcia-Fernandez, A.J., 2012. Razorbill (*Alca torda*) feathers as an alternative tool for evaluating exposure to organochlorine pesticides. Ecotoxicology 21, 183–190. https://doi.org/10.1007/s10646-011-0777-z.

Espin, S., Garcia-Fernandez, A.J., Herzke, D., Shore, R.F., van Hattum, B., Martinez-Lopez, E., Coeurdassier, M., Eulaers, I., Fritsch, C., Gomez-Ramirez, P., et al., 2016. Tracking pan-continental trends in environmental contamination using sentinel raptors-what types of samples should we use? Ecotoxicology 25, 777–801. https://doi.org/10.1007/s10646-016-1636-8.

Feare, C.J., 1984. The Starling. Oxford University Press, Oxford.

Fisichella, M., Morini, L., Sempio, C., Groppi, A., 2014. Validation of a multi-analyte LC-MS/MS method for screening and quantification of 87 psychoactive drugs and their metabolites in hair. Anal. Bioanal. Chem. 406, 3497–3506. https://doi.org/10.1007/s00216-014-7763-2.

Fuller, R.J., Glue, D.E., 1978. Seasonal activity of birds at a sewage-works. Br. Birds 71, 235–244.

Fuller, R.W., Snoddy, H.D., Krushinski, J.H., Robertson, D.W., 1992. Comparison of norfluoxetine enantiomers as serotonin uptake inhibitors *in vivo*. Neuropharmacology 31, 997–1000. https://doi.org/10.1016/0028-3908(92)90100-4.

Furness, R.W., Muirhead, S.J., Woodburn, M., 1986. Using bird feathers to measure mercury in the environment: relationships between mercury content and moult. Mar. Pollut. Bull. 17, 27–30. https://doi.org/10.1016/0025-326X(86)90801-5.

Garcia-Fernandez, A.J., Espin, S., Martinez-Lopez, E., 2013. Feathers as a biomonitoring tool of polyhalogenated compounds: a review. Environ. Sci. Technol. 47, 3028–3043. https://doi.org/10.1021/es302758x.

Gochfeld, M., 1980. Mercury levels in some seabirds of the Humboldt Current, Peru. Environ. Pollut. 22, 197–205. https://doi.org/10.1016/0143-1471(80)90014-8.

Gómez-Ramírez, P., Shore, R.F., van den Brink, N.W., van Hattum, B., Bustnes, J.O., Duke, G., Fritsch, C., García-Fernández, A.J., Helander, B.O., Jaspers, V., et al., 2014. An overview of existing raptor contaminant monitoring activities in Europe. Environ. Int. 67, 12–21. https://doi.org/10.1016/j.envint.2014.02.004.

Grabic, R., Fick, J., Lindberg, R.H., Fedorova, G., Tysklind, M., 2012. Multi-residue method for trace level determination of pharmaceuticals in environmental samples using liquid chromatography coupled to triple quadrupole mass spectrometry. Talanta 100, 183–195. https://doi.org/10.1016/j.talanta.2012.08.032.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42, 5807–5813. https://doi.org/10.1021/es8005173.

Hiemke, C., Hartter, S., 2000. Pharmacokinetics of selective serotonin reuptake inhibitors. Pharmacol. Ther. 85, 11–28. https://doi.org/10.1016/s0163-7258(99)00048-0.

Hughes, S.R., Kay, P., Brown, L.E., 2013. Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ. Sci. Technol. 47, 661–677. https://doi.org/10.1021/es3030148.

Jaspers, V.L.B., Voorspoels, S., Covaci, A., Lepoint, G., Eens, M., 2007. Evaluation of the usefulness of bird feathers as a non-destructive biomonitoring tool for organic pollutants: a comparative and meta-analytical approach. Environ. Int. 33, 328–337. https://doi.org/10.1016/j.envint.2006.11.011.

Jaspers, V.L.B., Covaci, A., Deleu, P., Neels, H., Eens, M., 2008. Preen oil as the main source of external contamination with organic pollutants onto feathers of the common magpie (*Pica pica*). Environ. Int. 34, 741–748. https://doi.org/10.1016/j.envint.2007.12.002.

Johnson, R.D., Lewis, R.J., Angier, M.K., 2007. The distribution of fluoxetine in human fluids and tissues. J. Anal. Toxicol. 31, 409–414. https://doi.org/10.1093/jat/31.7.409.

Kumar, A., Xagoraraki, I., 2010. Pharmaceuticals, personal care products and endocrine-disrupting chemicals in U.S. surface and finished drinking waters: a proposed ranking system. Sci. Total Environ. 408, 5972–5989. https://doi.org/10.1016/j.scitotenv.2010.08.048.

Lajeunesse, A., Smyth, S.A., Barclay, K., Sauve, S., Gagnon, C., 2012. Distribution of antidepressant residues in wastewater and biosolids following different treatment processes by municipal wastewater treatment plants in Canada. Water Res. 46, 5600–5612. https://doi.org/10.1016/j.watres.2012.07.042.

Lefebvre, M., Marchand, M., Horowitz, J.M., Torres, G., 1999. Detection of fluoxetine in brain, blood, liver and hair of rats using gas chromatography-mass spectrometry. Life Sci. 64, 805–811. https://doi.org/10.1016/S0024-3205(98)00622-5.

Love, D.C., Halden, R.U., Davis, M.F., Nachman, K.E., 2012. Feather meal: a previously unrecognized route for reentry into the food supply of multiple pharmaceuticals and personal care products (PPCPs). Environ. Sci. Technol. 46, 3795–3802. https://doi.org/10.1021/es203970e.

McNab, B.K., 1988. Food habits and the basal rate of metabolism in birds. Oecologia 77, 343–349. https://doi.org/10.1007/bf00378040.

Monteiro, S.C., Boxall, A.B., 2010. Occurrence and fate of human pharmaceuticals in the environment. Rev. Environ. Contam. Toxicol. 202, 53–154. https://doi.org/10.1007/978-1-4419-1157-5_2.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., et al., 2004. Diclofenac residues as the cause of vulture population decline in Pakistan. Nature 427, 630–633. https://doi.org/10.1038/nature02317.

Pohland, R.C., Bernhard, N.R., 1997. Postmortem serum and tissue redistribution of fluoxetine and norfluoxetine in dogs following oral administration of fluoxetine hydrochloride (Prozac). J. Forensic Sci. 42, 812–816.

QuintilesIMS Institute, 2016. Outlook for Global Medicines Through 2021.

R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Rothery, P., Wyllie, I.A.N., Newton, I.A.N., Dawson, A., Osborn, D.A.N., 2001. The timing and duration of moult in adult starlings *Sturnus vulgaris* in east-central England. Ibis 143, 435–441. https://doi.org/10.1111/j.1474-919X.2001.tb04944.x.

Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, L.B., Blazer, V.S., Norris, D.O., Vajda, A.M., 2010. Antidepressant pharmaceuticals in two U.S. effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. Environ. Sci. Technol. 44, 1918–1925. https://doi.org/10.1021/es9022706.

Shore, R.F., Taggart, M.A., Smits, J., Mateo, R., Richards, N.L., Fryday, S., 2014. Detection and drivers of exposure and effects of pharmaceuticals in higher vertebrates. Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci. 369.

Solheim, S.A., Sagerup, K., Huber, S., Byrkjedal, I., Gabrielsen, G.W., 2016. The black-legged kittiwake preen gland—an overlooked organ for depuration of fat-soluble contaminants? Polar Res. 35. https://doi.org/10.3402/polar.v35.29651.

Tukey, J., 1977. Exploratory Data Analysis. Addison-Wesley, Reading, MA.

van Zeeland, Y.R.A., Schoemaker, N.J., Haritova, A., Smit, J.W., van Maarseveen, E.M., Lumeij, J.T., Fink-Gremmels, J., 2013. Pharmacokinetics of paroxetine, a selective serotonin reuptake inhibitor, in Grey parrots (*Psittacus erithacus erithacus*): influence of pharmaceutical formulation and length of dosing. J. Vet. Pharmacol. Ther. 36, 51–58. https://doi.org/10.1111/j.1365-2885.2012.01391.x.

Whitlock, S.E., Pereira, M.G., Shore, R.F., Lane, J., Arnold, K.E., 2018. Environmentally relevant exposure to an antidepressant alters courtship behaviours in a songbird. Chemosphere 211, 17–24. https://doi.org/10.1016/j.chemosphere.2018.07.074.