The Consequences of Exposure to an Environmental Concentration of Antidepressant in the Eurasian Starling

Sophia Evelyn Whitlock

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University of York

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Abstract

Pharmaceuticals are vital to individual and societal health, yet there is growing concern regarding the effects they have on environmental health. Any pharmaceutical that is incompletely metabolised by humans can enter sewage systems following excretion. At wastewater treatment plants, many bird species are known to forage on invertebrates containing a mixture of pharmaceuticals. Psychotropic pharmaceuticals such as antidepressants, that are designed to modulate human behaviour, are predicted to elicit comparable effects in birds. In this thesis, the effects of a maximally relevant concentration of fluoxetine on ecologically relevant avian behavioural and physiological endpoints were assessed. Wild-caught Eurasian starling (*Sturnus vulgaris*), a species that forages at wastewater treatment plants, were exposed chronically to fluoxetine (2.7 µg day^{-1}) from winter to early summer, for 28 weeks. During the breeding season, male starlings sang less to and behaved more aggressively towards fluoxetine-treated than control females. Over the exposure period, control birds became less bold over time, whilst boldness was unchanging in fluoxetine-treated birds. No effects of treatment were observed on activity, exploration or neophobia. Controls regrew feathers of poorer quality during the exposure period than fluoxetine-treated individuals and the concentration of glucocorticoid metabolites in faecal samples increased over time in controls but decreased in fluoxetine-treated birds. Further, the leg skin temperature of fluoxetine-treated birds was unresponsive to changes in air temperature, whereas leg skin temperature varied with air temperature in control birds. Finally, fluoxetine was detected in all fluoxetine-treated bird tissue and feather samples analysed. In future, effects on free-living individuals and populations should be assessed, as should the effects of potentially additive environmentally relevant mixtures of antidepressants. My findings suggest that environmental concentrations of fluoxetine can alter multiple traits important for reproduction and survival in individual birds, and could consequently impact on exposed local populations.

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Author's Declaration

The work undertaken during this PhD was conducted not only at my host institution (University of York) but also at FERA Science, the Animal and Plant Health Agency and the Centre for Ecology and Hydrology.

For inclusion in this thesis, since I have written my thesis data chapters (Chapters 2-5) in research paper format as opposed to traditional thesis style, the chapters have been presented in a consistent format. The contributions of co-authors and others are shown in Table 0.1. Chapter 2 was published in an internationally peer-reviewed journal in July 2018 (online; see Table 0.1), whilst chapter 5 has been accepted for publication. The other data chapters (Chapters 3-4) are currently in preparation and will be submitted for publication to the target journals shown in Table 0.1.

Title	Chapter	Journal	Status	Contributions
Environmentally relevant exposure to an antidepressant alters courtship behaviours in a songbird	2	Chemosphere	Published online July 2018; volume 211, pages 17-24	**Supervision, review, editing:** Kathryn Arnold, Richard Shore, Gloria Pereira, Julie Lane **Statistical advice:** Flavie Vial **Radioimmunoassay advice:** Tom Pottinger
Environmentally relevant exposure to an antidepressant alters peripheral vasoconstriction but not stress-related measures	3	Target: Functional Ecology	In preparation	**Supervision, review, editing:** Kathryn Arnold, Richard Shore, Gloria Pereira, Katherine Herborn **Thermal imaging:** Katherine Herborn **ELISA advice:** Fiona Bellamy
Effects of an environmentally relevant concentration of fluoxetine on avian anxiety and stress responses	4	Target: Journal of Applied Ecology	In preparation	**Supervision, review, editing:** Kathryn Arnold, Richard Shore, Gloria Pereira, Julie Lane **ELISA advice:** Fiona Bellamy
Detecting fluoxetine and norfluoxetine in wild bird tissues and feathers	5	Environment International	Accepted for publication	**Supervision, review, editing:** Kathryn Arnold, Richard Shore, Gloria Pereira **Residues analysis assistance and data processing:** Darren sleep

Table 0.1 Shows the publication status of the papers contained in this thesis, the journals to which they are submitted or in which they are already published and contributions of others to these chapters.

I declare that the work contained in this thesis is my own and has not been submitted for an award at this or any other University. All sources are acknowledged as references.

Chapter 1: General Introduction

Background

Pharmaceuticals in the environment are a growing cause for concern. They are a threat to biodiversity and represent an additional stressor to wildlife populations that are already perturbed by a multitude of other anthropogenic stressors (Vörösmarty et al., 2010). Consumption of human pharmaceuticals is increasing, both globally and at home (QuintilesIMS Institute, 2016). In England, there were 1.7 million extra prescriptions in 2017 compared to the previous year; an increase of 0.15 % (HSCIC, 2018) and prescriptions are projected to rise further as human populations continue to grow, develop and age (Kookana et al., 2014). This has implications for concentrations of human pharmaceuticals in the environment, since patient excretion of parent pharmaceuticals and active metabolites is thought to be the biggest contributor to these contaminants (Daughton and Ternes, 1999, Heberer, 2002, Jones et al., 2005). Further, increasing medication of livestock is adding to global emissions of pharmaceuticals to the environment (Boxall et al., 2012). There has been a proliferation of research on the fate and consequences of pharmaceuticals in the environment over the last decade, made possible by increasingly sensitive analytical techniques able to detect the low concentrations present in the environment (Ferrer and Thurman, 2003).

The majority of research to date regarding the release of pharmaceuticals to the environment, alongside the subsequent environmental fate of these contaminants and their effects on wildlife, has studied freshwater ecosystems (Halling-Sørensen et al., 1998). This is unsurprising, since both human and veterinary medications are discharged into freshwater habitats by effluent from wastewater treatment plants (WWTPs), hospitals and pharmaceutical manufacturing plants (Brown et al., 2006). Discharges from municipal sewage treatment plants are particularly significant, as pharmaceuticals in human excreta are thought to be the biggest contributor to concentrations in the environment (Daughton and Ternes, 1999). By comparison to pharmaceutical contamination of freshwater ecosystems (Crane et al., 2006), there is comparatively little research into exposure routes in terrestrial habitats and the consequences for the species that inhabit them (Arnold et al., 2013, Shore et al., 2014). The focus of my thesis concerns a terrestrial exposure route, specifically exposure via foraging at WWTPs, as these sites are point sources of pharmaceutical pollution and are thus heavily contaminated sites.

Contaminants with the potential to alter behaviour, such as psychotropic pharmaceuticals, are rarely studied, despite the fact that changes to behaviour can impact on individual fitness, with potential repercussions for populations and food webs (Brodin et al., 2014, Arnold et al., 2013,

Saaristo et al., 2018). Psychotropic pharmaceuticals are defined as any medication that can affect mental state, emotions or behaviour. When investigating the effects of exposure to chemicals generally in organisms, ecotoxicology studies traditionally employ apical endpoints such as acute toxicity and reproduction. However, when studying the impacts of psychotropic pharmaceuticals in the environment on wildlife, sublethal alterations to behaviour are of particular interest, as these can still affect individual fitness (Bean et al., 2014, Brodin et al., 2014, Saaristo et al., 2017, Bertram et al., 2018, Brodin et al., 2017). Effects on behaviour are predicted because psychotropic pharmaceuticals are often designed specifically to elicit behavioural alterations in humans. Some psychotropic compounds are of particular concern as they are heavily prescribed and relatively persistent in the environment (Lajeunesse et al., 2012). Consequently, Chapter 1 concentrates firstly on psychotropic pharmaceuticals in general, before narrowing the focus to one behaviour-modulating compound, the selective serotonin reuptake inhibitor (SSRI) antidepressant fluoxetine (Prozac®), which is additionally the focus of my entire thesis. Fluoxetine has already been shown to alter behaviour in wildlife at environmental concentrations (Bean et al., 2014, Saaristo et al., 2018, Bertram et al., 2018, Martin et al., 2017).

The risk of adverse outcomes in wildlife exposed to pharmaceuticals can be attenuated by the presence of pharmaceutical mixtures in the environment. When organisms are exposed to several different compounds at once, sometimes even multiple members of the same class, additive or inhibitory effects may occur (Melvin et al., 2014, Backhaus, 2014). For example, exposure to six serotonin reuptake inhibitors in sewage effluent was found to increase the concentration of serotonin in the plasma of Goldfish (*Carassius auratus*) and to reduce anxiety behaviour (Simmons et al., 2017). However, whilst assessing the effect of realistic mixtures of pharmaceuticals in the environment on wildlife increases the "real-world" relevance of such work, compared to studying individual compounds, there are many challenges associated with the study of mixtures (Backhaus, 2014). For example, in multi-pharmaceutical mixtures, complex interactions can occur, causing inaccuracies in *in silico* predictions of the effects of such compounds on wildlife, as the magnitude of the effect can be quite different to that expected (Heys et al., 2016). Meanwhile in empirical *in vivo* or *in vitro* ecotoxicology toxicity studies, care must be taken to select a mixture that is representative of those found in the environment, e.g. in sewage. This in itself is challenging, as biomonitoring data need to be fed in so that a realistic mixture composition can be achieved (Backhaus, 2014). Therefore, my thesis is focused on an individual compound (fluoxetine) rather than a mixture.

To date, there is a great deal of literature available regarding the effects of psychotropic pharmaceuticals on mammals and to a lesser extent on fish, yet far fewer investigations into effects on birds have been conducted (Shore et al., 2014). This is of concern, since both the toxicity of pharmaceuticals and the level of sensitivity to them can vary between vertebrate taxa (Dorrestein and van Miert, 1988). Further, a review of the effects of fluoxetine on non-target organisms (e.g. wildlife such as birds), concluded that a greater understanding of such effects is required to improve environmental risk assessments (Brooks et al., 2003). Understanding the effects of pharmaceuticals in the environment on birds is important, as many common bird species are in decline across the UK. Also, certain species of bird can act as sentinels of environmental change (Shore et al., 2014), making them interesting yet mostly overlooked models for the investigation of environmental pharmaceutical exposure. Alongside the famous case of the near extinction of three species of *Gyps* vultures due to diclofenac residues (Oaks et al., 2004), avian research in this area to date has included the effects of fluoxetine on Eurasian starling (*Sturnus vulgaris*) behaviour and physiology (Bean et al., 2014), the effects of estrogenic contaminants on song and immune function (Markman et al., 2008) and nestling growth (Markman et al., 2011) in the Eurasian starling, and the biomagnification of diltiazem through a food web culminating in Osprey (*Pandion haliaetus*) (Lazarus et al., 2014). More research is now essential to better understand how pharmaceutical pollution impacts the physiology and behaviour of wild birds. Due to the paucity of existing data in this area and to the fact that WWTPs are important foraging grounds for a range of wild bird species, my thesis focuses on the impact of psychotropic pharmaceutical contaminants on wild birds. Specifically, my work aims to build on existing knowledge regarding a psychotropic contaminant of environmental concern, the antidepressant fluoxetine, which has already been shown to alter behaviour in the Eurasian starling at environmental concentrations (Bean et al., 2014). The main body of Chapter 1 is now concerned with introducing the themes that have shaped my thesis, which are as follows:

1.1 Exposure to pharmaceuticals in the terrestrial environment
1.2 Selection of a focal psychotropic pharmaceutical
1.3 The antidepressant fluoxetine
1.4 Assessing the effects of fluoxetine in captive birds
1.5 Monitoring fluoxetine exposure in wild birds

1.1 Exposure to pharmaceuticals in the terrestrial environment

Understanding so-called exposure routes, i.e. the pathways by which pharmaceuticals come into contact with wildlife, is key in order to predict which species are at risk from adverse effects. Risk

is itself a function firstly of hazard (i.e. the potential of a toxicant to cause harm) and of exposure. To understand hazard, information about the toxicity of the toxicant, its mode(s) of action (MoA; defined as changes effected at the cellular level) and its mechanism(s) of action (MOAs; defined as changes effected at the molecular level) are required. Exposure on the other hand, being a function of dose and time, concerns the concentration to which an individual is exposed and the duration of exposure. To assess the risk posed to terrestrial wildlife by psychotropic pharmaceuticals in the environment, knowledge about how and to what degree individuals are exposed is essential. Therefore, information about how pharmaceuticals enter the environment, their transport and environmental fate, and how they are taken up by biota is required.

1.1.1 Entry routes

Firstly, an appreciation of how pharmaceuticals enter the terrestrial environment (entry routes) is necessary. As mentioned earlier, pharmaceuticals excreted by humans into sewage systems are a major contributor to environmental pollution (Daughton and Ternes, 1999). Many pharmaceuticals and/or their active metabolites are not fully metabolised by human patients and are thus excreted into sewage systems, following which they are transported to WWTPs (Lienert et al., 2007). If pharmaceutical compounds are not effectively removed at WWTPs, they can be discharged into surface waters as effluent (Derksen et al., 2004, Jones et al., 2005), yet they can also enter terrestrial ecosystems via treated sewage sludge (biosolids), which is applied as fertiliser to agricultural land (Wu et al., 2010a, Jones-Lepp and Stevens, 2007). Other sources of pharmaceutical contamination in terrestrial habitats include the carcasses and waste of medicated livestock, the manure or faeces of livestock and medicated pets, and disposal of medication in landfill (Arnold et al., 2014, Shore et al., 2014). Therefore, wildlife species that forage at WWTPS or on land fertilised with biosolids, consume faeces/carcasses contaminated with veterinary medications or scavenge for food at landfill sites can be exposed to pharmaceuticals.

1.1.2 Environmental transport, fate and uptake by biota

Once pharmaceuticals have entered the terrestrial environment, knowledge about their subsequent transport and environmental fate is required. To illustrate this, I will discuss the fate of pharmaceuticals that enter the environment contained in biosolids. Biosolids (treated sewage sludge) from wastewater treatment processes are used to fertilise agricultural fields, pasture land and forestry plantations (Bramryd, 2001, Clarke and Smith, 2011). Those pharmaceuticals which are not effectively removed at WWTPs are thus transported from the freshwater aquatic to the wider terrestrial environment (Topp et al., 2008, Borgman and Chefetz, 2013). In the UK, biosolids can be applied at any time of year, but application is forbidden if fruit/vegetable crops are currently

growing on the field, according to UK regulations (DEFRA, 2006). Furthermore, for grassland, industrial, oil and cereal crops, biosolids can be applied during the growing period, with the provision that crops must not be harvested or the land grazed for at least three weeks following applications. However, the application of biosolids is limited by certain UK regulations, which stipulate that certain metals and toxicants cannot exceed certain concentrations in farmland soil. In 2010, 1,118,159 tonnes (or 79 %) of the 1,412,836 tonnes of biosolids produced in the UK were used on soils or in agriculture (DEFRA, 2012).

The pharmaceuticals in biosolids, depending on their physicochemical properties, may be transported to different matrices. For example, they can leach into groundwater, tile drainage, surface water or runoff (Topp et al., 2008, Clarke and Smith, 2011, Lapen et al., 2008). However, if the pharmaceuticals contained in biosolids tend to bind to soils by adsorption, they will persist longer in the soils to which the biosolids were applied. In the terrestrial environment, sorption is an important determinant of how persistent pharmaceuticals are likely to be in solid matrices such as soils, sediments or in biosolids themselves; or conversely how readily they will be retained in aqueous phases such as surface water. Sorption is a measure of how pharmaceuticals are partitioned between the aqueous and solid phases (the latter is known as the "sorbent") at equilibrium; for example in a soil (Tolls, 2001). The sorption coefficient (K_d) is defined as the ratio of the concentration of a contaminant adsorbed to solids (C_s) to the concentration in the water (C_{aq}) (Tolls, 2001). A higher sorption coefficient thus indicates that a contaminant is likely to be more persistent in soils, as it has a stronger propensity to bind to the sorbent. However, sorption is itself a complex process which can be influenced by factors such as sorbent pH and ambient temperature (Horsing et al., 2011). Therefore, the persistence of pharmaceuticals in the terrestrial environment can vary according to local conditions. Eventually, the pharmaceuticals contained in the biosolids that were applied to agricultural land will degrade. This can occur in a variety of different ways, for example by photolysis, hydrolysis or biotransformation by soil microbes (Topp et al., 2008), and can be influenced by factors such as soil composition and pH (Xu et al., 2009). Alternatively, the pharmaceuticals can be taken up by biota.

Pharmaceuticals can be taken up by biota from soils or surface water in agricultural land to which biosolids have been applied. For example, earthworms have been shown to take up pharmaceuticals in soils (Carter et al., 2014, Wu et al., 2015). Alternatively, pharmaceuticals may be taken up by crop plants; for example, certain compounds have been found to translocate to vegetables such as cabbage (Holling et al., 2012), soybean (Wu et al., 2010b) and cucumber (Shenker et al., 2011). Factors influencing the bioavailability of pharmaceuticals to biota, i.e. how

readily the compound can be absorbed by an organism, include sorption to solids, soil composition and whether the pharmaceutical is neutral or ionised (Wu et al., 2015, Carter et al., 2014). If pharmaceuticals are absorbed faster than they can be metabolised and/or excreted by an animal, the internal concentration in the organism will increase; a process known as bioaccumulation. Factors such as lipophilicity, defined as the affinity of a compound for fats, lipids, oils or non-polar solvents, determine the bioaccumulative potential of a contaminant (Du et al., 2014). More lipophilic compounds bioaccumulate more readily as they are more lipid soluble, so can partition to the lipid components of organisms. In the case of psychotropic medications, lipophilicity is also important because only compounds with high enough lipid solubility can cross the blood brain barrier. Once a pharmaceutical has been absorbed by an organism, it can in turn be absorbed by any organism that consumes it. In this way, pharmaceuticals can be transferred though the food web; so called "trophic transfer". Under certain circumstances, biomagnification may also occur. Biomagnification applies to situations where the concentration of a toxicant increases with increasing levels of a food chain. For example, one study found that Osprey (*Pandion haliaetus*) plasma contained concentrations of the calcium-channel blocker diltiazem 4.71 times greater than concentrations in the plasma of fish prey species (Lazarus et al., 2014). This illustrates the threat that pharmaceuticals can pose to birds that forage at pharmaceutical contaminated sites, for example piscivorous species of bird that consume fish from contaminated waters or insectivorous species that consume invertebrates from contaminated soils (Shore et al., 2014).

1.1.3 Wastewater treatment plants as exposure routes

Naturally, WWTPs, including sewage works, are themselves important exposure routes for certain wild vertebrate species, since these are key point sources from which pharmaceuticals enter the wider environment. Wildlife at risk of exposure via this route include certain bird and bat species, that are known to feed directly on invertebrates from trickling filter beds or consume flying insects that develop in wastewater treatment tanks during larval stages (Fuller and Glue, 1978, Fuller and Glue, 1980, Park and Cristinacce, 2006). During winter and the associated periods of harsh weather, sewage works are particularly important foraging grounds for certain species of bird (Fuller and Glue, 1978). Further, at WWTP sites, heavily prescribed pharmaceuticals that might degrade relatively quickly in the wider environment can behave as if they are persistent contaminants, because their continual usage by patients means they constantly flow into WWTPs; a special case known as "pseudo-persistence" (Ebele et al., 2017). The primary focus of my thesis will be exposure to psychotropic pharmaceuticals due to foraging on contaminated invertebrates from trickling filter beds at WWTPs, as this route is a particular threat due to the potential magnitude of exposure concentrations at these sites.

1.1.4 Species of bird at risk from pharmaceutical exposure

With populations of many bird species in steep decline, understanding the drivers of these declines, such as chemical contamination, is crucial (Vörösmarty et al., 2010, Saaristo et al., 2018). Since both plants and earthworms are known to take up pharmaceuticals from contaminated soils (Carter et al., 2014, Wu et al., 2010b, Holling et al., 2012), any bird species which consume these food sources from biosolid- or wastewater-contaminated sites risk exposure. This includes farmland species such as Grey Partridge (*Perdix perdix*) and Rook (*Corvus frugilegus*) that commonly forage on arable fields, which may receive applications of biosolids annually. Certain predatory birds that forage on farmland, such as the Red Kite (*Milvus milvus*), risk exposure, since earthworms can make up a significant portion of their diet. However, those species that forage on invertebrates at WWTPs are particularly at risk of exposure, due to the higher concentrations present at these sites. In countries where wastewater treatment wetlands are used as a method for treating sewage and wastewater, wading bird species also risk exposure to pharmaceuticals (Frederick and McGehee, 1994, Evans and Harris, 1994). Based on a survey of birds at 33 sewage works in the UK, the most commonly recorded species in spring and autumn were Eurasian starling (*Sturnus vulgaris*), Barn swallow (*Hirundo rustica*) and Northern lapwing (*Vanellus vanellus*), whilst in winter Eurasian starling, Northern lapwing and Black-headed gull (*Larus ridibundus*) were most abundant (Fuller and Glue, 1980). A further study at a single UK sewage works found that Eurasian starling and Pied wagtail (*Motacilla alba*) commonly forage directly on wastewater trickling filter beds during winter (Fuller and Glue, 1978). Eurasian starling are used as a model species in my thesis, due in part to their propensity to forage at WWTPs and also because they are a well-characterised model species (Asher and Bateson, 2008).

1.2 Selection of a focal psychotropic pharmaceutical

As mentioned at the beginning of this chapter, I decided to select a single psychotropic pharmaceutical for investigation in my thesis rather than a mixture. This section therefore details the selection of this focal compound for further empirical study.

1.2.1 Selection based on prioritisation

Pharmaceuticals are a diverse and varied family of chemicals and it is therefore not feasible to empirically study the environmental impact of every pharmaceutical. To identify candidate pharmaceuticals to investigate experimentally or to monitor in the environment, it is necessary to consult prioritisation literature – studies that have ranked pharmaceuticals according to their potential to cause environmental harm. There is a wide array of prioritisation methods in the literature, taking many different parameters into account, thus the corresponding results are also

rather varied. Such methods typically score pharmaceuticals based on a range of criteria, which can vary between prioritisations, before using the resultant scores to rank the compounds. The prioritisation/ranking of psychotropic pharmaceuticals in five prioritisation papers (Besse and Garric, 2008, Dong et al., 2013, Kumar and Xagoraraki, 2010, Ortiz de Garcia et al., 2013, Roos et al., 2012) is shown in Table 1.1.

One method, applied to circumstances in French surface waters, has incorporated both mammalian and human pharmacological data (Besse and Garric, 2008) in order to select unranked priority compounds. This method first calculated predicted environmental concentrations (PECs) for 120 parent compounds and 30 metabolites. Those selected for inclusion in the study were the 100 most widely prescribed pharmaceuticals in France, plus those detected in aquatic ecosystems, those reported to be highly ecotoxic in the aquatic environment and those reported to be persistent in the aquatic environment. Data on criteria such as consumption (i.e. quantity of a pharmaceutical used by a population in one country over one year), the fraction of a pharmaceutical excreted (calculated according to the metabolic pathways of each pharmaceutical), the fraction of a pharmaceutical emitted to surface waters after WWTP treatment and wastewater volume generated per person per day were fed into the PEC calculations. Next, the compounds were reviewed on a case-by-case basis and were assessed using pharmacological, mammalian toxicological and physicochemical data, alongside data on environmental transport and fate. In the third and final step, priority compounds in the same priority pharmacological/chemical class (i.e. compounds with the same mode of action (MoA)) were selected. The final prioritisation contained 40 molecules, including psychotropic medications with serotonin-reuptake inhibiting (SSRI), anticonvulsive and analgesic MoAs (see Table 1.1). Interestingly, several of the included psychiatric pharmaceuticals (tramadol, cyamemazine) had not been detected in surface waters at the time the paper was published.

Reference	Method	Psychotropic Pharmaceuticals	Ranked?
Ortiz de Garcia et al (2013)	OPBT Total Ranking (utility function)	**3.** Sertraline; **7.** N-desmethylsertraline, norfluoxetine; **11.** Escitalopram, fluoxetine, paroxetine, N-desmethyl escitalopram; **17.** Fluvoxamine; **18.** Alprazolam, sertraline carbamoyl glucutronide, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine	Yes
	OPBT Partial Ranking	**6.** Metabolite of paroxetine; **7.** Carbamazepine 10,11 epoxide; **8.** Sertraline, N-desmethyl sertraline; **9.** Escitalopram; **10.** Fluoxetine, sertraline carnamoyl glucuronide; **11.** N-desmethyl escitalopram; **12.** Carbamazepine, fluvoxamine; **13.** Carbamazepine 10,11 dihydrodiol; **14**. Lorazepam; **16.** Norfluoxetine, 2-hydroxy carbamazepine, 3-hydroxy carbamazepine; **17.** Paroxetine; **18.** Alprazolam, bromazepam, gabapentin	Yes
Besse & Garric (2008)	PEC-based; parent drugs	Carbamazepine, Cyamemazine, Fluoxetine, Oxazepam, Sertraline, Tramadol, Valproic acid	No
	PEC based; metabolites	Demethyltramadol, Norfluoxetine	No
Dong et al (2013)	All endpoints	**9.** Tramadol HCl; **14.** Bupropion; **18.** Levetiracetam; **19.** Risperidone; **20.** Citalopram HBr	Yes
	Human endpoints	**11.** Clonidine; **12.** Topiramate; **15.** Risperidone; **16.** Lamotrigine; **19.** Oxycodone HCl	Yes
	Rodent endpoints	**4.** Levetiracetam; **11.** Tramadol HCl; **13.** Gabapentin; **15.** Risperidone; **16.** Lamotrigine; **18.** Citalopram HBr	Yes
	Aquatic endpoints	**7.** Bupropion; **10.** Tramadol HCl; **11.** Hydroxyzine pamoate; **20.** Trazadone HCl	Yes
Kumar et al (2010)	Overall Score	**31.** Risperidone; **33.** Norfluoxetine; **37.** Carbamazepine; **41.** Diphenhydramine; **49.** Fluoxetine	Yes
	Occurrence	**4.** Carbamazepine; **21.** Meprobamate; **23.** Phenytoin; **35.** Diphenhydramine; **37.** Norfluoxetine	Yes
	Ecological Effects	**8.** Fluoxetine; **22.** Diphenhydramine; **33.** Risperidone; **50.** Norfluoxetine	Yes
	Human health effects	**10.** Carbamazepine; **17.** Diazepam; **18.** Phenytoin; **37.** Risperidone; **38.** Norfluoxetine	Yes

Table 1.1 (part 1): Table shows psychotropic pharmaceuticals appearing in prioritisations from five different studies (Besse and Garric, 2008, Dong et al., 2013, Kumar and Xagoraraki, 2010, Ortiz de Garcia et al., 2013, Roos et al., 2012). For ranked prioritisation methods, the position of the compound in the ranking is displayed. The method used for each prioritisation is also displayed. Note that OPBT is occurrence, persistence, bioaccumulation, toxicity; PBT is persistence, bioaccumulation, toxicity; PEC is predicted environmental concentration; FPM is fish plasma model; PNEC is predicted no effect concentration; MEC is measured environmental concentration; PNEC is predicted no effect concentration; Cooper aq env is the Cooper aqueous environment method (Cooper et al., 2008); CEC is critical environmental concentration; QSAR is quantitative structure-activity relationship. Only pharmaceuticals in the top 20 were considered for ranked lists in Roos et al., Ortiz de Garcia et al. and Dong et al., whereas the top 50 were considered for Kumar et al.

Reference	Method	Psychotropic Pharmaceuticals	Ranked?
Roos et al (2012)	FPM	**2.** Fluphenazine; **4.** Loratidine; **11.** Sertraline; **14.** Dextropropoxyphene; **15.** Meclozine; **16.** Clomipramine; **17.** Duloxetine; **18.** Levomepromazine	Yes
	Sold kg/year	**13.** Valproic acid; **14.** Gabapentin; **15.** Carbamazepine; **16.** Tramadol	Yes
	PEC/PNEC	**3.** Sertraline; **12.** Amitriptyline; **13.** Fluoxetine; **15.** Chlorprothixene; **20.** Propofol	Yes
	MEC/PNEC	**4.** Fluvoxamine; **6.** Sertraline; **8.** Fluoxetine; **11.** Citalopram; **16.** Carbamazepine; **18.** Mirtazapine; **19.** Loratidine	Yes
	Cooper Aq env	**1.** Fluoxetine; **10.** Diazepam; **11.** Paroxetine;**12.** Amitriptyline; **13.** Carbamazepine; **14.** Risperidone; **15.** Codeine; **16.** Phenobarbital	Yes
	CEC	**4.** Loratidine; **5.** Clemastine; **6.** Azelastine, buprenorphine; **12**. Flupentixol; **13.** Meclozine; **17.** Haloperidol; **19.** Levomepromazine; **20.** Pizotifen	Yes
	PBT	Bromocriptine; Citalopram; Clemastine; Ketotifen; Nortriptyline; Tropisetron	No
	QSAR	**6.** Haloperidol; **18.** Pizotifen	Yes
	logP	**5.** Fluphenazine	Yes

Table 1.1 (part 2): Table shows psychotropic pharmaceuticals appearing in prioritisations. See previous page for full table legend.

Another method for the prioritisation of prescription pharmaceuticals applied in the US (Dong et al., 2013) used a so called "Toxic Load" calculation, whereby mass loading (calculated based on the mass prescribed per year, the fraction excreted unchanged, and the fraction emitted from WWTPs unchanged) for each pharmaceutical was divided by its individual toxicity threshold (various toxicity data were used for this parameter, for example: rat LD50, daphnid LD50, adult human minimum initial dose). Since the study fed data on human, rodent and aquatic toxicity endpoints into the mass toxic load calculation, the compounds were ranked according to their toxicity over all endpoints and also over human, rodent and aquatic endpoints respectively. The approach was applied to the 200 most heavily prescribed pharmaceuticals in the US in 2009 and the output was ranked, with the results for the top 20 priority pharmaceuticals reported. The reported psychotropic priority compounds differed greatly from those reported by (Besse and Garric, 2008), although the opiate analgesic tramadol was identified by both methods. The psychotropic pharmaceuticals contained within the top 20 pharmaceuticals ranked by this study are displayed in Table 1.1.

Another prioritisation method, for ranking pharmaceuticals and personal care products (PPCPs) in US surface waters (Kumar and Xagoraraki, 2010) used a comprehensive ranking system based on four criteria (occurrence, treatment, ecological effects, health effects) and seven weighted

attributes. Under the occurrence criterion were the attributes prevalence (frequency of detection in water from various aquatic environments) and magnitude (concentration in water from various aquatic environments). The treatment criterion had just one attribute, related to the removal efficiency at a WWTP. Bioaccumulation (scored based on lipophilicity) and ecotoxicity effects (toxicity data such as LC50 for aquatic species such as fish and daphnids) were the attributes under the ecological effects criterion. Finally, under the health effects criterion were the attributes pregnancy category and then effects sub-attributes, the latter concerning factors such as mutagenicity, carcinogenicity and central nervous system activity. 100 compounds comprising pharmaceuticals, personal care products and endocrine disrupting chemicals, were assessed and ranked according to three of the criterions (occurrence, ecological effect, health effects) and overall. The antidepressant fluoxetine ranked highly in the ecological effects category (see Table 1.1). Although this prioritisation method was very comprehensive in principal, performance would have been improved by balanced water data from across the US, as there were many data absences.

A Spanish study used quite a different approach to rank pharmaceuticals and personal care products (PPCPs) (Ortiz de Garcia et al., 2013). Firstly, 96 parent compounds and metabolites were selected for study on the basis of human consumption, occurrence in Spanish aquatic environments and results of previous risk assessment studies. These 96 were then assessed according to OPBT indices: occurrence, persistence, bioaccumulation, toxicity. Data such as human consumption, prescription quantities, human excretion and WWTP removal efficiency fed into the occurrence criterion, whilst Quantitative study of Structure and Activity Relationships ((Q)SAR) were used to calculate PBT indices. A partial ranking (not based on geographical factors) and a total ranking, including occurrence, were then proposed. These rankings were decided upon using the Decision Analysis by Ranking Techniques (DART) tool, a technique recommended by the European Commission. Many SSRI antidepressants and anticonvulsive medications features heavily in the final rankings (see Table 1.1).

A comparative study from Sweden fed data on 582 pharmaceutically active compounds into nine different existing ranking systems (Roos et al., 2012). The nine methods were as follows: 1) fish plasma model; a method that compares human therapeutic plasma concentrations to calculated steady state plasma concentrations in fish, 2) predicted environmental concentration (PEC)/predicted no effect concentration (PNEC); where PNEC is the concentration below which adverse effects are not likely to occur, 3) Swedish sales statistics (kg $year^{-1}$), 4) aquatic environment ranking; takes factors such as surface water concentrations, environmental half-life and aquatic organism toxicity into account ((Cooper et al., 2008); Cooper aq env in Table 1.1), 5) critical

environmental concentration (CEC); similar to FPM but does not take exposure/PEC into account, 6) PBT score ranking (persistence, bioaccumulation, toxicity), 7) QSAR (quantitative structure-activity relationship models); common mode-of-action (narcosis) assumed, 8) logP values; octanol-water partition coefficient, relates to lipophilicity, 9) pregnancy categories; related to the risk posed by a pharmaceutical to a developing child *in utero*. For each of these 9 methods, the 32 most highly ranked compounds were reported. The performance of these nine systems was then assessed by comparing the results of each for seven pharmaceutical compounds known to be hazardous in the environment. This investigation found that risk-based methods were the most effective at correctly identifying the seven known hazardous substances, with the fish plasma model (FPM) also performing well. As one would expect, the nine different methods in this study resulted in a wide range of psychotropic pharmaceuticals being identified, with SSRIs and anticonvulsive medications again appearing frequently, as well as tricyclic antidepressants and benzodiazepines (see Table 1.1).

Due to the differing methods and results of these five prioritisation studies, I have used the results from all five to determine which psychotropic pharmaceuticals were most frequently identified and thus highest priority for empirical investigation. The top five pharmaceuticals, in terms of frequency of appearance in the five papers (see Table 1.1), are shown in Table 1.2 below.

Pharmaceutical name	No. rankings listing pharmaceutical	No. prioritisations listing pharmaceutical	UK prescription items in 2016 (thousands)	Pharmaceutical class
Carbamazepine	9	4	2 370.7	Anticonvulsive
Fluoxetine	8	4	6 594.1	SSRI antidepressant
Risperidone	8	3	1 722.4	Antipsychotic
Sertraline	6	3	11 222.9	SSRI antidepressant
Tramadol	5	3	7 331.6	Opiate analgesic

Table 1.2: Shows the top five most frequently occurring pharmaceuticals in the five prioritisation papers reviewed (Besse and Garric, 2008, Dong et al., 2013, Kumar and Xagoraraki, 2010, Ortiz de Garcia et al., 2013, Roos et al., 2012). Specifically, table shows how many rankings within the five papers each pharmaceutical was listed in (out of 22 rankings total across the five papers), how many of the prioritisation papers the pharmaceutical was listed in (out of 5 papers), 2016 UK prescription data from the HSCIC Prescription Cost Analysis (HSCIC, 2018) and the class of the pharmaceutical. The compounds are displayed in descending order of occurrence in the lists.

1.2.2 Selection based on occurrence at WWTPs and in the wider terrestrial environment

Prioritisation methods often take observed concentrations of pharmaceuticals and occurrences in the environment into account. However, the data such studies employ often pertain only to the aquatic environment. For my thesis, an important criterion for selecting a focal pharmaceutical for

further study is whether there is evidence that it occurs at measurable concentrations in the terrestrial environment, particularly at WWTPs where birds forage. Surveys conducted to monitor the presence of multiple pharmaceuticals in the environment are useful for providing information about the prevalence of certain compounds. However, such surveys of the occurrence of pharmaceuticals in the UK, particularly in soils, sewage sludge or treated biosolids, are few and limited. There are some data available on the occurrence of pharmaceuticals in UK freshwaters. A survey of UK groundwater, part of a national monitoring programme, reported concentrations of only two pharmaceuticals: the antiepileptic carbamazepine and the tricyclic antidepressant trimipramine, both at low $\mu g\ L^{-1}$ concentrations (Stuart et al., 2011). UK drinking water data are also available from a survey (Boxall et al., 2011), but samples were analysed for only 17 pharmaceuticals. Of these 17, the psychotropic compounds caffeine, carbamazepine and the active metabolite of carbamazepine (carbamazepine-10,11-epoxide) were detected in both treated and source drinking water at the $ng\ L^{-1}$ level. A more comprehensive UK-based study investigated concentrations of 55 pharmaceuticals in WWTP influent, effluent and surface waters over five months (Kasprzyk-Hordern et al., 2009); yet even this larger study only reported concentrations of five psychotropic medicines (codeine, tramadol, carbamazepine, gabapentin, amitriptyline; detected at concentrations of $ng\ L^{-1}$ to $\mu g\ L^{-1}$). To select psychotropic pharmaceuticals that pose a risk to terrestrial ecosystems, concentrations in raw sewage sludge and treated biosolids are important. One sewage sludge survey detected fluoxetine (Jones et al., 2014) at hundreds of $\mu g\ kg^{-1}$ level in UK samples, however the samples were not analysed for any other psychotropic pharmaceuticals. The paucity of UK survey data illustrates two common problems: firstly, that compounds selected for analysis in any particular study or survey are limited and variable, and secondly, by consequence, that psychotropic pharmaceuticals are not always selected for analysis. Due to the dearth of UK based data, it is necessary to use data from other comparably developed countries, such as EU members or North America, to build a picture of which psychotropic pharmaceuticals are likely to be the most widespread at WWTPs and in the wider terrestrial environment in the UK. For example, in a US survey of contaminants in biosolids destined for land application, $\mu g\ L^{-1}$ concentrations of fluoxetine, diphenhydramine and carbamazepine were reported (Kinney et al., 2006), whilst a study focused on psychotropic pharmaceuticals and conducted in Korea reported contamination of sewage sludge by 16 psychotropic medications at the $ng\ g^{-1}$ level (Subedi et al., 2013). Another survey of sewage sludge in Spain reported concentrations of carbamazepine and caffeine at concentrations of $\mu g\ kg^{-1}$ (Nieto et al., 2010). By leveraging survey data from a range of different countries with comparably advanced healthcare systems, psychotropic compounds that are frequently detected can be identified.

Another issue, which is considered by some prioritisations, is how efficiently individual psychotropic pharmaceuticals are removed at WWTPs, thereby ending up in biosolids that will enter the wider terrestrial environment. For example, in one study evaluating the removal of 14 antidepressants/antidepressant metabolites and carbamazepine from five WWTPs in Canada, mean removal rates varied from around 60 % for the antidepressant mirtazapine to under 10 % for the desmethyl metabolite of the serotonin norepinephrine reuptake inhibitor (SNRI) venlafaxine (Lajeunesse et al., 2012). Another study investigated the effectiveness of anaerobic digestion, a common sewage treatment method, for removal of five SSRIs (Bergersen et al., 2012). This found large differences in removal rates, with paroxetine reduced by 98 % after 24 days but, at the other end of the scale, fluoxetine only reduced by 32 %. In contrast, another study found that 21 days of composting sewage sludge led to a greater decrease in fluoxetine (57 % recovered) than of paroxetine (61 % recovered) (Vasskog et al., 2009). This huge variation, even within members of the same pharmaceutical class, illustrates the potential for certain pharmaceuticals to concentrate as the raw sewage is converted to biosolids. This indicates that WWTP influent and effluent water concentrations are not always reliable indications of the concentrations that will end up in treated biosolids destined for land application.

Another important factor in selecting a focal compound for my study is how persistent psychotropic pharmaceuticals are in biosolids, sludge or once applied to agricultural land. For example, all three of the psychotropic pharmaceuticals investigated in one persistence study were found to be persistent in soils, due to low rates of leaching and other dissipation processes (Wu et al., 2010c). SSRIs such as fluoxetine (Redshaw et al., 2008) and sertraline (Li et al., 2013), antihistamines such as diphenhydramine (Topp et al., 2012), benzodiazepines such as diazepam (Redshaw et al., 2008) and antiepileptics such as carbamazepine (Wu et al., 2010c) have all been found to persist for many days in soils, once applied as biosolids. The implications in terms of exposure risk are significant, since those pharmaceuticals which persist in soil for a long time may present chronic exposure risks to organisms. Further, at WWTPs themselves, many heavily prescribed pharmaceuticals exhibit "pseudo-persistence" as they are being continually replenished on site by incoming influent waters (Ebele et al., 2017). If psychotropic pharmaceuticals are widespread in sludge, inefficiently removed at WWTPs and are persistent, then they merit further investigation.

1.2.3 Final selection of focal pharmaceutical

Based on the results in Table 1.2 and the considerations I have discussed in sections 1.2.1 and 1.2.2 of this chapter, I now discuss selection of a focal psychotropic pharmaceutical for further study in my thesis, based on the five candidates in Table 1.2. This section includes more detailed information

about these five candidates, particularly the selected focal pharmaceutical fluoxetine, in terms of their occurrence and persistence at WWTPs and in the terrestrial environment, and also regarding their occurrence in prey items and free-living vertebrates.

Fluoxetine: The pharmaceutical selected as a priority for further investigation in my thesis is the SSRI antidepressant fluoxetine. Fluoxetine appeared frequently in the prioritisations reviewed (Table 1.1) and is the third most heavily prescribed pharmaceutical identified in Table 1.2. Further, norfluoxetine, the active metabolite of fluoxetine, appears in eight of the 22 of the rankings shown in Table 1.1. There is existing evidence that fluoxetine can be taken up by the prey items of insectivorous birds, indicating that such birds foraging at WWTPs could be exposed. For, example it has been detected in earthworm (*Eisenia fetida*) samples from UK WWTPs at concentrations of up to 53.8 ng g^{-1} (see (Bean et al., 2017), Supporting Data S1). Further, an uptake and depuration study has shown that fluoxetine has a pore-water-based bioconcentration factor (BCF) of 30.8-43.76 in *E. fetida,* compared to 1.14-2.2 for the antiepileptic carbamazepine (Carter et al., 2014, Carter et al., 2016), another of the pharmaceuticals commonly identified in the prioritisations. Although relatively small (<100), the BCF for fluoxetine is nonetheless many times greater than that of carbamazepine. Similar results were reported in another earthworm species, *Lumbricus terrestris*, with $BCF_{pore\text{-}water}$ 16.87 for fluoxetine compared to just 1.72 for carbamazepine (Carter et al., 2016), indicating that fluoxetine is more likely to bioaccumulate in the prey species of insectivorous birds than carbamazepine.

At WWTPs, fluoxetine has been detected in influent and effluent, typically at concentrations in the order of ng L^{-1} through to hundreds of ng L^{-1} (Vasskog et al., 2006, Vasskog et al., 2008, Metcalfe et al., 2010, Lajeunesse et al., 2012). Fluoxetine has also been widely detected in sewage sludge/biosolids at the ng kg^{-1} level (Niemi et al., 2013) through to the µg kg^{-1} (Lajeunesse et al., 2011, Jones et al., 2014, Kinney et al., 2006) or even mg kg^{-1} level (Kinney et al., 2006). Importantly, fluoxetine can also accumulate in the tissues of biota and reach the brain: the intended site of drug action. For example, in three species of fish (Channel catfish, *Ictalurus punctatus;* Black crappie, *Pomoxis nigromaculatus;* Bluegill, *Lepomis macrochirus*) from an effluent-dominated stream, fluoxetine and its active metabolite norfluoxetine were detected in tissues at concentrations of ng g^{-1}, with highest concentrations in brain tissue (Brooks et al., 2005). In a separate study, fluoxetine and norfluoxtine were detected in brain tissue from another species of fish (White sucker, *Catostomus commersoni*) sampled from two effluent-impacted streams (Schultz et al., 2010), although concentrations were roughly an order of magnitude lower than those Brooks et al. detected (low ng g^{-1}). In terms of persistence within the terrestrial environment, the half-life of

fluoxetine in agricultural soils appears to be at least 60 days, since the compound consistently failed to degrade during 60 day experiments (Monteiro and Boxall, 2009) and could still be detected in samples in its ionised form after >200 days during another experiment (Redshaw et al., 2008). A further study has experimentally estimated the half-life to be >1000 days (Walters et al., 2010). At WWTPs, firstly due to its high prescription rates (see Table 1.2) and also since patients typically take a daily dose over a long time period (Joint Formulary Committee), fluoxetine can be considered a persistent contaminant due to pseudo-persistence.

Finally, since fluoxetine exerts its effect by increasing serotonin levels in the brain and since serotonin is responsible for modulating many physiological and behavioural processes (Mohammad-Zadeh et al., 2008), a wide range of adverse effects are predicted to result from exposure. In addition, there is existing evidence that environmentally relevant concentrations of fluoxetine can alter avian behaviour and physiology (Bean et al., 2014). Therefore, based on my review of prioritisation literature, UK prescription data, prevalence in the terrestrial environment and at WWTPs, evidence of both uptake into prey species and free-living vertebrates at environmentally relevant concentrations and limited existing evidence of effects on wild birds, fluoxetine was selected as the focal contaminant for investigation in my thesis.

Sertraline: The second choice for further investigation was the SSRI antidepressant Sertraline, which appeared less frequently in the reviewed prioritisations than fluoxetine (another SSRI), with fluoxetine the 2nd most listed pharmaceutical and sertraline the 4th (see Table 1.2). However, sertraline is now more heavily prescribed than fluoxetine in the UK (see Table 1.2), indicating a need for further assessment of environmental harm. Although less sertraline is excreted as the unchanged parent in humans than fluoxetine, concentrations (ng g^{-1} level) of sertraline fourfold greater than those of fluoxetine have been reported in treated biosolids (Niemi et al., 2013, Lajeunesse et al., 2012). Further, concentrations of sertraline up to fivefold higher than those of fluoxetine have also been reported in sewage influent/effluent waters, although in a minority of WWTPs concentrations of fluoxetine were marginally (<twofold) higher (Vasskog et al., 2006, Vasskog et al., 2008). Sertraline has also been found to be persistent in soil, with a mean half-life of 48, 64 and 85 days in loam, sandy loam and clay loam soils respectively (Li et al., 2013). In biota, sertraline has been found to accumulate in the muscle, liver and brain of several fish species (Channel catfish, *Ictalurus punctatus;* Black crappie, *Pomoxis nigromaculatus;* Bluegill, *Lepomis macrochirus*) in an effluent dominated stream in the US, at slightly higher ng g^{-1} concentration levels than fluoxetine (Brooks et al., 2005). In relation to birds, there has been an investigation into the effects of an environmentally relevant concentrations of fluoxetine on the behaviour of starlings

(Bean et al., 2014) but no such work has been carried out for sertraline. To date, there has been far less research into the effects of sertraline contamination on the environment and on vertebrate wildlife than there has been for its cousin fluoxetine, suggesting that this SSRI warrants further investigation. However, it was ultimately beyond the scope of my thesis to conduct an investigation into the effects of exposure to sertraline on wild birds.

Other compounds considered: The antiepileptic carbamazepine, prescribed primarily to treat epilepsy but also used to treat bipolar disorder, was listed frequently in the pharmaceutical prioritisations reviewed (Table 1.2). Carbamazepine has been commonly detected in biosolids, in a variety of different countries including the US, Spain, Korea and Canada (Kinney et al., 2006, Nieto et al., 2010, Subedi et al., 2013, Miao et al., 2005). Concentrations into the hundreds of μg kg^{-1} have been detected in treated biosolids (Miao et al., 2005) and carbamazepine can also be taken up by certain crop plants (Wu et al., 2010b, Wu et al., 2012, Shenker et al., 2011, Carter et al., 2014, Holling et al., 2012) and by earthworms (Carter et al., 2014). This suggests it may present an exposure risk to birds foraging on farmland. Furthermore, there is evidence that carbamazepine can accumulate in fish tissue, as one study found that Tilapia living in sand filters at a wastewater reclamation plant contained concentrations into the hundreds of ng g^{-1} in muscle tissue and plasma, with lower ng g^{-1} levels reported in liver (Garcia et al., 2012). Yet I discounted carbamazepine for further investigation as it is prescribed primarily as an antiepileptic and thus its potential to modulate behaviour is predicted to be less severe than for the SSRI antidepressants. Risperidone, although flagged as being a potentially hazardous contaminant in prioritisations, was discounted from being an appropriate pharmaceutical for my project, since there are currently few data available regarding its environmental prevalence and impacts on biota. Tramadol was also discounted, firstly because it was fifth of the psychotropic substances identified from prioritisation lists and secondly because available data on its prevalence in the environment were sparse and pertained mostly to aquatic rather than terrestrial contexts (Rua-Gomez and Puttmann, 2012a, Rua-Gomez and Puttmann, 2012b).

1.3 The antidepressant fluoxetine

To predict how exposure to fluoxetine in the environment might pose a risk to wildlife, including birds, a basic understanding of its clinical indications, physico-chemical, pharmacokinetics and pharmacodynamics are required. This enables the formulation of hypotheses that reflect the situations in which fluoxetine in the environment is most likely to pose a risk to wild birdss, as well as how avian behaviour or physiology might be altered by exposure.

1.3.1 Clinical indications

Fluoxetine is a commonly prescribed selective serotonin reuptake inhibitor (SSRI) antidepressant used to treat conditions such as major depression, bulimia nervosa and obsessive-compulsive disorder in humans. Very common side effects of treatment with fluoxetine include insomnia, headache, diarrhoea, nausea and fatigue whilst common side effects include appetite and weight loss, restlessness, nervousness, sleep problems, dizziness, personality changes, loss of libido and sexual dysfunction among others. Fluoxetine is typically delivered orally in humans. For major depression and obsessive-compulsive disorder, 20 mg daily is the initial dose for adults, increasing up to 60 mg per day if required, whilst 60 mg per day is the standard dose for bulimia nervosa in adults. Fluoxetine is also used to treat menopausal symptoms in women, with a recommended dose of 20 mg daily. (Joint Formulary Committee). These therapeutic effects and side effects should be taken into account when predicting effects in exposed wild birds.

1.3.2 Physico-chemical properties

Fluoxetine is weakly basic, with a pKa of 10.06 (Brooks et al., 2003) and the pH of the environment can affect how much fluoxetine is taken by aquatic vertebrates. For example, the bioconcentration factors of fluoxetine in Japanese medaka (*Oryzias latipes*) body and liver tissues were found to increase by several orders of magnitude between pH 7 and pH 9, due to the greater proportion of fluoxetine present as the lipophilic, non-ionised form as pH tends towards the pKa of fluoxetine (Nakamura et al., 2008). Based on experimental work by (Kwon and Armbrust, 2008), the sorption coefficient (log K_{oc}) of fluoxetine was found to range from 4.09 to 5.18 across two sediments and two soils, indicating “very strong” (>4.5) to “strong” (3.5-4.5) sorption in these soils/sediments, according to US EPA classification of log K_{oc} values (U.S. EPA, 2003). This may help to explain why fluoxetine is so persistent in soils (Redshaw et al., 2008). At pH levels relevant to soils in the environment, more fluoxetine will be present in its ionised, cationic form and this helps to explain the relatively low *n*-octanol-water partition coefficient log K_{ow} (1.22) and strong sorption in (Kwon and Armbrust, 2008). The N-desmethylated metabolite of fluoxetine, norfluoxetine, is an active metabolite and is also an inhibitor of serotonin reuptake. Both fluoxetine and norfluoxetine are chiral and fluoxetine is sold as a racemic mixture. The S- enantiomer of fluoxetine is a slightly more potent inhibitor of serotonin reuptake than the R- enantiomer whilst in norfluoxetine, the S-enantiomer has been found to be 20x more potent than the R- enantiomer for blocking serotonin reuptake (Fuller et al., 1992). Exposure to fluoxetine enriched with one enantiomer is a possibility under certain conditions (Evans et al., 2017) and may have implications for exposed wildlife; for example S-fluoxetine has been shown to be almost ten times more toxic to the Fathead minnow (*Pimephales promelas*) than R-fluoxetine in terms of the EC10 for growth (Stanley et al., 2007).

Understanding these physico-chemical properties and how they interact with the environment allows better predictions of uptake into biota and effects on biota to be made.

1.3.3 Pharmacokinetics of fluoxetine

The following section (1.3.3) is based on mammalian literature unless otherwise stated because such literature is plentiful, since fluoxetine is a human pharmaceutical. Pharmacokinetics can be considered in terms of absorption, distribution, metabolism and elimination, sometimes referred to as ADME. Understanding the pharmacokinetic properties of a drug in mammals should enable more accurate predictions to be made about the pharmacokinetics of the same drug in wild birds (LaLone et al., 2014).

Absorption is the process by which the pharmaceutical enters blood circulation and all oral exposure routes involve an absorption phase. Following oral ingestion, fluoxetine travels through the oesophagus to the stomach and is absorbed in the small intestine. It then crosses intestinal mucosa and enters circulation via the hepatic portal vein. The hepatic portal vein enters the liver and pharmaceuticals can be broken down to metabolites at this point, before they have a chance to enter systemic circulation, in an effect called "first-pass metabolism". Orally administered fluoxetine is almost fully absorbed from the gastrointestinal tract but undergoes hepatic first-pass metabolism, resulting in an oral bioavailability below 90 % (Altamura et al., 1994, Hiemke and Hartter, 2000). In humans, peak plasma concentration of 15-55 µg is achieved in 6-8 hours after one dose of 30-40 mg (Aronoff et al., 1984), although this may be delayed by 3-5 hours if the dose is taken with food (Lemberger et al., 1985).

In the distribution step, the pharmaceutical travels through the systemic circulation and is distributed around the body, although the physicochemical blood-brain barrier excludes many compounds from entering the brain. This barrier, which stops the blood circulation from coming into direct contact with neurons, comprises capillary endothelial cells and astrocytes. Since fluoxetine is designed to alter the neurotransmission of serotonin in the brain, it is also designed to cross the blood-brain barrier (Pardridge, 1998). Fluoxetine is lipophilic and has a highly variable volume of distribution (V_d) between 14-100 L kg^{-1} (Hiemke and Hartter, 2000), which is the highest V_d of any SSRI (Catterson and Preskorn, 1996). This indicates that fluoxetine is widely distributed to tissues throughout the body.

Metabolism is the process by which a molecule is converted to a more hydrophilic metabolite and occurs by oxidative metabolism followed by conjugation, to increase water solubility, which facilitates excretion by the kidneys. The enzymes responsible for hepatic metabolism are located in

hepatocytes on the membrane of the endoplasmic reticulum. Fluoxetine is metabolised by cytochrome P450 enzymes (CYPs), a superfamily of proteins which are responsible for the biotransformation of many pharmaceuticals. Enzymes known to catalyse the metabolism of fluoxetine and norfluoxetine are CYP2D6, CYP2D9, CYP2C19 and CYP3A4. In humans, genetic polymorphisms of all these enzymes except CYP3A4 exist, which may cause variability in the pharmacokinetics of these compounds between patients (Scordo et al., 2005). Fluoxetine is transformed to norfluoxetine by N-demethylation. Other major metabolites in humans include inactive glucuronides of fluoxetine and norfluoxetine (Altamura et al., 1994). However, note that in birds, a recent study on the Eurasian starling exposed to an environmentally relevant concentration of fluoxetine (1.58 μg $weekday^{-1}$) found that only norfluoxetine, and not the other inactive metabolites commonly detected in humans, could be detected in faeces (Bean et al., 2017).

Finally, the pharmaceutical is excreted from the body by the process of elimination. Fluoxetine is excreted both into urine (renally) by the kidneys and also via faeces. For renal excretion, 2-10 % is reportedly excreted as the parent compound (Lemberger et al., 1985, Benfield et al., 1986) whilst in the faeces, 15-16 % is reportedly excreted unchanged (Lienert et al., 2007). At therapeutic concentrations fluoxetine has a long half-life ($t_{1/2}$) of 1-4 days, whilst the active metabolite norfluoxetine has a half-life of 7-15 days (Lemberger et al., 1985). This contrasts with other SSRI parent compounds, which have elimination half-lives of around one day (Preskorn, 1997).

1.3.4 Pharmacodynamics of fluoxetine

Understanding the pharmacodynamics of a pharmaceutical, i.e. how the drug affects an organism, is vital for understanding the physiological and behavioural effects that could result from exposure. Fluoxetine is designed to increase levels of the neurotransmitter serotonin in the brain. Serotonin (5-HT) is a monoamine neurotransmitter that is thought to regulate mood and low serotonin is linked to depression. The SSRI antidepressant fluoxetine works primarily by inhibiting the reuptake of serotonin at the pre-synaptic cleft, which increases levels of serotonin (5-HT) in the brain, as shown in Figure 1.1. This is proposed to occur primarily via downregulation of the serotonin transporter protein SERT (Benmansour et al., 2002, Andersen et al., 2014). This reduction in transporter activity slows reuptake from the synaptic cleft, thereby increasing serotonin levels. The effects of fluoxetine on 5-HT receptors may also contribute to therapeutic effects. For example, fluoxetine is a 5-HT_{2C} receptor antagonist, meaning that it can bind to and block the activity of these receptors (Ni and Miledi, 1997). Fluoxetine is also an agonist of the 5-HT_{2B} receptor (Peng et al., 2014, Diaz et al., 2012) and this upregulation is thought to be important in modulating serotonergic activity. Another contribution to therapeutic effects occurs via desensitisation of 5-HT_{1A} and 5-HT_4

receptors (Vidal et al., 2009, Czachura and Rasmussen, 2000, Lesch et al., 1991). Interestingly, subchronic treatment with fluoxetine increases stimulation of 5-HT_{1A} receptors but with chronic treatment, these receptors become downregulated and desensitised (Czachura and Rasmussen, 2000). This is thought to contribute to the delay in onset of therapeutic effects observed in patients treated with fluoxetine and also the fact that some side effects occur mainly in the first few weeks of therapy. In addition, chronic fluoxetine treatment induces neurogenesis in some regions of the brain (Malberg et al., 2000, Sachs and Caron, 2015) and some effects of treatment have been reported to persist long after cessation of treatment (Csoka et al., 2008). Given the delay in onset of therapeutic effects and the potential for neurogenesis to occur after longer treatment periods, I have decided to investigate the effects of chronic exposure on behaviour and physiology in my thesis, as this is predicted to increase the likelihood of the occurrence of therapeutic mode-of-action effects.

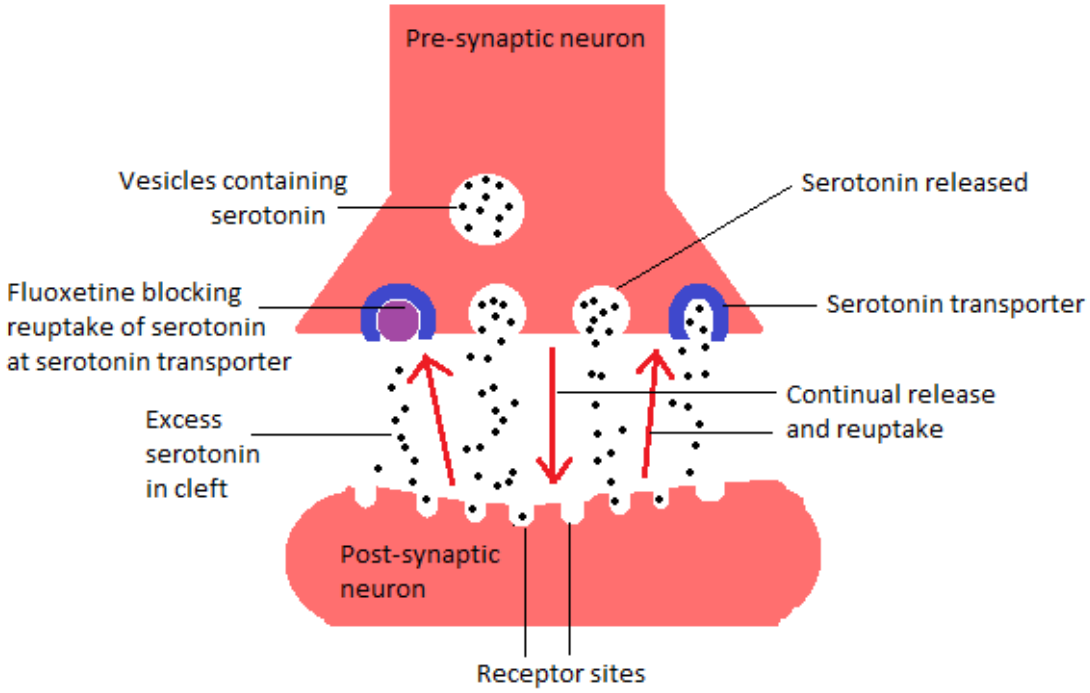

Figure 1.1 Diagram illustrating the serotonin-reuptake inhibiting mechanism of fluoxetine, leading to increased levels of serotonin in the synaptic cleft.

Aside from alteration of serotonergic neurotransmission, fluoxetine has been shown to increase circulating allopregnanolone in the brain (Fry et al., 2014, Pinna et al., 2003) and this may also contribute to therapeutic effects. Allopregnanolone is a positive modulator of the $GABA_A$ receptor, where it influences the effects of the neurotransmitter γ-amininobutyric acid (GABA).

Allopregnanolone exerts effects on mood and beahaviour, and reduced levels of allopregnanolone are associated with depression and anxiety (Schule et al., 2014). Interestingly, there is evidence that the S- enantiomers of fluoxetine and norfluoxetine can modulate allopregnanolone levels at doses an order of magnitude below those required to block serotonin uptake (Pinna et al., 2003). Indeed, fluoxetine in the environment has recently been identified as a potential neuroendocrine disruptor (Leon-Olea et al., 2014) and in mammalian studies has been reported to affect endogenous levels of various hormones (Kumsar et al., 2014, Abreu et al., 2014). Thus, studying the endocrine effects of fluoxetine makes a pertinent addition to the study of serotonin-mediated behavioural effects.

1.4 Assessing the effects of fluoxetine in captive birds

1.4.1 Existing evidence of effects

Few studies to date have investigated the effects of fluoxetine exposure at any concentration in birds. Two studies on two species of sparrow treated males with fluoxetine to investigate the role of the serotonergic system in male aggressive behaviour during the breeding season (Sperry et al., 2003, Sperry et al., 2005). However, in these studies the dose administered was 10 mg kg^{-1} delivered by injection rather than orally. Therefore, the treatment in these two studies cannot be considered environmentally relevant. Nevertheless, the first of these studies, which investigated whether serotonin modulates male aggression in captive male song sparrows (*Melospiza melodia morphna*), found that fluoxetine reduced male aggression in aggressively responding males but not activity levels (Sperry et al., 2003). In the second study, male wild American tree sparrows (*Spizella arborea*) were captured during the breeding season, injected with one dose of fluoxetine and then released. This study found that fluoxetine reduced male territorial aggression, although the effect only became apparent after 6 days post-initiation of territorial behaviour (Sperry et al., 2005). This provides some indication that fluoxetine has the potential to alter behaviour in birds. There is one existing study that has investigated whether fluoxetine affects birds at an environmentally relevant concentration. An aviary experiment in which Eurasian starling (*Sturnus vulgaris*) were exposed to 1.58 µg $weekday^{-1}$ over several months found that fluoxetine-treated birds did not feed as frequently as controls during the key foraging periods around sunrise and sunset and also that fluoxetine-treated birds seemed to habituate differently to stress compared to controls, based on the relationship between concentrations of corticosterone metabolites in faecal samples and body mass loss over two days of isolation (Bean et al., 2014). This study provides evidence that environmentally relevant concentrations of fluoxetine can alter avian behaviour and physiology when birds are exposed chronically, although the study found no effect on boldness, exploration or activity levels (endpoints relating to personality and anxiety-related behaviour). Some of the

findings of these studies may have been overly conservative due to low sample sizes, yet it should be noted that there is a trade-off between achieving the appropriate statistical power for detection of effects versus reducing the number of animals used in such experiments on ethical grounds (Burden et al., 2015).

Aside from adverse or therapeutic effects, some appreciation of the pharmacokinetics of fluoxetine in birds is needed. There is a plethora of literature considering pharmacokinetics in rodents and of course, humans. However, less is known about how psychotropic pharmaceuticals are absorbed, distributed, metabolised and eliminated in birds. In the veterinary literature, there is one pharmacokinetics study investigating the pharmacokinetic properties of another pharmaceutical from the SSRI family: the antidepressant paroxetine (van Zeeland et al., 2013). This study showed that to some extent, Grey parrots (*Psittacus erithacus erithacus*) respond similarly to humans following paroxetine treatment. For example, the pharmacokinetics of paroxetine varied widely between individuals in both humans and parrots, and the plasma time-concentration curve following intravenous administration was comparable between the two species. However, there were some appreciable differences, such as the far shorter plasma half-life in parrots (5.4 h in parrots compared to 21 h in humans). Also, the paroxetine was more bioavailable to the parrots when delivered in an aqueous solution rather than as a commercially formulated suspension, designed for humans. Whilst fluoxetine, like paroxetine, is an SSRI medication, there are differences in their pharmacokinetic profiles. Nevertheless, the paroxetine example shows that whilst there are some key biological differences between birds and mammals which cause differences in pharmacokinetics, similarities also exist. There is one study that has investigated the pharmacokinetics of an environmental concentration of fluoxetine (1.58 µg weekday^{-1}) in Eurasian starling (*Sturnus vulgaris*) (Bean et al., 2017). This study measured fluoxetine concentrations in avian tissues at 2, 26, 50 and 74 hours post-exposure and found that after chronic dosing, the final dose (1.58 µg) was distributed to and could be reliably detected in brain, liver, kidney or faeces at 2 hours post-exposure but not at 26, 50 or 74 hours. The half-lives estimated by Bean et al. were 3.1 h for brain, 8.3 h for kidney, 8.4 h for liver and 10.8 h for muscle. However, Bean et al. did not detect fluoxetine in any plasma sample analysed in the study, even at two hours post exposure. This supports the findings in Grey parrots and indicates that faster ADME of pharmaceuticals is likely in birds.

1.4.2 Predicting effects from existing data

Given the paucity of avian data, it is worth considering whether effects in humans, other mammals or other taxa can be used to predict the effects of fluoxetine exposure in birds. There have been

efforts to develop methods which can "read across" the effects of exposure to contaminants between different species or taxa. For example, the fish plasma model (FPM) assumes that pharmaceutical receptors are conserved and compares measured therapeutic plasma concentrations in humans to a predicted steady state plasma concentration in fish (Huggett et al., 2003). This model has been subsequently applied in later research (Schreiber et al., 2011), where it was validated as a useful tool for prioritisation. However, the extrapolation between human and fish pharmacokinetics and knowledge gaps regarding the details of pharmaceutical modes/mechanisms of action means that the model cannot be totally relied upon (Rand-Weaver et al., 2013). Other methods, including the Molecular Target Similarity Approach (LaLone et al., 2013), use the degree of cross-species homology to estimate whether the effects seen in humans are likely to be conserved (Brown et al., 2014). So, the more similar a drug target sequence is between humans and another organism, the more likely the mode/mechanism of action is to be conserved. This seems to be a useful approach on a broad scale but unexpected and extreme effects, as those observed in Gyps Vultures (Oaks et al., 2004), cannot be ruled out. Also reading-across from one species to another can be inaccurate, as pharmaceuticals can have wildly different effects even in closely related species. In some instances, variation in pharmacokinetics amongst species in one class (Baert and De Backer, 2003, González Canga et al., 2009) can be substantial. This has been illustrated by the fact that the Pied Crow is insensitive to diclofenac at concentrations which would be acutely toxic to *Gyps* vultures (Naidoo et al., 2011). The wealth of mammalian pharmacokinetic data from pharmaceutical development does however make "reading across" an attractive strategy for prioritisation (LaLone et al., 2014). So even though these comparison/read-across methods may be helpful in identifying problematic contaminants and in some cases predicting effects, pinpointing the exact effects of a contaminant on a particular wild bird species is not possible at the present time.

1.4.3 Endpoint selection

To assess the effects of exposure to an environmentally relevant concentration of fluoxetine on captive birds (in this case Eurasian starling), appropriate endpoints must be selected. Traditional toxicology tests such as acute toxicity tests or standard reproduction tests are not well suited to assessing the subtle, sublethal effects that a behaviour modulating contaminant such as fluoxetine is likely to exert (Arnold et al., 2013). Endpoints should instead be chosen with the therapeutic action of fluoxetine or its unwanted side-effects in mind and should also be endpoints with important consequences for fitness (Brodin et al., 2014). The latter is important to ensure that ecotoxicology studies do indeed have ecological relevance, to place the findings of captive *in vivo* studies in context within wild populations. Given that fluoxetine is designed to alter behaviour at

low concentrations, and given the consequences behavioural alterations can have for fitness and even populations (Brodin et al., 2014, Arnold et al., 2014, Saaristo et al., 2018), behavioural endpoints are particularly appropriate for fluoxetine.

To facilitate the selection of appropriate endpoints, clinical literature can be reviewed and used to predict effects in birds, based on the read-across hypothesis (LaLone et al., 2014, Brown et al., 2014). However, such literature must be used with caution, since concentrations in the environment are several orders of magnitude below those typically employed in studies of clinical relevance and some pharmaceuticals exhibit non-monotonic dose-response curves; i.e. a lower dose may not translate to an effect of lesser magnitude. This has been shown to be the case in some studies investigating the effects of environmental concentrations of fluoxetine on fish (Saaristo et al., 2017, Barry, 2013). To help solve this issue, existing vertebrate ecotoxicology, in which environmentally relevant concentrations of fluoxetine have been administered, can be reviewed (see Table 1.3) to ascertain which behaviours are most likely to be altered by environmental concentrations. However, it is important to note that sensitivity to fluoxetine appears to vary widely between different fish species in aquatic ecotoxicology studies (Sumpter et al., 2014). Thus, predicting the likely effects of environmental concentrations of fluoxetine on birds based on results from studies involving fish is likely to be of limited accuracy.

Reference	Endpoint	Species	Concentration	Exposure length	Effect details (compared to control)
(Bean et al., 2014)	Feeding	Eurasian starling (*Sturnus vulgaris*)	1.58 µg per weekday	22 weeks	Less feeding during crepuscular periods
(Bean et al., 2014)	Stress physiology	Eurasian starling (*Sturnus vulgaris*)	1.58 µg per weekday	22 weeks	Different relationship between faecal corticosterone metabolites and body mass (under isolation)
(Bertram et al., 2018)	Copulation	Mosquitofish (*Gambusia holbrooki)*	400 ng L^{-1}	30 days	Increased male copulatory behaviour in absence of competitor
(Bertram et al., 2018)	Sperm count	Mosquitofish (*Gambusia holbrooki)*	40 and 400 ng L^{-1}	30 days	Higher male sperm count
(Martin et al., 2017)	Activity	Mosquitofish (*Gambusia holbrooki)*	25 ng L^{-1}	28 days	More active
(Martin et al., 2017)	Anti-predator behaviour	Mosquitofish (*Gambusia holbrooki)*	25 and 226 ng L^{-1}	28 days	Entered predator strike zone more rapidly and froze for less time
(Saaristo et al., 2017)	Anti-predator behaviour	Wild guppy (*Poecilia reticulata*)	16 ng L^{-1}	28 days	Froze for longer and spent more time under cover
(Saaristo et al., 2017)	Activity	Wild guppy (*Poecilia reticulata*)	16 ng L^{-1}	28 days	Less active
(Saaristo et al., 2017)	Exploration	Wild guppy (*Poecilia reticulata*)	4 ng L^{-1}	28 days	Male guppies less exploratory
(Pelli and Connaughton, 2015)	Anti-predator behaviour	Wild guppy (*Poecilia reticulata*)	30 and 500 ng L^{-1}	28 days	Delayed escape response
(Pelli and Connaughton, 2015)	Survival	Wild guppy (*Poecilia reticulata*)	30 and 500 ng L^{-1}	35 days	Decreased juvenile survival
(Pelli and Connaughton, 2015)	Growth	Wild guppy (*Poecilia reticulata*)	30 and 500 ng L^{-1}	35 days	Juveniles shorter and thinner

Table 1.3 (part 1): Effects of environmentally relevant concentrations of fluoxetine in various vertebrates. Table shows the endpoint, species, exposure concentration (s) and exposure length that elicited the effect and some details of the effect for each study therein. Note that concentrations pertaining to fish studies represent the latent concentration of fluoxetine in the water, unless stated otherwise, whereas the dose quantity and frequency is given for birds. All water concentrations are environmentally relevant, although note that the general limit for concentrations to be considered as such is 540 ng L^{-1} (Brooks et al., 2003) and given that this concentration was recorded in an effluent-dominated stream, this is higher than typical concentrations are predicted to be in surface waters. However, effects resulting from exposure to 1 µg L^{-1} water concentration have also been included for completeness (concentration starred), with the caveat that such high concentrations would only be expected in highly contaminated areas.

Reference	Endpoint	Species	Concentration	Exposure length	Effect details (compared to control)
(Foran et al., 2004)	Development	Japanese medaka (*Oryzias latipes*)	100 and 500 ng L^{-1}, 1 µg L^{-1} *	28 days	Higher incidence of developmental abnormalities in offspring
(Foran et al., 2004)	Reproductive physiology	Japanese medaka (*Oryzias latipes*)	100 and 500 ng L^{-1}	28 days	Increased levels of oestradiol in plasma
(Barry, 2013)	Anti-predator behaviour	Arabian killifish (*Aphanius dispar*)	300 ng L^{-1}	7 days	Greater reduction to swimming speed following predator alarm chemical exposure, slower to return to pre-exposure speed
(Weinberger and Klaper, 2014)	Reproductive behaviour	Fathead minnow (*Pimephales promelas*)	1 µg L^{-1} *	28 days	Males spent more time cleaning nest
(Weinberger and Klaper, 2014)	Anti-predator behaviour	Fathead minnow (*Pimephales promelas*)	1 µg L^{-1} *	28 days	Decreased startle reaction to mock predator
(Barry, 2014)	Anti-predator behaviour	Arabian toad (*Bufo arabicus*)	300 ng L^{-1}	14 days	Tadpole swimming speed decreased
(Abreu et al., 2014)	Stress physiology	Zebrafish (*Danio rerio*)	1 µg L^{-1} *	15 minutes	Impaired stress response (reduced whole body cortisol concentration increase following exposure to stressor)
(Mennigen et al., 2017)	Reproductive physiology	Goldfish (*Carassius auratus*)	540 ng L^{-1}	14 days	In females: decreased circulating oestradiol, increased circulating luteinising hormone and ovarian aromatase mRNA
(Mennigen et al., 2010)	Metabolism	Goldfish (*Carassius auratus*)	540 ng L^{-1}	28 days	Decreased circulating glucose
(Schultz et al., 2011)	Reproductive physiology	Fathead minnow (*Pimephales promelas*)	28 ng L^{-1}	21 days	Induced vitellogenin
(Dzieweczynski and Hebert, 2012)	Aggression	Siamese fighting fish (*Betta splendens*)	540 ng L^{-1}	5 hours	Male-male aggression
(Dzieweczynski et al., 2016a)	Boldness	Siamese fighting fish (*Betta splendens*)	500 ng L^{-1}	24 hours, then variable	Decreased male boldness
(Dzieweczynski et al., 2016b)	Boldness	Siamese fighting fish (*Betta splendens*)	500 ng L^{-1}	8 days	Decreased female boldness

Table 1.3 (part 2): Effects of environmentally relevant concentrations of fluoxetine in various vertebrates. See previous page for full table legend.

Behaviours that impact directly on survival (such as feeding and predator avoidance) and on reproduction (such as mating or reproductive success) are particularly important for individual fitness and are directly ecologically relevant (Brodin et al., 2014, Arnold et al., 2014, Wilson and Godin, 2009, Arroyo and Razin, 2006). Since fluoxetine is prescribed for anxiety disorders and causes sexual dysfunction as a major side effect, I have focused on endpoints designed to measure anxiolytic effects on birds (with corresponding implications for survival) and sexual effects (with corresponding implications for reproductive success). With this in mind, and also using the ecotoxicology findings presented in Table 1.3, I selected endpoints to use in my aviary study. The endpoints are shown in Table 1.4 and additionally include some physiological measures, which complement my chosen behavioural endpoints.

Therapeutic action or side effect	Endpoint	Ecological relevance
Anxiolytic, appetite changes, personality changes	Neophobia	Innovating to acquire food (Webster and Lefebvre, 2001), anxiety (Baugh et al., 2017)
Anxiolytic, personality changes	Boldness	Anti-predator behaviour (Wilson and Godin, 2009), dispersal (Atwell et al., 2012), feeding (Jolles et al., 2013)
Anxiolytic, personality changes	Exploration	Home range size (Minderman et al., 2010), dispersal (Atwell et al., 2012)
Anxiolytic, lethargy	Activity	Anti-predator behaviour (Archer, 1973), feeding (Bonter et al., 2013)
Weight changes	Mass loss	Flight efficiency (Freed Leonard, 1981), anti-predator behaviour (Walters Benjamin et al., 2017)
Anxiolytic	Faecal corticosterone metabolites	Integrated basal and acute stress (Goymann, 2009)
Anxiolytic	Feather regrowth	Basal stress (DesRochers et al., 2009), flight efficiency (Swaddle et al., 1999)
Anxiolytic	Peripheral skin temperature	Basal stress (Jerem et al., 2018)
Sexual dysfunction	Male courtship song	Mating success (Eens et al., 1991)
Sexual dysfunction	Intersex courtship behaviour	Mating success (Smith and Sandell, 2005)
Sexual dysfunction	Circulating testosterone	Reproductive success (Pinxten et al., 2003)
Sexual dysfunction	Circulating oestradiol	Reproductive success (Searcy and Capp, 1997)

Table 1.4: Selected endpoints that are used in my thesis during an aviary study to assess the effects of an environmental concentration of fluoxetine on wild birds. The therapeutic action or side effect (in humans) to which each endpoint corresponds, along with the ecological relevance of each endpoint, is shown.

Finally, since most vertebrate toxicology and ecotoxicology studies to date have focused on fish and mammals rather than birds, it is important that the methods used to assess chosen endpoints have relevance to birds. To this end, I have based my behavioural methods on assays developed for use specifically in birds throughout my thesis.

1.4.4 Aviary experiment overview

All of the data chapters that form my thesis were components of the same aviary experiment, which ran for approximately 9 months between mid-October 2015 and mid-July 2016. This aviary experiment involved catching wild Eurasian starlings (*Sturnus vulgaris*), bringing them into captivity and exposing them to an environmentally relevant concentration of fluoxetine. The same individuals were used throughout. The decision to use wild-caught starlings was taken to optimise the ecological relevance of my study, as the behaviour and phenotype of captive bred animals are not necessarily representative of wild individuals (Feenders et al., 2011, Carrete and Tella, 2015). Other steps that were taken towards the same goal included: a) delivering the dose via spiked waxworms (Bean et al., 2014, Markman et al., 2008) to simulate foraging on contaminated invertebrates in the wild, b) keeping the starlings in outdoor aviaries to maximise similarity to environmental conditions, c) selecting endpoints with direct ecological relevance in mind (see section 1.4.3).

Details regarding methodological aspects of the aviary study, and the data and sample collection protocols therein, are provided in the methods sections of the data chapters and in the appendices of my thesis. For visualisation of the aviary study as a whole, a flow chart is provided in Figure 1.2 which shows all the key phases of the experiment, including when data (behavioural data and avian samples) were collected, in chronological order and to which chapter of my thesis those data pertain.

October 2015: Starlings captured; acclimation (wash-out period) begins

12/11/2015: Starlings moved to permanent home aviaries

23/11/2015-24/12/2015: Baseline (pre-treatment) data collected*
Data: Faecal corticosterone metabolite levels
Chapter: 4

30/11/2015-28/12/2015: Dosing commenced*

07/12/2015-04/01/2016: Tail feathers plucked*
Data: Quality of wild-grown feathers and fluoxetine concentrations therein
Chapters: 4 and 5

11/01/2016-12/02/2016: Chronic (after 6 weeks treatment) exposure data collected*
Data: Anxiety-related behaviours (boldness, neophobia, activity, exploration), faecal corticosterone metabolite levels
Chapter: 4

22/02/2016-25/03/2016: Chronic (after 12 weeks treatment) exposure data collected*
Data: Anxiety-related behaviours (activity, exploration), faecal corticosterone metabolite levels
Chapter: 3

29/03/2016-05/04/2016: Chronic exposure (after 13-18 weeks treatment, depending on aviary) data collected
Data: Leg skin and head temperature (thermal imaging)
Chapter: 3

28/04/2016-13/05/2016: Chronic exposure (after 17-23 weeks treatment, depending on aviary) data collected
Data: Courtship behaviours (including male song), aggressive behaviours, circulating sex hormone levels (testosterone and oestradiol)
Chapter: 2

10/06/2016-12/07/2016: Chronic (27-28 weeks treatment) exposure data*
Data: Anxiety-related behaviours (boldness, neophobia, activity, exploration), faecal corticosterone metabolite levels, fluoxetine tissue concentrations
Chapters: 4 and 5

Figure 1.2: Experimental timeline for the aviary study, which underpins this thesis, showing important points/periods in chronological order. Where applicable, data collected at each point/period and corresponding chapter(s) in which the data appear are shown. Note that for entries marked with an asterisk (*), data and sample collection were staggered over five weeks (reflecting the staggered onset of dosing between the five home aviaries over five weeks).

1.5 Monitoring fluoxetine exposure in wild birds

Whilst *in vivo* laboratory studies with captive birds can provide information about adverse effects resulting from exposure to environmental concentrations of fluoxetine, knowledge is required regarding how widespread exposure to fluoxetine is in wild populations. Monitoring the presence of fluoxetine using avian plasma samples is likely to be challenging due to differences in avian metabolism, which mean that fluoxetine is apparently cleared from plasma rapidly (van Zeeland et al., 2013, Bean et al., 2017). Consequently, an exposed individual would likely need to be sampled within a narrow window following feeding at a contaminated site to allow detection of fluoxetine in plasma. Birds could potentially be sampled destructively, although such an approach raises ethical concerns and still necessitates that samples be taken before the fluoxetine is eliminated from tissues. Although in the case of avian tissues, fluoxetine appears to remain detectable for hours or even several days following exposure to an environmentally relevant concentration (Bean et al., 2017), providing a wider window for detection. This may allow the prevalence of fluoxetine in wild bird populations to be assessed to some extent, on a presence/absence basis, using samples from tissue banks (Gómez-Ramírez et al., 2014). However, monitoring exposure in this way is limited by the fact that samples are required to be collected and stored soon after death, before the fluoxetine in tissues has been metabolised.

The feathers of birds have previously been used to monitor the presence of heavy metals and persistent organic contaminants, particularly in seabirds and raptors, and contaminants tend to be stable over long periods of time once sequestered in the feather matrix (Espín et al., 2016, Garcia-Fernandez et al., 2013, Abdullah et al., 2015). Therefore, one exciting proposition is monitoring concentrations of target pharmaceuticals and their metabolites deposited in feathers. This could enable non-invasive sampling and monitoring of priority pharmaceuticals such as fluoxetine in wild bird populations. The analysis of chicken feather meal by LC-MS/MS has previously shown that various pharmaceuticals are deposited in feathers, including fluoxetine (Love et al., 2012). However problems identified in this area include ensuring that the feather surface is properly cleaned, to avoid biased results, and also the need to link plasma concentrations to feather deposition if exposure is to be properly quantified (Garcia-Fernandez et al., 2013, Bortolotti, 2010). Nevertheless, on a presence/absence basis, feathers represent an attractive solution for monitoring exposure to fluoxetine in wild populations, although note that they reflect exposure corresponding specifically to the time period during which the feather grew. Using feathers for biomonitoring would help to elucidate the magnitude of the risk fluoxetine in the environment poses to wild birds and allow crude predictions regarding the likelihood of adverse effects to be made.

1.6 Aims and objectives

The aims of the work presented in my thesis are as follows:

1) Determine whether chronic exposure to the antidepressant fluoxetine can modulate ecologically relevant behavioural endpoints, selected based on therapeutic and side effects of fluoxetine in humans, at concentrations encountered by birds foraging on invertebrates at wastewater treatment plants.
2) Assess whether mode-of-action related effects on physiology are elicited in birds exposed to an environmentally relevant concentration of fluoxetine, using endpoints selected based on effects reported in other vertebrates.
3) Determine the feasibility of feathers and other avian samples for assessing the extent of fluoxetine exposure in wild populations.

The above aims are assessed through the following objectives:

1) Determine whether chronic exposure to an environmentally relevant concentration of fluoxetine can affect avian courtship behaviours and reproductive physiology, in line with reported sexual dysfunction side effects of fluoxetine in mammals. Specifically, investigate effects on male mate choice and female attractiveness, and on sex hormones (Chapter 2).
2) In line with the anxiolytic action of fluoxetine in humans, explore whether chronic exposure to the same environmentally relevant concentration of fluoxetine alters basal stress, by measuring avian skin temperature non-invasively using thermal imaging (Chapter 3).
3) Assess effects on individual stress and anxiety–related behaviours and on stress hormone (corticosterone) in birds (Chapters 3 and 4) following chronic exposure to the same environmentally relevant concentration of fluoxetine.
4) Test whether perturbations to avian stress physiology are evident during the first weeks of exposure to the same concentration of fluoxetine by assessing differences in feather quality between fluoxetine-treated and control individuals (Chapter 4).
5) Assess the viability of various avian samples for biomonitoring fluoxetine by measuring concentrations in avian organ tissues, plasma and feathers following chronic exposure to the same environmentally relevant concentration, and evaluating the persistence of fluoxetine in these sample types (Chapter 5).

1.7 Thesis structure

My thesis comprises six main chapters. Note that chapters 2-5 have been written in research paper format rather than in the style of a traditional thesis.

Chapter 1 provides a general introduction to my thesis.

Chapter 2 demonstrates empirical evidence of fluoxetine-mediated disruption to courtship in Eurasian starling, following chronic exposure to an environmentally relevant concentration. The results of a courtship experiment, including the recording and analysis of male song, are conveyed. Measurements of sex hormones in plasma samples are also reported and potential mechanistic details of the observed treatment effects discussed.

Chapter 3 concerns work carried out to assess the impact of fluoxetine on basal stress in Eurasian starling following chronic exposure to an environmentally relevant concentration, using a combination of non-invasive techniques; primarily thermal imaging. The thermal imaging results, and whether or not they measure basal stress, are discussed.

Chapter 4 reports the results of an aviary experiment designed to assess whether chronic exposure to an environmentally relevant concentration of fluoxetine alter can anxiety-related behaviours or physiology in Eurasian starling. Various endpoints, selected based on the therapeutic action of fluoxetine, are employed. The effects observed after 6 weeks' (winter) and 27 weeks' (summer) chronic exposure are compared and discussed, whilst perturbations to physiology caused during the first few weeks of exposure are assessed using feather quality indices.

Chapter 5 reports on concentrations of fluoxetine and its active metabolite norfluoxetine in various samples harvested from Eurasian starling exposed chronically to an environmentally relevant concentration of fluoxetine during an aviary experiment. Samples analysed include internal organ tissues, plasma and feathers, with a focus on the latter. Concentrations of fluoxetine in a set feathers from wild-living Eurasian starling are also reported. The applicability of each type of sample for biomonitoring is discussed.

Chapter 6 summarises the main findings of my thesis. The implications of my findings for wild birds exposed to fluoxetine via foraging at WWTPs are discussed. Finally, challenges associated with understanding the risk antidepressants in the environment pose to wild birds are identified and discussed, and recommendations for future work are made.

1.8 Ethical note

The work described in Chapters 2-5 was approved by the ethics committees at the University of York and the Animal and Plant Health Agency, and subsequently conducted under Home Office Licence (PPL 60/4213). The Eurasia starling were captured under licences from Natural England and the British Trust for Ornithology.

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Chapter 2: Environmentally relevant exposure to an antidepressant alters courtship behaviours in a songbird

2.1 Abstract

Pharmaceuticals in the environment are a recently identified global threat to wildlife, including birds. Like other human pharmaceuticals, the antidepressant fluoxetine (Prozac®) enters the environment via sewage and has been detected at wastewater treatment plants. Birds foraging on invertebrates at these sites can be exposed to pharmaceuticals, although the implications of exposure are poorly understood. We conducted experiments to test whether chronic exposure to a maximally environmentally relevant concentration of fluoxetine (2.7 µg day^{-1}) altered courtship behaviour and female reproductive physiology in wild-caught starlings (*Sturnus vulgaris*), a species commonly found foraging on invertebrates at wastewater plants. When paired with a female over two days, males sang less and were more aggressive towards fluoxetine-treated females than controls. Fluoxetine-treated females were initially aggressive towards males, becoming significantly less aggressive by the second day. In contrast, control females expressed intermediate levels of aggression throughout. We found no effect of female treatment on female courtship behaviour. Female body condition, circulating testosterone and circulating oestradiol were unaffected by treatment and did not account for male preference. Our findings suggest that exposure to an antidepressant reduced female attractiveness, adding to growing evidence that environmental concentrations of pharmaceuticals can alter important traits related to individual fitness and population dynamics.

2.2 Introduction

Chemical contaminants are a driver of global biodiversity loss, representing an additional stressor to wildlife already under pressure from factors such as habitat loss and climate change (Novacek and Cleland, 2001). In recent years, pharmaceuticals that contaminate the environment have been identified as a potential risk to wildlife, including birds (Shore et al., 2014, Arnold et al., 2014). An extreme example of this threat was demonstrated by the deaths in India of *Gyps* vultures from diclofenac residues in cattle carcasses, which led to local population collapse (Oaks et al., 2004). Direct mortality as a result of exposure to pharmaceuticals at environmental concentrations is apparently rare, yet such contaminants can instead exert sublethal effects on wildlife (Shore et al., 2014). Psychotropic pharmaceuticals, such as antidepressants, are designed to alter behaviour at low doses and so have the potential to modulate wildlife behaviours, with implications for individual fitness and even population persistence (Brodin et al., 2014). To date, few studies have explored the behavioural effects of exposure to psychotropic pharmaceuticals, such as antidepressants, in wild terrestrial vertebrates, including birds (Bean et al., 2014).

A widely prescribed antidepressant of the selective serotonin reuptake inhibitor class, Fluoxetine (Prozac®), has been identified as a contaminant of environmental concern (Kumar and Xagoraraki, 2010). Prescriptions of fluoxetine have been rising in the UK, increasing by 19 % between 2011 and 2016 to 6.59 million items per year (HSCIC, 2018). Since approximately 24 % of fluoxetine is excreted as the parent compound by human patients (Lienert et al., 2007), fluoxetine has been detected at wastewater treatment plants in influent and effluent water at the ng L^{-1} level (Lajeunesse et al., 2012). However, one recent UK-based study reported a far greater concentration of 1310 ng L^{-1} in sewage influent (Bean et al., 2017). Fluoxetine has also been detected in sewage sludge at the µg kg^{-1} level (Jones et al., 2014) and treated sewage sludge is used as fertiliser on agricultural land, representing an important entry route to the terrestrial environment (Redshaw et al., 2008). Due to its high sorption coefficient, fluoxetine can persist in soils for many months (Redshaw et al., 2008, Arnold et al., 2014), during which time it can be incorporated into crops (Wu et al., 2010) and invertebrates (Carter et al., 2014). At WWTPs, birds and bats which forage directly on invertebrates at filter beds (Fuller and Glue, 1978, Bean et al., 2017) or airborne insects that spend their larval stages in wastewater tanks (Park and Cristinacce, 2006), risk exposure to comparatively high concentrations of pharmaceutical contaminants, such as fluoxetine. For example, earthworms (*Eisenia fetida*) taken from trickling filter beds at wastewater treatment plants contained up to 53.8 ng g^{-1} fluoxetine (Bean et al., 2017). Yet most studies to date have focused on aquatic ecosystems and species, whilst comparatively little research has investigated the impact of terrestrial exposure routes on free-living vertebrates, including birds (Arnold et al., 2014).

The consequences of such exposure in wild birds are also poorly understood. The evolutionarily ancient serotonergic system, including the primary target of fluoxetine (SERT), is well-conserved across vertebrates (Lillesaar, 2011). In line with the read-across hypothesis (Huggett et al., 2003), we might predict effects similar to those observed in humans in birds and mammals, following exposure to fluoxetine. Sexual dysfunction is a common side effect of fluoxetine in humans, causing delayed ejaculation in men, anorgasmia in women and decreased libido in both sexes at therapeutic dosages (typically 20-60 mg day^{-1}) (Higgins et al., 2010), with similar effects in rodents (after 10 mg kg^{-1} injected daily) (Matuszczyk et al., 1998, Uphouse et al., 2006, Sarkar et al., 2008). Fluoxetine has also been shown to increase circulating testosterone in depressed female human patients at therapeutic doses (Kumsar et al., 2014). However, it is challenging to extrapolate to free-living vertebrates using such data, as clinical studies often employ dosages several orders of magnitude higher than environmental concentrations. In fish, reproductive behavioural and physiological responses to environmentally relevant concentrations of fluoxetine have proven highly variable between exposure concentrations, and between and within species (Sumpter et al., 2014). Effects on the frequency of certain male courtship behaviours have been observed at ~0.5 µg L^{-1} in some species (e.g. Eastern mosquitofish (*Gambusia holbrooki*) (Bertram et al., 2018)) but not others (e.g. Siamese fighting fish (*Betta splendens*) (Dzieweczynski and Hebert, 2012)), and a far lower exposure concentration (40 ng L^{-1}) has been found to increase sperm count and reduce body condition in male Eastern mosquitofish (Bertram et al., 2018). In goldfish (*Carassius auratus*), a 14-day exposure to 0.54 µg L^{-1} fluoxetine was shown to decrease circulating oestradiol in females (Mennigen et al., 2017), whilst another study found no effect of fluoxetine (dose range 0.1 – 100 µg L^{-1}) on oestradiol in female Fathead minnows (*Pimephales promelas*) exposed for 4 weeks (Weinberger and Klaper, 2014). The same study showed that fluoxetine altered male but not female mating behaviour in Fathead minnows (Weinberger and Klaper, 2014). To put these exposures in context with environmental concentrations, the median concentration in the effluent of 162 UK wastewater treatment plants was found to be 23 ng L^{-1} (5^{th} percentile 5 ng L^{-1} , 95^{th} percentile 69 ng L^{-1}) (Gardner et al., 2012), although concentrations ranging into the hundreds of nanograms have occasionally been reported in treated wastewater (Metcalfe et al., 2010). Therefore, the exposures in these studies can be considered to reflect worst-case exposure scenarios within the aquatic environment. Nevertheless, fluoxetine exposure has the potential to alter sexual behaviour and sex hormone levels in free-living vertebrates.

Sexual behaviour, or courtship, and sex hormones have been well studied in both free-living and captive birds (Eens et al., 1991, Pinxten et al., 2003, Dawson, 2008), albeit rarely in the context of ecotoxicology, although see (Markman et al., 2008). In songbirds, male song is known to vary

according to environmental stressors such as food availability (Ritschard and Brumm, 2012) and has previously proven a sensitive endpoint for studying the effects of certain contaminants (Markman et al., 2008) and anthropogenic disturbances (Kempenaers et al., 2010). Male song is under strong sexual selection pressure and is a signal of male quality that females use to make mate choice decisions (Eens et al., 1991). However, some degree of mutual mate choice is predicted in species with biparental care (Edward and Chapman, 2011), such as the Eurasian starling (*Sturnus vulgaris*). Male starlings are selective in their mate choice and can choose females based on factors such as plumage iridescence or age (Komdeur et al., 2005). Males exercising mate choice might be expected to invest less time singing to less attractive or lower quality females; as observed in Bengalese finches (*Lonchura striata domestica*) (Heinig et al., 2014). If fluoxetine alters female reproductive behaviour or physiology in starlings and thereby alters female attractiveness, this could alter signalling of individual quality (Markman et al., 2008) by females, with associated consequences for male courtship responses and male mate choice. This could impact on individual fitness by reducing reproductive success, with predicted negative impacts at the local population level (Brodin et al., 2014).

The aim of this study was to assess whether a maximally environmentally relevant concentration of fluoxetine affected courtship behaviour or physiology in a songbird, in terms of male responses to fluoxetine-treated and control females respectively. We first investigated whether female treatment affected the following behavioural measures: a) male courtship song; b) male aggressive or courtship behaviour. We then tested whether female treatment altered female aggressive or courtship behaviour. We also determined whether treatment altered the following physiological measures in females: circulating testosterone, circulating oestradiol or body condition index. Finally, we explored whether female circulating testosterone, circulating oestradiol, body condition index, aggression or courtship behaviour accounted for variation in male behaviours.

2.3 Methods

2.3.1 Ethics statement

This work was carried out under a Home Office Licence (PPL 60/4213) and approved by ethics committees at both the University of York and at the Animal and Plant Health Agency. The birds were captured under licences from Natural England and the British Trust for Ornithology.

2.3.2 Capture and husbandry

In October 2015, we captured 24 wild Eurasian starlings (*Sturnus vulgaris*) in North Yorkshire, UK and moved them to our experimental facility. The birds were uniquely marked on arrival with a numbered leg ring (AC Hughes, Hampton Hill, UK). DNA sexing (Avian Biotech, Truro, UK) confirmed

that there were 16 females and 8 males. The birds were given four weeks to acclimate to captivity, which also allowed excretion of non-persistent contaminants. See Appendix A1 for husbandry details.

2.3.3 Experimental Treatment

All birds (males and females) were dosed from late November 2015 for 28 weeks, simulating foraging at WWTPs during winter and spring (Fuller and Glue, 1978). Eurasian starling are known to be particularly common visitors to wastewater treatment plants in the UK during spring, autumn and winter, with foraging groups of Eurasian starling (>100 individuals) recorded at 82 % of sewage works during a winter survey of birds at 33 UK wastewater treatment plants (Fuller and Glue, 1978). WWTPs are particularly important foraging grounds during periods of cold weather, as they provide reliable access to food sources (Fuller and Glue, 1978).

All starlings (i.e. both sexes) were allocated to either the control or fluoxetine-treatment group (12 per treatment) by stratified random allocation, with home aviary as the stratum. Dosing involved handfeeding every fluoxetine-treated bird each weekday with a spiked waxworm (*Galleria mellonella;* UK Waxworms Ltd, Sheffield, UK) (Bean et al., 2014, Markman et al., 2008) injected with 3.8 µg d^{-1} fluoxetine dissolved in deionised water. This was equivalent to an average daily dose throughout the dosing period of 2.7 µg d^{-1}, which was estimated to correspond to a maximal environmentally relevant dose. Our dose was calculated by assuming 100 % of the diet (50 g d^{-1} (Feare, 1984)) consisted of contaminated invertebrates containing fluoxetine at levels of 53.8 ng g^{-1}, which was the highest concentration of fluoxetine found in earthworms (*Eisenia fetida)* taken from UK WWTPs (Bean et al., 2017). This equated to a mean daily dose of 0.03 mg kg^{-1} bodyweight (using the seven day dose of 2.7 µg d^{-1}; n = 12), which is an order of magnitude less than the human therapeutic dose (0.32 mg kg^{-1} bodyweight, assuming a dose of 20 mg and bodyweight of 62 kg (Walpole et al., 2012)). A subset of randomly selected injected waxworms, analysed to confirm dose rates, contained a mean concentration of 3.71 µg per worm (15 % RSD, 76 % recovery, n = 10; see Appendix A7 for methods). Control birds were sham-dosed with a waxworm injected instead with deionised water only. For further details of experimental treatment, see Appendix A2.

2.3.4 Courtship Experiment

We conducted a courtship experiment, based on a design that has been previously used to study Eurasian starling courtship (Markman et al., 2008, Eens et al., 1993), over two weeks from 29th April 2016, reflecting the UK breeding season (Markman et al., 2008, Pinxten et al., 2003). Each male (eight in total; four fluoxetine-treated and four control) participated in two replicate trials (one per week): one trial with a control female, the other with a fluoxetine-treated female. The

primary objective of these trials was to assess the effect of female treatment and phenotype on male courtship responses, under the assumption that female phenotype was a driver of male behaviour. The order of female presentation and the order in which each male was tested in the first week were randomised. Each male underwent two trials, one in each of two test arenas. Each female (8 per treatment) was paired with only one male. We allocated birds randomly to pairs, within the constraint that pairs comprised visually unfamiliar individuals from non-neighbouring home aviaries.

Two visually, but not aurally, occluded outdoor courtship arenas were used, each containing a wooden nest box with an attached perch from which males could sing and two swinging perches in close proximity to the nest box (see Appendix A, Figure A1). We affixed a hidden condenser shotgun microphone (RØDE NTG2; RØDE Microphones, London, UK) to the top of the nest box in each arena. Nesting materials were provided on the floor and there was *ad libitum* access to food and water. A window into the arena allowed behavioural observation.

Each trial began at 14:00 on the first day and ended at 11:00 on the second day. Two pairs were tested simultaneously (one pair per arena). Before a trial started, the focal individuals were weighed and introduced to the arena. The pair was given 15 minutes to settle before sound recording began. After an additional 15 minutes the observational period began, with 30 minutes of observational data collected per pair. After 2.5 hours of sound recording, the microphones were removed and the pairs remained in the arenas overnight. The following morning, the microphones were reinstated, and the song and behavioural data collection protocol repeated. After the 2.5 hour recording period, the focal birds were captured and immediately blood sampled by jugular venepuncture for quantification of testosterone and oestradiol, before being returned to their home aviaries. For further details of the courtship experiment, see Appendix A3.

To assess the effect of fluoxetine on female attractiveness, male singing directed at fluoxetine-treated and control females was compared. Song was recorded using one solid state recorder per microphone (Marantz PMD 660; Marantz Europe, Eindhoven, The Netherlands). We used a sound analysis package (RavenPro (2011)) and followed protocols used previously (Markman et al., 2008) to analyse the recordings for number of male song bouts and total male time singing (in seconds). To be classed as song, each bout had to be longer than five seconds and contain complex or composite phrases, as opposed to simple calls. Separate bouts were defined as being at least 1.5 seconds apart.

To compare female aggressive and courtship behaviours between fluoxetine-treated and control females, and to compare male aggression and courtship behaviours directed to females of different

treatments, we counted occurrences of certain behaviours during each 30 minute observation window. Aggressive behaviour per individual was defined as the sum count of the following behaviours: displacement (accounted for approximately two thirds of the aggressive behaviours), chasing, tugging of feathers, pecking, clawing. Courtship behaviour per individual was defined as the sum count of the following behaviours: approaches (within two body lengths), perching on nest box, entering nest box, carrying of nesting material; plus singing and displaying for males only.

2.3.5 Physiological Measures

Plasma samples (collected at the end of each courtship trial) were analysed for testosterone by radioimmunoassay (RIA), following the assay protocol described in (Pottinger and Pickering, 1985). The intra-assay coefficient of variation was 8.96 % and the assay detection limit was 62.5 pg ml^{-1}. Circulating oestradiol was determined from plasma samples using an enzyme-linked immunosorbent assay (ELISA) kit (DKO003 Estradiol ELISA; DiaMetra, Milan, Italy). The intra-assay coefficient of variation for the oestradiol assay was 17.48 %, which is rather poor; probably due to limited sample volume. As such, our oestradiol data should be regarded as approximate rather than absolute. See Appendix A5 and A6 for hormone analysis methods.

To calculate an index of body condition, we could not use a method based on the residuals from a regression of mass against length as described in (Peig and Green, 2010), as the regression of body mass against length (tarsus) was not statistically significant in our sample. Instead, we used an alternative method, known as Fulton's index (K), posited to perform favourably compared to more sophisticated techniques in a recent critical appraisal (Peig and Green, 2010). This is calculated by the following formula ($K = M/L^3$; M = mass in kg, L = length in m) and has previously been used to describe body condition in birds (Saino and Møller, 1996, Møller and Erritzøe, 2003). We used the body mass of each individual (to the nearest 0.1 g) and the tarsus length (to the nearest 0.1 mm) for the calculation. Finally, we scaled the condition index by dividing by 1000, to ensure that our mixed models would converge when the body condition indices were included as predictors.

2.3.6 Statistical Analyses

All statistical analyses were performed using the software R, version 3.3.1 (R Core Team, 2016). Using R package lme4 (Bates et al., 2015), we constructed generalised linear mixed models (GLMMs) to assess the effect of treatment and other predictors on the following response variables: number (count) of male song bouts, count of male aggressive behaviours, count of male courtship behaviours, count of female aggressive behaviours, count of female courtship behaviours. We included male or female ID as a random factor, depending on whether the response variable pertained to male or female focal birds respectively. We initially included the following fixed effects

in each model: treatment (both sexes), day of experimental period (as ordered factor in ascending order; also controlled for changing photoperiod), trial phase (i.e. first or second day of trial), body condition index (both sexes), circulating testosterone (focal sex only), circulating oestradiol (focal sex only) female treatment*trial phase interaction, male treatment*female treatment interaction. For each response variable, we fitted a GLMM with either Poisson or (if overdispersed) negative binomial error structure (see Appendix A4) with a log link function. The model was reduced by iteratively removing the least significant term (based on Wald's *Z* test (Bolker et al., 2009)). Corrected Aikake's Information Criterion (AIC_c) (Burnham and Anderson, 2004) was used to select the minimum adequate model. To check whether the data met the assumptions of the models, diagnostic tests were conducted using R package DHARMa (Hartig, 2017).

We employed a linear mixed model (LMM) to analyse the continuous behavioural response variable, total male time singing (square root transformed). We transformed the response variable to improve the normality and spread of the distribution of the model residuals, in order to ensure that the assumptions of the model were met. Fixed and random effects were specified as per GLMMs. The LMM was reduced and minimum adequate model selected as per GLMMs, except that least significant predictors were identified using likelihood ratio tests. The LMM was checked for homogeneity of variance and normality of residuals.

To test whether female behavioural or physiological traits influenced male behaviours, we constructed five further mixed models. Number of male song bouts, count of male aggressive behaviours and count of male courtship behaviours were modelled using negative binomial GLMMs, whilst male total time singing (square root transformed) was modelled as an LMM. We included the following fixed effects in the models: female courtship behaviour, female aggressive behaviour, female circulating oestradiol, female circulating testosterone and female body condition index; whilst male ID was included as a random factor. Modelling followed the same process described previously for LMMs and GLMMs.

Finally, the effect of treatment on female body condition index, circulating testosterone and circulating oestradiol were assessed via Mann-Whitney U-test, with median and interquartile range reported. Our significance level for *p* values was $\alpha = 0.05$ throughout.

2.4 Results

Male treatment did not explain variation in any response variable and was removed from all of the final models below.

2.4.1 Male singing behaviour

There was a significant interaction between female treatment and trial phase (i.e. whether day one or two of the trial) on both the number of male song bouts (Figure 2.1a and Table 2.1a) and male total time singing (Figure 2.1b and Table 2.2). During the second day of the trial, males sang significantly more to control than fluoxetine females, both in terms of number of song bouts (Figure 2.1a and Table 2.1a) and total male time singing (Figure 2.1b and Table 2.2). Males also spent significantly more time singing during the second day than the first day of the trial to control females (Figure 2.1b and Table 2.2).

When we tested whether female behavioural and physiological traits explained variation in male song, we found no effect of any predictor on number of male song bouts or total male time singing ($p > 0.2$ in all cases). Full minimum adequate model outputs are reported in Appendix A, Tables A1 and A2.

Predictor	Coef. β	SE (β)	***z***	***p***
a) Number of male song bouts				
Intercept	1.97	0.55	3.59	<0.001
Female treatment	-0.83	0.28	-2.94	0.003
Trial phase	0.42	0.30	1.42	0.157
Female treatment*trial phase	-1.13	0.41	-2.75	0.006
b) Count of male aggressive behaviours				
Intercept	-0.90	0.75	-1.20	0.229
Female treatment	0.90	0.41	2.18	0.030
Day of experimental period	0.15	0.06	2.27	0.023
c) Count of male courtship behaviours				
Intercept	1.53	0.32	4.78	<0.001
Trial phase	0.66	0.24	2.73	0.006
d) Count of female aggressive behaviours				
Intercept	-0.74	0.45	-1.62	0.105
Female treatment	-0.63	0.59	-1.07	0.285
Trial phase	-0.48	0.31	-1.56	0.120
Female treatment*trial phase	-1.67	0.44	-3.78	<0.001
e) Count of female courtship behaviours				
Intercept	0.69	0.33	2.11	0.035
Female circulating oestradiol	0.01	0.01	2.68	0.007

Table 2.1: Summary of GLMM minimum adequate model outputs for the response variables: a) number of male song bouts; b) count of male aggressive behaviours; c) count of male courtship behaviours; d) count of female aggressive behaviours, e) count of female courtship behaviours. *n* = 32 per model. Table shows: coefficient estimates (β), standard errors (SE(β)), Wald's *z*-score (= β /SE(β)) and significance level *p*. GLMMs had negative binomial error distributions except for d), which had Poisson.

Predictor	Coef. β	SE (β)	*T*	χ^2	*p*
Total male time singing					
Intercept	16.06	4.06	3.96	-	-
Female treatment	-6.40	1.91	-3.35	15.05	<0.001
Trial phase	3.17	1.91	1.66	10.02	0.007
Fem. treatment*trial phase	-8.40	2.70	-3.11	8.14	0.004

Table 2.2: Summary of LMM minimum adequate model output for the response variable total male time singing (square root transformed, *n* = 32). Table shows: coefficient estimate (β), standard error (SE(β)), t-statistic, Chi-Square statistic (χ^2) and significance level *p*.

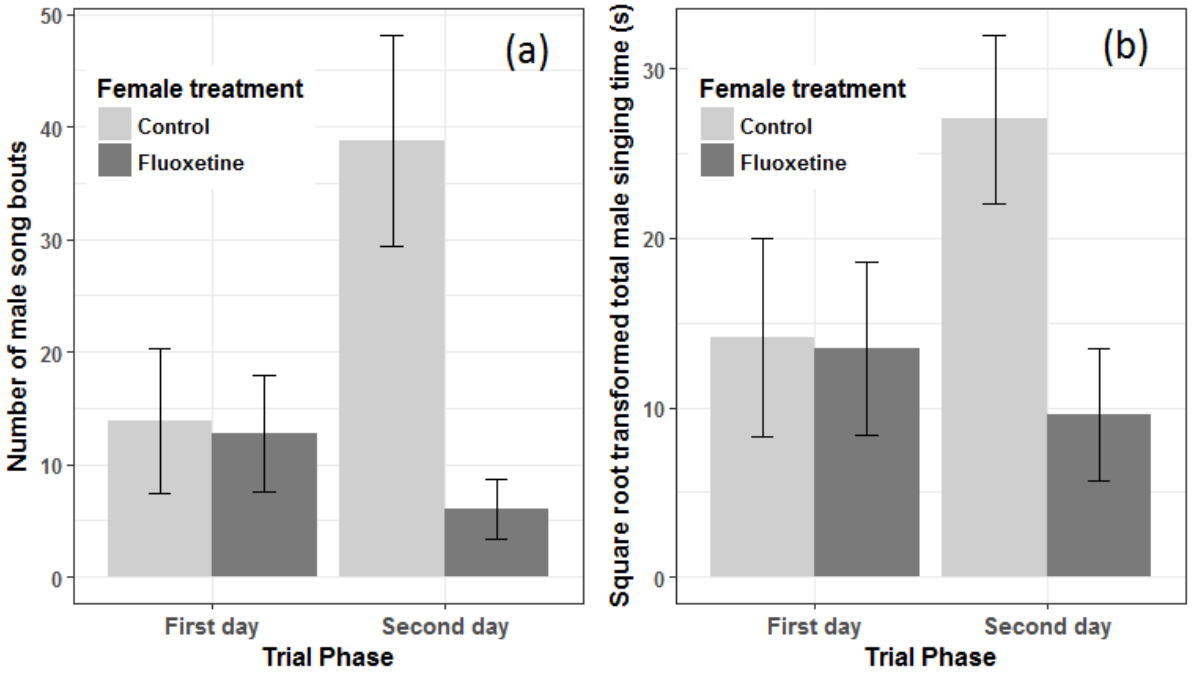

Figure 2.1. Male song behaviour shown as mean ± S.E.: (a) number of male song bouts and (b) total male time singing (s), by female treatment and trial phase (*n* = 8 females per bar). Data collected after 17-23 weeks of treatment (dependant on aviary, due to staggered onset of dosing between aviaries).

2.4.2 Male aggressive and courtship behaviours

Males displayed significantly more aggressive behaviours towards fluoxetine-treated females than controls (Figure 2.2a and Table 2.1b) and displayed more aggressive behaviours with increasing calendar date (Table 2.1b). Males displayed more courtship behaviours on the second compared to the first day (Table 2.1c). However, we found no effect of female treatment on male courtship behaviours.

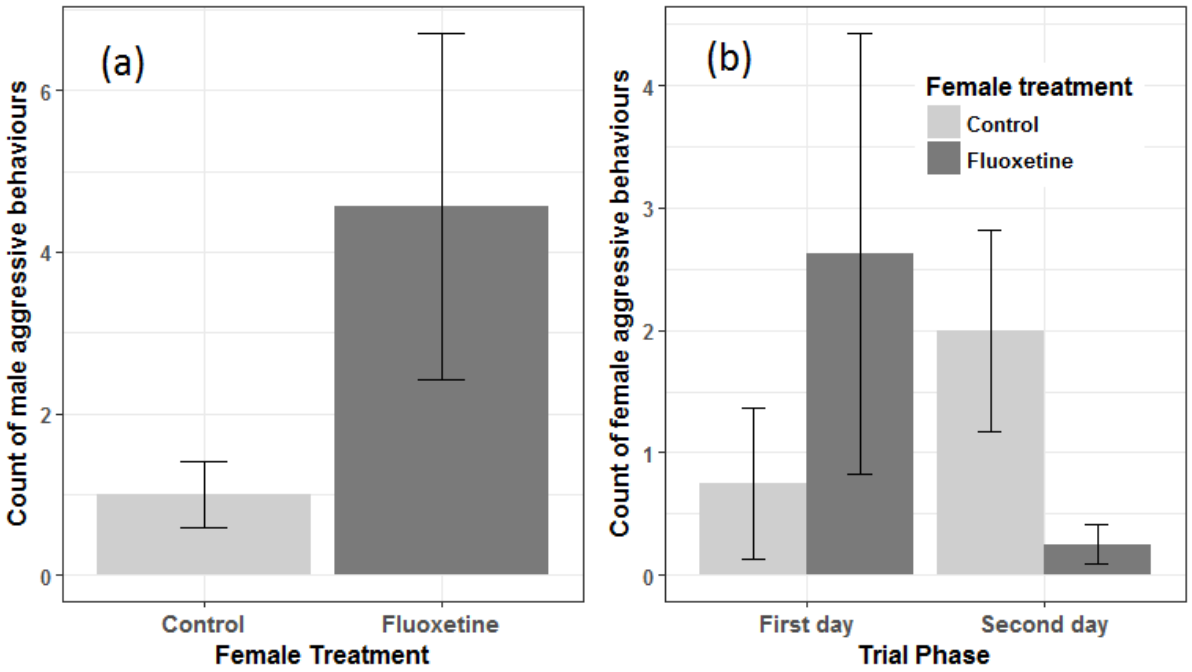

Figure 2.2. Male and female courtship interactions shown as mean ± S.E.: (a) count of male aggressive behaviours, (b) count of female aggressive behaviours. Note that displacements accounted for approximately two thirds of aggressive behaviours. (a) is split only by female treatment, as there was no significant effect of trial phase on these responses. In (a), *n* = 16 observations per bar; in (b), *n* = 8 observations per bar. Data collected after 17-23 weeks of treatment (dependant on aviary due to staggered onset of dosing between aviaries). NB. Different scaling on y-axes.

Variation in male aggressive behaviours were not explained by any female behavioural or physiological traits, although male aggressive behaviour had a borderline significant positive relationship with female aggressive behaviour (β = 0.17, SE(β) = 0.09, z = 1.90, p = 0.06). Male courtship behaviours were explained by some female traits independent of experimental treatment; males directed more courtship behaviour at females who also expressed high levels of courtship behaviour compared with females that courted less (β = 0.14, SE(β) = 0.05, z = 2.98, p = 0.003). Complete minimum adequate model outputs are reported in Appendix A, Tables A1 and A2.

2.4.3 Female aggressive and courtship behaviours

There was a significant interaction between female treatment and trial phase (i.e. whether day one or day two of the trial) on female aggression (Figure 2.2b and Table 2.1d), as fluoxetine-treated females were less aggressive during the second day of the trial compared to the first, whilst control females displayed intermediate levels of aggression throughout. There was a significant positive relationship between circulating oestradiol and female courtship (Table 2.1e), irrespective of female treatment. No other female traits explained variation in female behaviours.

2.4.4 Physiological measures

There was no effect of treatment on female circulating testosterone (Mann-Whitney test: U = 29.5, p = 0.91; fluoxetine-treated: median = 0.61 ng ml^{-1}, IQR = 1.01 ng ml^{-1}, n = 7; control: median = 0.84 ng ml^{-1}, IQR = 0.70 ng ml^{-1}; n = 8), female circulating oestradiol (Mann-Whitney test: U = 20, p = 0.60; fluoxetine-treated: median = 27.55 pg ml^{-1}, IQR = 37.33 pg ml^{-1}; control: median = 12.19 pg ml^{-1}, IQR = 42.44 pg ml^{-1}; n = 7 per group) or female body condition index (Mann-Whitney test: U = 38, p = 0.57; fluoxetine-treated: median = 3.11, IQR = 0.12; control: median = 3.17, IQR = 0.27; n = 8 per group).

2.5 Discussion

This study investigated whether a maximally environmentally relevant concentration of fluoxetine altered courtship behaviour and female attractiveness in a model songbird. We found that males directed more song bouts and spent more time singing to control than fluoxetine-treated females, particularly during the second day of the trial. Also, males behaved more aggressively towards fluoxetine-treated females than controls. Moreover, fluoxetine-treated females were more aggressive towards males on the first day of trials but became comparatively less aggressive on the second day. In contrast, control females showed intermediate levels of aggression across the two days of each trial. Male courtship behaviour increased significantly on the morning of the second day compared to the afternoon of the first day but unexpectedly was not affected by female treatment, as was male singing. This could be because the observation period (30 minutes) was insufficient to detect any effect on male courtship, whereas singing was recorded for 2.5 hours. Females and males also appeared to match their levels of courtship behaviours to each other. The observed effects were apparently independent of male treatment, since neither male treatment nor the male treatment*female treatment interaction were significant in any of the relevant mixed models. Overall, our data show a clear effect of female fluoxetine treatment on sexually selected male behaviours.

One important function of male song is to attract females (Eens et al., 1991). Copulation attempts by male starlings are typically preceded by song (Eens and Pinxten, 1990), therefore the higher number of song bouts and longer time spent singing towards control females suggests that males found control-treated females more attractive than fluoxetine-treated females. Male starlings have previously been shown to increase their song rate to females in the late morning compared to the evening (Pinxten and Eens, 1998) and we observed such an increase in singing when males were paired with control females but not when they were paired with fluoxetine-treated females. This again demonstrates reduced courtship activity towards the apparently unattractive fluoxetine-treated females.

The reduced male singing and increased aggression towards fluoxetine-treated females were not explained by females' body condition, circulating testosterone, circulating oestradiol, aggressive or courtship behaviours. To date, we have not been able to fully explain how fluoxetine-treatment altered the attractiveness of females to males. Certainly, the aggression of fluoxetine-treated females towards males decreased over time, whilst control female aggression was intermediate throughout and thus more consistent, although female aggression was not significantly related to male song variables. There was a positive relationship between female courtship behaviour and circulating oestradiol as might be expected (Searcy and Capp, 1997) and levels of male and female courtship behaviours were correlated within pairs. However, we found no effect of female treatment on female courtship behaviour or circulating oestradiol.

The effect of female treatment on male song could have been mediated by males interpreting behavioural cues and we did find evidence that fluoxetine-treatment altered female aggressive behaviour. If fluoxetine treatment also altered female behaviours relating to more general side effects of fluoxetine, such as lethargy (Uphouse et al., 2006) or changes to personality (Tang et al., 2009), this could have indirectly affected female attractiveness. Alternatively, other sexual behavioural cues than those measured in the present study might have better characterised the observed effects of fluoxetine on female attractiveness. For example, fluoxetine has been found to reduce sexual receptivity behaviours in female rats (Guptarak et al., 2010, Sarkar et al., 2008). If similar effects were observed in female starlings they might translate to reduced female receptivity to copulation, with potential consequences for breeding success, since copulations are generally female solicited (Eens and Pinxten, 1995).

In addition to behaviour, morphological or plumage cues might have been affected by fluoxetine treatment, although the contribution of visual cues to behavioural responses was not assessed in this experiment. For example, male starlings are known to select females based on their throat

feather length and iridescence. (Komdeur et al., 2005). However, this could be a signal of age rather than quality. We did not include female age in our models, as accurate ageing of starlings is challenging, but we estimated that most of our females were first year birds, with two older birds per treatment group. Therefore, it is unlikely that age accounted for the difference in male song responses towards females of different treatments. Sexually selected ornaments have been shown to be sensitive to environmental perturbations, such as exposure to contaminants (Lifshitz and St Clair, 2016). For example, exposure to pollutants can alter the expression of carotenoid and melanin pigmentation due to oxidative stress and/or endocrine disruption pathways (Lifshitz and St Clair, 2016). However, further investigation would be required in order to assess whether fluoxetine disrupts avian courtship by altering the expression of sexually selected ornaments.

During our courtship experiment, fluoxetine-treated females were initially aggressive but became less aggressive in the second compared to the first day of the trial. Generally, since there is intersexual conflict within the starling mating system, alterations to female aggression could have fitness costs (Sandell, 1998). The offspring of polygynous males receive less parental care than the offspring of monogamous males (Sandell et al., 1996). Therefore, displaying high levels of female-female aggression during the breeding season could enable a female to maintain a monogamous status (Sandell, 1998). The observed disruption to fluoxetine-treated female aggression levels was not associated with changes in testosterone levels or an effect of treatment on female testosterone, or indeed on oestradiol. This contrasts with female rats, where there is some evidence that sexual dysfunction from fluoxetine treatment results from disruption of the neuroendocrine axis (Sarkar et al., 2008, Uphouse et al., 2006, Matuszczyk et al., 1998), although the doses used in these studies were several orders of magnitude higher than our dose. Environmentally relevant concentrations of fluoxetine administered subchronically have been reported to cause endocrine disruption in fish (Mennigen et al., 2017) but since our study involved a chronic rather than subchronic exposure, direct comparison with these studies is difficult. Since we observed no endocrine effects of chronic fluoxetine treatment in females, the reduction in attractiveness could have been mediated instead by altered neurotransmission (Higgins et al., 2010). 5-HT_{1A} receptors have been suggested to play a role in fluoxetine-induced sexual dysfunction in female rats (Guptarak et al., 2010). Birds possess 5-HT_{1A} receptors (Dennis et al., 2013), presenting the possibility that female birds exposed to fluoxetine could likewise experience inhibition of sexual behaviour. In mammals, sexual dysfunction can occur following even an acute or subchronic dose (Guptarak et al., 2010, Sarkar et al., 2008), indicating a need to assess the effects of shorter exposures in passerines. In general, further work in this area should now focus on elucidating the mechanism, in terms of alterations to neurotransmission in fluoxetine-exposed

females, that results in reduced attractiveness. Such work should again collect behavioural courtship data but should also investigate whether key mode of action related targets, such as serotonin transporter (SERT) and relevant serotonin receptors (e.g. 5-HT_{1A}), are differentially expressed in fluoxetine-treated compared to control female brain tissue during the breeding season. Finally, generating a dose-response curve, ranging from low environmental concentrations through to high human dose equivalent concentrations, could be beneficial in furthering the current level of understanding of the effects of fluoxetine on behaviour and other ecologically relevant traits, and the implications of exposure in the environment. However, determining traditional threshold concentrations at which effects become apparent could be challenging for two reasons. Firstly, fluoxetine has already been shown to exhibit a non-monotonic dose-response relationship at environmental concentrations in other vertebrates (Martin et al., 2017, Saaristo et al., 2017). Secondly, a trait such as 'courtship' consists of different behaviours with different underlying mechanisms and responses are likely to be context dependent. Thus, the utility of a dose response curve in defining 'safe' environmental concentrations is likely to be limited for contaminants with sublethal effects.

In this study, we have shown that environmental concentrations of fluoxetine can alter courtship interactions in a songbird, with clear effects on male song responses towards fluoxetine-treated females. Indeed, courtship behaviour, particularly birdsong, has promise as an ecologically relevant endpoint, since song is known to signal individual quality and responds sensitively to environmental stressors, such as food availability (Ritschard and Brumm, 2012). Moreover, male song has already been successfully employed to assess the effects of exposure to environmental contaminants in wild birds in a previous study, which showed that cocktails of sewage-derived oestrogenic contaminants disrupted sexual signalling in Eurasian starlings (Markman et al., 2008). Our study was limited somewhat by low sample size. Nevertheless, we still feel our results are important because although our weight-corrected dose for each starling was only around 10 % of the human therapeutic daily dose, we still found evidence that fluoxetine treatment altered avian courtship. Interestingly, we found no physiological evidence of endocrine disruption as a mechanism for behavioural changes. This builds on evidence from another study that environmental concentrations of fluoxetine can alter avian behaviour (Bean et al., 2014), as well as reproductive and other behaviours in aquatic vertebrates (Weinberger and Klaper, 2014, Bertram et al., 2018). If the behavioural effects reported in this experiment are reflected in the wild, disrupted signalling of female quality may result, biasing male mate choice away from fluoxetine-exposed females. Such apparently subtle, sublethal effects, resulting from environmental concentrations of

pharmaceuticals, have potential to impact on exposed female fitness and even on local population dynamics (Brodin et al., 2014).

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Chapter 3: Environmentally relevant exposure to an antidepressant alters peripheral vasoconstriction but not stress-related measures

3.1 Abstract

Anthropogenic stressors, such as pollutants, have the potential to alter individual stress responsiveness or basal stress levels; essential traits for individual survival. The antidepressant fluoxetine is a prevalent pharmaceutical pollutant, since human patients excrete a portion of each dose unchanged. At wastewater plants, wild birds forage on sewage-contaminated invertebrates containing fluoxetine, particularly during winter. We exposed wild-caught Eurasian starling (*Sturnus vulgaris*) chronically to an environmentally relevant dose of fluoxetine (2.7 µg day^{-1}; roughly 10 % of the human therapeutic dose) over winter and collected data during early-mid spring. In line with the anxiolytic action of fluoxetine, we hypothesised that exposed birds would exhibit altered anxiety-related behaviour, lower faecal corticosterone metabolite concentration and higher skin temperature than control birds, since stress can cause raised core temperature accompanied by lowered peripheral temperature. However, anxiety-related behaviours were unaffected by treatment and there was only a borderline effect of treatment on faecal corticosterone metabolite levels. Unexpectedly, control birds did not have cooler legs than fluoxetine-treated individuals, and leg temperature in fluoxetine-treated birds was less responsive to air temperature than in controls. Neither faecal corticosterone metabolite levels, exploration nor activity explained variation in skin temperature. Instead, the effects seen are likely a physiological effect of fluoxetine treatment on vasoconstriction, which has previously been observed in mammals. The legs are important for avian thermoregulation. Therefore, if such effects are seen in the wild, they may impact on individual survival, since inability to regulate body temperature appropriately during harsh weather represents a serious physiological perturbation.

3.2 Introduction

Stress is a broad and complex concept that encompasses a range of behavioural and physiological responses to real or perceived threats (stressors), resulting in perturbation of homeostasis (McEwen and Wingfield, 2003). Such stressors could for example be environmental, due to predation, or social in nature. The hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system are an integral part of the stress response and control the execution of adaptive physiological and behavioural responses to stressors. For example, exposure to an environmental stressor activates the HPA axis, resulting in the secretion of glucocorticoids (principally corticosterone in birds) (Cockrem and Silverin, 2002). Increased circulating corticosterone helps birds respond to stressors under acute stress by mobilising energy stores and increasing blood glucose levels, whilst behavioural responses to acute stress include increased anxiety-related behaviour and alterations to activity levels, for example in preparation for escape (McEwen and Wingfield, 2003). When an individual is not acutely stressed, comparatively lower basal concentrations of corticosterone circulate, although these basal levels can be attenuated by periods of chronic stress (Cyr and Michael Romero, 2007). Under both acute and chronic stress, important processes that are non-essential to survival, such as reproduction and immune function, can be supressed (Segerstrom and Miller, 2004, Wingfield and Sapolsky, 2003). Thus, alterations to the stress response or increased exposure to stressors caused by anthropogenic factors could have far-reaching implications for individual fitness and consequently for populations.

Various methods have been employed to assess basal (i.e. baseline levels) and acute stress in birds, including measuring glucocorticoid levels, anxiety-related behaviours and thermal imaging of body temperature. Circulating glucocorticoid concentrations increase as a function of stressor intensity and are therefore a physiological marker of stress (Armario et al., 1986). Glucocorticoids can be measured in blood but levels can be elevated as a result of stress induced by capturing and handling, introducing bias (Millspaugh and Washburn, 2004). Alternatively, faecal samples can be analysed for glucocorticoid metabolites which provides a non-invasive and integrated measure of corticosterone levels over a period of time (Goymann, 2009) that is representative of basal and/or acute stress. However, faecal glucocorticoid levels can vary with time of day, body condition and individual variation in hormone metabolism (Millspaugh and Washburn, 2004, Goymann, 2009) and so assessment of stress is strengthened when glucocorticoid measurements are complemented by direct observations of anxiety-related behaviours. These include individual exploration of a novel environment (Huang et al., 2016); exploration has been found to correlate with risk taking behaviour (van Oers et al., 2004) and to be inversely related to faecal corticosterone metabolites, i.e. higher exploration is associated with lower HPA axis activity (Carere et al., 2003). During a novel

environment trial, the number of features visited and movement frequency are the most commonly recorded measures, with the latter also considered to be a measure of activity or locomotion (Huang et al., 2016). Activity can also be an index of acute stress, since acutely stressed individuals may respond by becoming more active (escape response), or conversely by becoming less active (freezing response) (Archer, 1973).

Thermal imaging (infrared thermography) is another non-invasive method that can be used to measure skin temperature and thus assess either acute or basal stress in endotherms. One response to acute stress in endotherms is stress-induced hyperthermia, in which core temperature rises whilst skin temperature decreases at certain locations due to peripheral vasoconstriction (Cabanac and Aizawa, 2000, Vinkers et al., 2013). Although stress-induced hyperthermia typically lasts no longer than an hour following exposure to an acute stressor, chronically increased core temperature can result from exposure to chronic (Ushijima et al., 2006) or social stress (Hayashida et al., 2010). In birds, thermal imaging can be used to measure the skin cooling associated with stress-induced peripheral vasoconstriction rather than measuring core temperature. Advantages of the technique include the ability to collect data continuously and to avoid biases induced by capture and handling stress (Jerem et al., 2015, McCafferty Dominic and Marsden, 2012). Measurement of skin temperature by thermal imaging has been successfully applied to assess acute handling stress in hens (*Gallus gallus domesticus*) and skin temperature was found to be positively associated with the intensity of the stressor (Herborn et al., 2015). Eye skin temperature, as measured by thermal imaging, has also been shown to correlate negatively with basal circulating glucocorticoids and positively with body condition in undisturbed wild Blue tits (*Cyanistes caeruleus*) (Jerem et al., 2018). Thus, thermal imaging appears to be a viable and non-invasive method for assessing both basal and acute stress. However, a recent study in hens found that chronic stress actually increased basal peripheral temperature (Herborn et al., 2018). Therefore, the use of thermal imaging to assess basal stress should be supported by the use of other measures of stress physiology or behaviour. In the case of leg skin, since bird legs are important in heat dissipation and conservation (Ward et al., 1999, Martineau and Larochelle, 1988), differences in skin temperature could reflect thermoregulatory status rather than basal stress (Jerem et al., 2018). This should be taken into consideration if differences in leg skin temperature cannot be linked to stress physiology.

The presence of pharmaceuticals in the environment poses a potential risk to wildlife and ecosystems (Arnold et al., 2014). The antidepressant fluoxetine, one of several selective serotonin reuptake inhibitor (SSRI) medications, is of particular concern since it has been widely detected in the environment (Hughes et al., 2013) and is designed to alter behaviour (Brodin et al., 2014). Certain species of bird forage and consume invertebrate prey living directly on filter beds at

wastewater treatment plants (WWTPs) (Fuller and Glue, 1978). Such prey can contain fluoxetine since approximately 24 % of a dose is excreted unchanged by patients (Lienert et al., 2007), leading to contamination of influent, effluent and sludge at WWTPs (Metcalfe et al., 2010, Lajeunesse et al., 2012). Fluoxetine is commonly prescribed to treat certain anxiety disorders and has similarly been shown to alter stress-related behaviours and physiology in non-target species at environmental concentrations. For example, wild guppies (*Poecilia reticulata*) exposed to 4 and 16 ng L^{-1} fluoxetine froze for a longer period of time than control fish following a simulated bird strike (Saaristo et al., 2017), whilst Zebrafish (*Danio rerio*) exposed for 15 minutes to 1 µg L^{-1} fluoxetine showed an impaired acute stress response, exhibiting lower whole body cortisol levels compared to untreated fish (Abreu et al., 2014). In birds, chronic exposure to 0.92 µg day^{-1} fluoxetine has been shown to alter the stress response in Eurasian starling (*Sturnus vulgaris*), by disrupting the relationship between corticosterone levels and stress-induced body mass loss (Bean et al., 2014). Within a clinical context, antidepressants are prescribed to patients to normalise hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis, which is associated with depression (Pariante and Miller, 2001). However, the effect of fluoxetine on corticosterone levels is unclear. In one human study, acute and subchronic administration of fluoxetine increased circulating corticosterone concentrations (Weber et al., 2006) whereas in rodents, treatment with fluoxetine has been shown to reduce HPA axis activity, for example by lowering basal secretions of corticosterone (Brady et al., 1992) and increasing glucocorticoid receptor expression (Yau et al., 2002, Pariante et al., 2003). In fact, clinical findings may be of limited value in terms of understanding the environmental effects of pharmaceuticals as clinical doses are generally orders of magnitude greater than environmental concentrations. There is a clear need to understand mode-of-action related effects of environmentally relevant concentrations of fluoxetine on wildlife as alterations to basal stress levels and acute stress responsiveness have implications for behaviours such as predator avoidance and energy expenditure, and thus potentially for individual survival (McEwen and Wingfield, 2003, Brodin et al., 2014). Further, the effects of fluoxetine in birds could differ to effects observed in mammals.

The aim of this study was firstly to assess whether chronic exposure to an environmentally relevant concentration of fluoxetine altered various measures of stress physiology and behaviour, specifically: a) faecal corticosterone metabolite concentration, b) exploration, c) activity, d) peripheral skin temperature. We hypothesised that exposure to fluoxetine would result in lower faecal glucocorticoid metabolite concentrations, higher exploration, altered activity levels and lower peripheral body temperature. We then aimed to determine whether any detected changes in faecal corticosterone metabolite concentration, exploration, activity and body condition were

related to peripheral body (skin) temperature and thus whether lower skin temperature was indicative of higher basal stress levels.

3.3 Methods

3.3.1 Ethics statement

This study was approved by ethics committees from the Animal and Plant Health Agency and the University of York and carried out under Home Office Licence number PPL 60/4213. The capture of the birds was carried out under licences from Natural England and the British Trust for Ornithology. Between capture and the start of the experiments described in the current study, the average change in starling body mass was an increase of 5.8 g (range: 4.2 g loss – 15.4 g gain).

3.3.2 Husbandry

All 27 Eurasian starlings (*Sturnus vulgaris*) were captured from the same roost site in North Yorkshire, UK, in October 2015 and immediately transported to the experimental facility, upon which each bird was weighed (nearest 0.1 g) and marked on the right leg with a pale blue, numbered leg ring (AC Hughes; Hampton Hill, UK). The birds were acclimated to captivity for four weeks, also allowing pharmaceuticals to which they may have been exposed in the wild to be excreted. After acclimation, the starlings were allocated to one of five single sex home aviaries (two male and three female aviaries), which measured 6.9 m L x 2.5 W x 2 m H. These were outdoor aviaries, so that birds were exposed to natural variations in temperature, weather and daylength. Food and water were provided *ad libitum* throughout. Further details of capture and husbandry are available in Appendix A1.

3.3.3 Experimental Treatment

Individuals within each home aviary were assigned randomly to the control or the fluoxetine treatment group. Experimental treatment began on 30th November 2015 in the first home aviary and each aviary started experimental treatment in a successive week, so that the onset of treatment was staggered across five weeks. In all cases, observational measurements and sample collections were likewise staggered, so that all such measurements were made during the same week of treatment for all birds. Fluoxetine-treated birds were hand-fed each weekday with a live waxworm (*Galleria mellonella;* UK Waxworms Ltd, Sheffield, UK) injected with 3.8 µg fluoxetine; control birds were sham-dosed with a waxworm injected only with deionised water (Bean et al., 2014). This fluoxetine weekday dose was equivalent to a calculated daily dose over the whole week of 2.7 µg day^{-1} and represented an environmentally relevant exposure based on consumption of contaminated invertebrate prey at WWTPs (see Chapter 2.3.3 and Appendix A2). The mean body mass of the starlings during the dosing period was 77.4g and so the 2.7 µg daily dose was equivalent

to 0.035 mg kg^{-1} bodyweight, roughly 10 % of the human therapeutic dose. The fluoxetine concentration in the fluoxetine-injected waxworms was verified by measuring residue levels in a randomly selected subset. This revealed an average concentration of 3.71 µg fluoxetine $waxworm^{-1}$ (15 % relative standard deviation, 76 % recovery, *n* = 10; see Appendix A7).

3.3.4 Faecal Corticosterone Metabolite Assay

Faecal samples were collected from birds from each home aviary after 12 weeks of treatment (collection period 22nd February – 25th March 2016, during which the temperature ranged between 0 °C and 10 °C). To collect each sample, the focal bird was kept in an individual cage, with *ad libitum* access to food and water, for two hours on two separate days (four hours total). On one day, faecal samples were collected before the individual participated in behavioural trials (activity and exploration) and on the other day, samples were collected after participation in behavioural trials. The order of the days (collection before or after behavioural trails) was randomised. The elapsed time between the two collection days varied between one and four days per individual but this did not significantly affect results, as subsequent analysis showed that the number of days between collections was unrelated to the concentration of faecal corticosterone metabolites on the second day (Spearman's rank correlation: R_s = -0.42, p = 0.102). A single, integrated measure of faecal corticosterone metabolites was calculated for each individual across the week by averaging the measurements taken across the two days.

The ELISA analysis of corticosterone metabolites was carried out according to kit instructions, using a kit previously validated for faeces from a range of species (Bean et al., 2014) (AC-14F1 Corticosterone EIA; IDS Limited, Tyne and Wear, UK). The mean recovery was 78.7 %, the mean intra-assay CV was 20.1 % and the mean inter-assay CV was 21.0 %. See Appendix B2 for further details.

3.3.5 Individual Exploration and Activity Behaviour

Individual exploration and activity data were collected after 12 weeks of treatment. Each individual underwent two eight minute trials during the same week, similar to those described in (Minderman et al., 2009). In each trial, the focal individual was released from a start cage into a visually occluded, outdoor trial arena with eleven perches and the number of flights (as measure of activity) and number of unique perches visited (as measure of exploration) were recorded. The mean of each measure over the two trials was then computed for each individual, to give an integrated, single measure across the week, which could be used to assess the relationship with body temperature. This was deemed to be appropriate as our statistical models showed that trial replicate did not account for variation in either exploration or activity (see results).

3.3.6 Skin Temperature

Thermal imaging (infrared thermography) data were collected in each of the five home aviaries, on two separate days one week apart (29th March and 5th April 2016). Before data collection started, all feeders were removed from the focal aviary except for one metal floor feeder. This remaining feeder was repositioned close to the mesh aviary wall under the roofed end of the home aviary, to shelter it from rain or solar radiation, both of which can affect temperature measurements (McCafferty et al., 2015, Marder, 1973). The thermal camera (FLIR A65sc, 640 x 512 resolution, 7.5 fps, sensitivity 0.05 oC; FLIR Systems Inc., Oregon, USA) was then positioned on the outside of the aviary wall, 60 cm away from and at the same height as the ground feeder, so that feeding birds would be within the camera's field of view. A separate video camera recorded the birds at the feeder simultaneously with the thermal image recording, to allow later identification of individuals from leg rings. Air temperature and humidity data were collected every five minutes during the recording period for subsequent inclusion in statistical models, as both can affect skin temperature (Lin et al., 2005, Nilsson et al., 2016). Other confounding factors noted for each focal bird were: distance from the camera, head orientation to the camera, body position, behaviour (whether still, feeding or walking), latency to approach the feeder, number of other birds at the feeder; see Appendix B4 for details. Recordings lasted 45 minutes or until all birds in the aviary had been filmed feeding.

To measure body temperature, multiple images were collected of each bird where possible (*n* = 23 birds; mean: 15 images per bird, minimum: 1 image per bird, maximum: 52 images per bird), with intervals of at least 5 seconds between images. The maximum head temperature and the maximum temperature of the skin of the right leg (see Appendix B4) were recorded from thermal images as measures of peripheral body temperature (Figure 3.1).

Figure 3.1: Thermal image of starlings perched on a ground feeder. Most of a passerine's body is well insulated by feathers but the skin of the legs and around the eye is exposed, radiating most heat. In this image, the yellow colour (highest temperature) is around the eye and foot, whilst the feather-covered body is mid-dark blue, indicating cooler temperatures.

Leg temperature data for four individuals were excluded (see Appendix B4). We used the maximum temperature of the whole head rather than the canthus of the eye, which has previously been used as a measure of peripheral body temperature in passerines (Jerem et al., 2015, Jerem et al., 2018), as the latter was too small to measure reliably in the images. Leg skin temperature, as a major site of heat dissipation in passerines, could additionally indicate thermoregulatory differences (Martineau and Larochelle, 1988, Jerem et al., 2018). Using maximum head and maximum right leg temperature, we also computed a head-leg temperature differential, to investigate the effect of treatment and other factors on peripheral vasoconstriction. For each image, the number of minutes since sunrise was also recorded, as circadian rhythm is known to affect body temperature (Refinetti and Menaker, 1992).

3.3.7 Statistical Analyses

Statistical analyses were performed with software R, version 3.4.2 (R Core Team, 2016) and package 'lme4' (Bates et al., 2015) was used to fit all mixed models. The effect of treatment on faecal corticosterone metabolite concentration was assessed via Mann-Whitney U-test. Our critical significance level for *p* values was $\alpha = 0.05$ throughout.

Prior to modelling, univariate tests were carried out to identify potentially problematic correlations between pairs of predictors and variance inflation factors (VIFs) were calculated for the predictors in each full (i.e. before reduction) model, in order to minimise multicollinearity. Exploratory

analysis revealed that head position and head angle were correlated. Therefore, if both predictors were included in a minimum adequate model, one of them was removed based on corrected AIC (AIC_c). Further, sex and home aviary were conflated with each other due to our experimental design, (home aviaries were single sex), meaning we could not include both predictors in any one model. Therefore, we ran models which included one of each of these predictors (sex, home aviary) independently and selected the most appropriate of the two predictors for inclusion based on significance under Wald's z (generalised linear mixed models) or likelihood ratio tests (linear mixed models). If both sex and home aviary were significant in minimum adequate models, selection was based on AIC_c (although note that it was not necessary to use this selection criterium; see Appendix B5). Further details of the model selection process, including procedures to check model assumptions were met, are contained in the Appendix B5.

The effect of treatment on exploration and activity respectively was investigated using two separate generalised linear mixed models (GLMMs). Individual ID was specified as a random effect, whilst the following variables were included as fixed effects in each model: treatment, sex, home aviary, trial replicate (out of two), trial arena (out of two), test order (i.e. whether an individual underwent the behavioural trial or faeces collection protocol first) and body condition (divided by 1000 to ensure model convergence; see Appendix B3 for details). For each response, we fitted a GLMM with a log link function and a Poisson or negative binomial (if overdispersed) error structure.

Using separate linear mixed models (LMMs), we tested the effect of various predictors (treatment, mean faecal corticosterone metabolite concentrations (natural logarithm transformed to improve spread), latency to approach the feeder (one added to all points followed by natural logarithmic transformation to improve spread), number of birds at the feeder, activity and exploration on the following response variables: head temperature, leg temperature, head-leg temperature differential. In addition to the relevant predictor, the following confounding variables were also included in each model as fixed effects: air temperature, air humidity, minutes since sunrise, home aviary, sex, body condition (see Appendix B3 for details), behaviour (still, walking or feeding). In addition, certain measures of body position were included, as this can affect temperature measurements (Bengoudifa and Mieusset, 2007). See Appendix B4 and B5 for full details of body position measures. In all LMMs, bird ID was specified as a random effect.

3.4 Results

3.4.1 Faecal corticosterone metabolites

There was no significant difference in mean faecal corticosterone metabolite concentration between the treatment groups when including all the data points (Mann-Whitney U-test, U = 49, *p*

= 0.193; fluoxetine-treated: median = 527 ng g^{-1}, IQR = 256 ng g^{-1}, *n* = 7; control: median = 982 ng g^{-1}, IQR = 466 ng g^{-1}, *n* = 10). However, the effect of treatment became significant once the outlier in the fluoxetine-treated group at 1675 ng g^{-1} was removed (Mann-Whitney U-test, U = 49, *p* = 0.042; fluoxetine-treated: median = 516 ng g^{-1}, IQR = 204 ng g^{-1}, *n* = 7; control: as above). See Figure 3.2a.

3.4.2 Individual Exploration and Activity Behaviour

Treatment did not explain variation in exploration (Figure 3.2b) and was dropped during the model reduction process. The last remaining predictor in the minimum adequate model was trial replicate (*p* = 0.093). For complete minimum adequate model output, see Appendix B, Table B1. Similarly, treatment did not explain variation in activity (Figure 3.2c) and was dropped during the model reduction process. Body condition was negatively related to activity (GLMM: z = -2.52, *p* = 0.012). For complete minimum adequate model output, see Appendix B, Table B1.

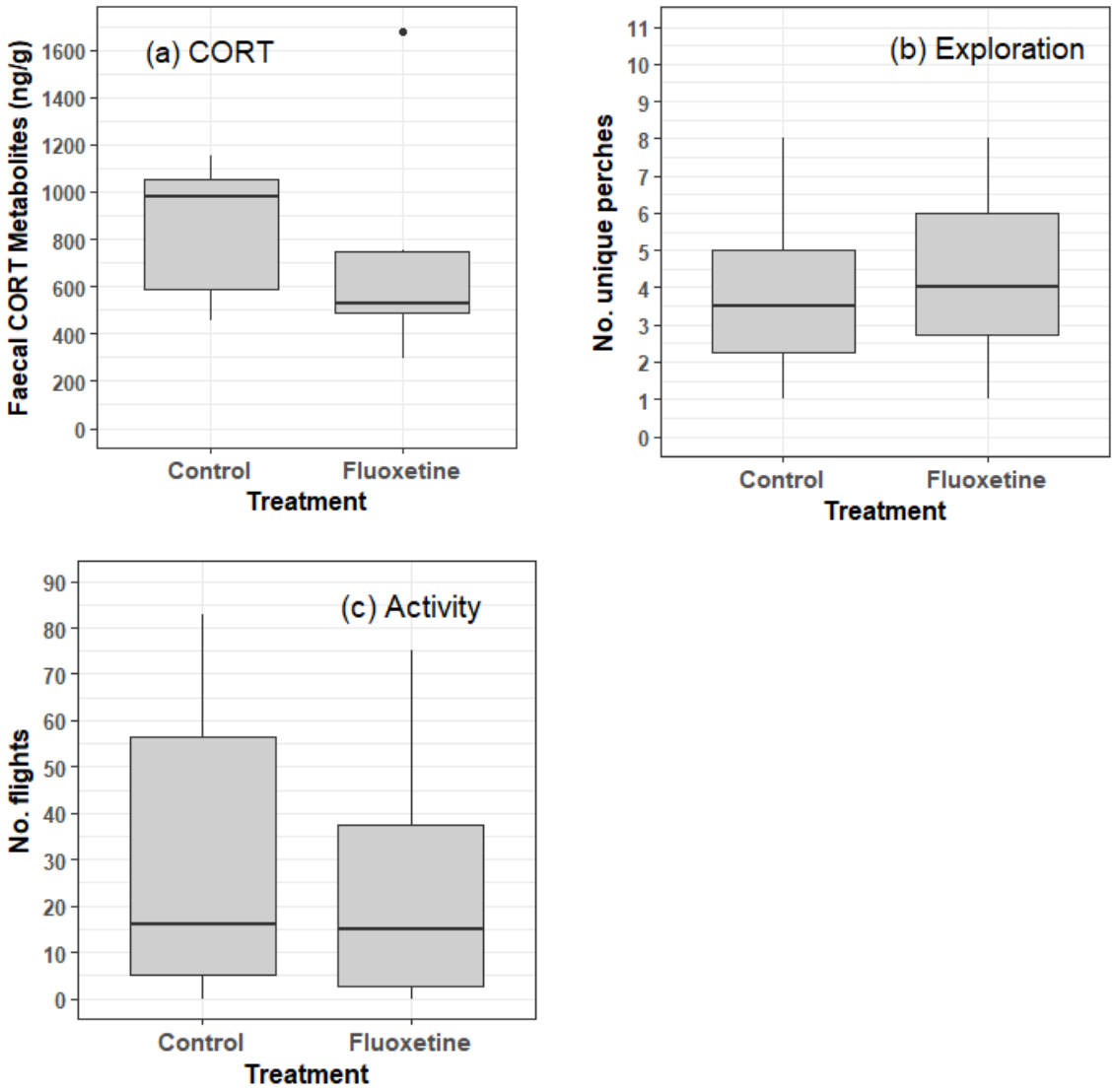

Figure 3.2: Plots showing the effect of treatment on a) faecal corticosterone metabolites (*n* = 10 control, *n* = 7 fluoxetine-treated birds), b) exploration (*n* = 13 control, *n* = 14 fluoxetine-treated birds), c) activity (*n* = 13 control, *n* = 14 fluoxetine-treated birds). Boxes correspond to upper and lower quartiles and points shown outside of whiskers are outliers (1.5*IQR, as defined by Tukey (Tukey, 1977)). Data collected after 12 weeks of treatment.

3.4.3 Skin temperature

When controlling for confounding factors, control bird right leg skin temperature increased linearly with air temperature (Table 3.1a, Figure 3.3a), whilst head-leg temperature differential decreased with increasing air temperature in controls (Table 3.1c, Figure 3.3b). In fluoxetine-treated birds, leg skin temperature did not vary over the air temperature range sampled (Table 3.1a, Figure 3.3a) and the leg skin of the fluoxetine-treated birds also remained unchanged and low relative to head temperature, even when ambient air temperature increased (Table 3.1c, Figure 3.3b).

We found no effect of treatment on head temperature and treatment was dropped as a non-significant variable during the model reduction process (Table 3.1b, Figure 3.4).

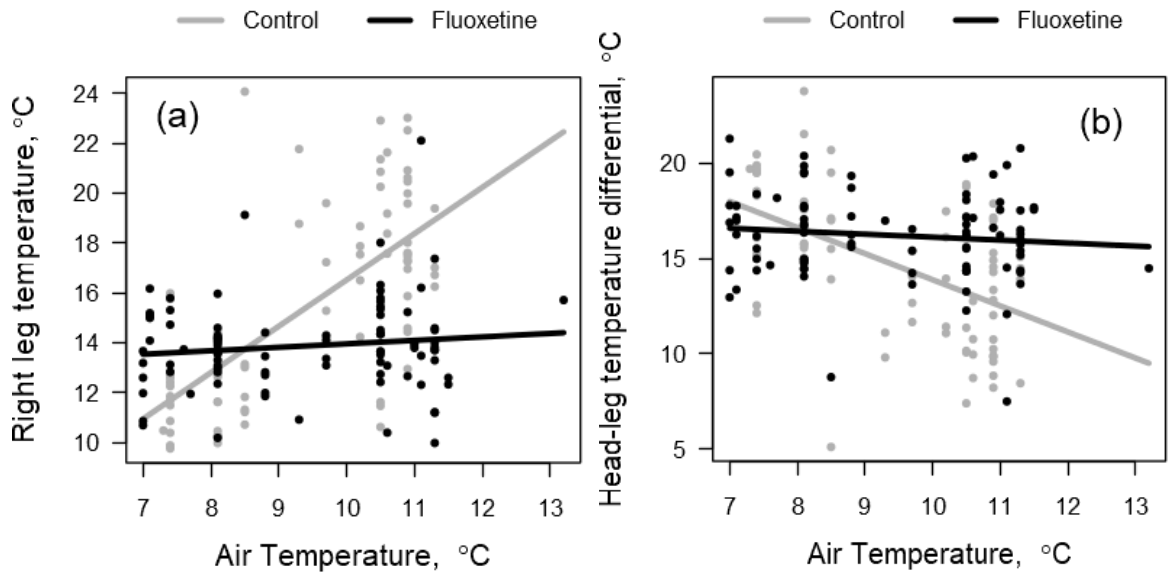

Figure 3.3: Plotted LMM model outputs showing the interaction between treatment and air temperature for responses: a) right leg temperature, b) head-leg temperature differential. Each graph contains *n* = 167 observations from 8 control and 11 fluoxetine-treated birds. Data collected after 13-18 weeks of treatment (dependant on aviary due to staggered onset of dosing between aviaries).

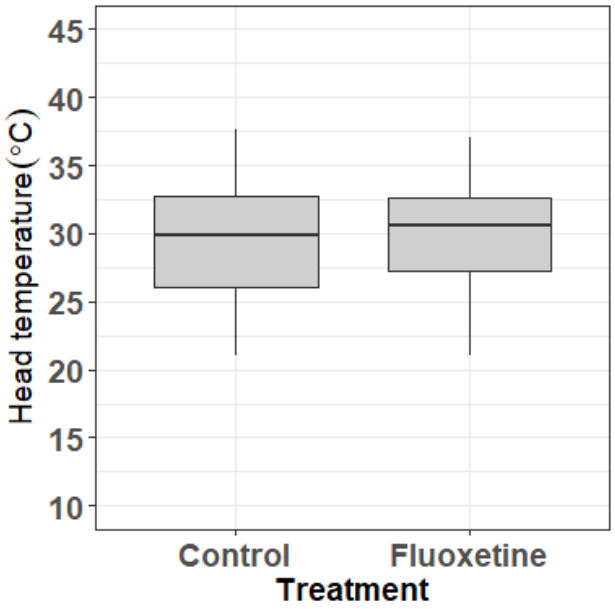

Figure 3.4: Plot showing the effect of treatment on head temperature (*n* = 353 observations from 10 control and 13 fluoxetine-treated birds). Boxes correspond to upper and lower quartiles and points shown outside of whiskers are outliers (1.5*IQR, as defined by Tukey (Tukey, 1977)). Data collected after 13-18 weeks of treatment (dependant on aviary due to staggered onset of dosing between aviaries).

Fixed Effects	Coef. β	SE (β)	t	χ^2	P
a) Response: Right leg temperature					
Intercept	-8.56	4.80	-1.78	-	-
Treatment	21.73	6.81	3.19	13.87	<0.001
Air temperature	6.10	1.69	3.61	23.71	<0.001
Treatment*air temperature	-7.46	2.19	-3.41	11.76	<0.001
Minutes since sunrise	0.02	0.01	3.46	12.78	<0.001
Behaviour (still)	-1.05	0.57	-1.86	12.74	0.002
Behaviour (walking)	0.93	0.57	1.64		
Aviary (E)	7.91	2.17	3.64	25.63	<0.001
Aviary (C)	-5.33	2.45	-2.17		
Aviary (B)	-2.59	1.81	-1.43		
Aviary (A)	-2.18	2.12	-1.03		
b) Response: Head temperature					
Intercept	18.43	1.14	16.17	-	-
Air temperature	0.23	0.08	2.78	7.60	0.006
Minutes since sunrise	0.04	0.00	13.19	144.85	<0.001
Behaviour (still)	-0.82	0.24	-3.43	53.09	<0.001
Behaviour (walking)	-1.89	0.26	-7.41		
Aviary (E)	6.30	0.88	7.16	46.70	<0.001
Aviary (C)	-0.81	0.89	-0.91		
Aviary (B)	-2.29	0.68	-3.36		
Aviary (A)	-1.96	0.71	-2.75		
Head tilt	-1.32	0.35	-3.77	13.59	<0.001
Head angle (90°)	-1.38	0.34	-4.07	36.77	<0.001
Head angle (120°)	-0.61	0.21	-2.92		
c) Response: Head-leg temperature differential					
Intercept	20.16	2.87	7.04	-	-
Air temperature	-0.76	0.27	-2.87	17.82	<0.001
Treatment	-7.01	3.66	-1.92	9.53	0.009
Treatment*air temperature	0.86	0.37	2.34	5.70	0.017
Behaviour (still)	-0.13	0.68	-0.20	31.57	<0.001
Behaviour (walking)	-3.26	0.67	-4.87		
Aviary (E)	-3.54	1.62	-2.18	12.17	0.016
Aviary (C)	3.32	2.14	1.55		
Aviary (B)	0.07	1.38	0.05		
Aviary (A)	0.85	1.68	0.50		
Head tilt	-2.86	0.78	-3.65	14.18	<0.001
Head position	2.39	1.03	2.32	5.52	0.019

Table 3.1: Minimum adequate model outputs for LMMs investigating the effect of treatment and confounding variables on the following responses: a) right leg temperature (n = 167 observations from 8 control and 11 fluoxetine-treated birds), b) head temperature (n = 353 observations from 10 control and 13 fluoxetine-treated birds), c) head-leg temperature differential (n = 167 observations from 8 control and 11 fluoxetine-treated birds). Table shows: coefficient estimate (β), standard error of β, t-statistic, Chi-Square statistic (χ^2) and significance level p.

3.4.4 Relationship between skin temperature and individual behaviour

Neither individual activity nor individual exploration were significantly related to right leg temperature (Appendix B, Table B2a), head temperature (Table 3.1b) or head-leg temperature differential (Appendix B, Table B2b). They were therefore dropped from each respective model during the model reduction process. For head temperature, this resulted in a minimum adequate model identical to that derived from assessing the effect of treatment (Table 3.1b).

3.4.5 Relationship between skin temperature and faecal glucocorticoid metabolites

Mean faecal glucocorticoid metabolite concentration was unrelated to right leg temperature (Appendix B, Table B3a), head temperature (Appendix B, Table B3b) and head-leg temperature differential (Appendix B, Table B3c) and was dropped from each respective model during model reduction.

3.5 Discussion

Contrary to our expectations, we found no effect of treatment on our measures of anxiety-related behaviours (exploration, activity). We did observe lower corticosterone metabolites in the faeces of fluoxetine-treated birds compared to controls, as predicted, but only following exclusion of an outlying data point. Our hypothesis that control birds would have lower peripheral skin temperature, due to higher basal stress compared to fluoxetine-treated birds, was also not supported. In fact, leg skin temperature and head-leg temperature differential were unresponsive to changes in air temperature in fluoxetine-treated birds across the ambient temperature range sampled. Our thermal imaging observations therefore do not appear to be related to stress behaviour and physiology but instead appear to be an unexpected physiological side effect caused by exposure to fluoxetine.

Fluoxetine is designed to change anxiety-related behaviours in humans suffering from mental health conditions (Benfield et al., 1986). However, our environmentally relevant dosage was equivalent to ~10 % of a human therapeutic dose, perhaps explaining the lack of any such effect on anxiety-related behaviours in our study. Our findings are in line with those reported in an earlier study, in which Eurasian starling exposed chronically to a lower concentration of fluoxetine (0.92 µg day^{-1}), did not exhibit differing exploration or activity scores compared to controls (Bean et al., 2014). As more general measures of anxiety-related behaviours, our exploration and activity results differ from effects found in several species of fish at environmentally relevant concentrations. For example, chronic exposure to fluoxetine has been shown to inhibit anti-predator behaviour in Eastern mosquitofish (*Gambusia holbrooki*) at 25 and 226 ng L^{-1} (Martin et al., 2017), and in Wild guppies (*Poecilia reticulata*) at 16 ng L^{-1} (Saaristo et al., 2017). The same studies also found that

fluoxetine altered activity levels, although exposed Mosquitofish became more active (Martin et al., 2017), whilst activity levels decreased in exposed guppies (Saaristo et al., 2017). Further, male guppies exposed to 4 ng L^{-1} for 28 days became less exploratory, although this effect was not observed in females or at the other two concentrations studied (Saaristo et al., 2017). The inconsistency between the findings in these studies and ours may relate to differences in the magnitude of the dose administered and/or differences in responses to fluoxetine between birds and fish. Alternatively, they may relate to the manner of dose delivery, since fish are typically exposed constantly during an exposure study, via the ambient concentration in the water, whilst the birds in our study were exposed once per weekday via diet.

The treatment effect we observed on anxiety-related physiology (faecal corticosterone metabolite concentration) was weak, only becoming significant with the exclusion of an outlier. In fish, fluoxetine has been shown to reduce glucocorticoid levels at environmentally relevant concentrations (Abreu et al., 2014, Giacomini et al., 2016), whilst in mammals, clinically relevant dosages of fluoxetine have been found to reduce endogenous glucocorticoid levels in some circumstances but actually to increase them in others (Weber et al., 2006, Brady et al., 1992, Yau et al., 2002, Pariante et al., 2003). Again, our environmentally relevant exposure may have been too low to observe a well-defined treatment effect on corticosterone. Alternatively, our sample size may have been too low, resulting in insufficient statistical power. Such issues may have been compounded by the fact that faecal corticosterone metabolites have been assumed to reflect circulating corticosterone levels, whereas in reality, variation in factors such as diet and individual metabolism create extra noise in the data (Goymann, 2009). Indeed, our data exhibited a high degree of such noise, as our coefficient of variation was 20.1 % (intra-assay) to 21.0 % (inter-assay). Nonetheless, we found that fluoxetine-treated birds exhibited lower faecal corticosterone metabolites as predicted. Our findings are in line with an earlier study, which showed an effect of fluoxetine on faecal corticosterone metabolites and found that chronic exposure (0.92 µg day^{-1} $bird^{-1}$) disrupted the relationship between corticosterone metabolites and body mass loss during isolation-induced stress in starlings (Bean et al., 2014). Although the effect observed in this study was indirect, it provides further indication that exposure to environmental concentrations of fluoxetine can interfere with endogenous corticosterone.

We had originally hypothesised that fluoxetine-treated birds would exhibit higher skin temperature than controls due to a lesser degree of sympathetically-mediated vasoconstriction, owing to lower basal stress levels (Herborn et al., 2015, Oka et al., 2001, Jerem et al., 2018). However, based on mean leg temperature, fluoxetine-treated birds had cooler leg skin than controls. Previous work has shown that the temperature of the canthus of the eye is linked to energy reserves and

circulating glucocorticoid levels in Blue tits (*Cyanistes caeruleus*), with lower canthus temperature indicative of increased basal stress (Jerem et al., 2018). If cooler leg skin temperature was linked to increased basal stress, we would have expected leg skin temperature and faecal corticosterone metabolite concentrations to be negatively correlated, and body condition and leg skin temperature to be positively correlated, based on the findings in Blue tits (Jerem et al., 2018). However, no such relationships were found. It is possible that this was because faecal corticosterone metabolite concentrations in our study were actually representative of acute rather than basal stress, since our measured concentrations were high compared to those previously measured in Eurasian starlings under isolation stress (Bean et al., 2014). Passerines rapidly metabolise and excrete corticosterone (Goymann, 2009), and it may be that faeces collected before behavioural trials was an integrated measure of basal and acute stress, whilst faeces collected after behavioural trials largely represented acute stress. Similarly, our activity and exploration observations may also have been related to acute rather than the basal stress presumably represented by the infrared thermography measurements, as our measures of anxiety-related behaviour were recorded during trials conducted in isolation (potentially stressful for Eurasian starlings which are social birds) and in a novel environment. These differences, alongside the fact that the thermal imaging and behaviour/corticosterone data were collected in separate weeks, represent limitations that may have hampered our ability to explain differences in skin temperature based on activity, exploration and faecal corticosterone metabolites.

Previous studies suggest that the effect of fluoxetine on anxiety-mediated body temperature attenuation depends on the type of stressor(s) applied. In rodents, the core temperature rise due to stress-induced hyperthermia, which occurs concomitantly with a drop in peripheral temperature (Cabanac and Aizawa, 2000, Vinkers et al., 2013), is commonly used as a measure of anxiety and employed to assess the efficacy of anxiolytic medicines (Olivier et al., 2003). In telemetered rats and mice, chronic but not acute treatment with fluoxetine (10-15 mg kg^{-1}) has been found to reduce the intensity of the acute stress-induced hyperthermia response (Conley and Hutson, 2007). In contrast, an earlier study found no effect of chronic fluoxetine treatment (10-20 mg kg^{-1}) on stress-induced hyperthermia in mice, possibly because different acute stressors were employed and also because rectal probes were used to measure body temperature, representing an additional stressor (Lecci et al., 1990). In the present study, we did not expose the focal birds to an acute stressor before collecting thermography data, as we were concerned with measuring basal skin temperature. This may explain why we did not find evidence of changes to skin temperature driven by the anxiolytic action of fluoxetine, comparable to findings in telemetered rodents (Conley and Hutson, 2007). Whilst there was an unexpected treatment effect on leg skin temperature, we found

no such effect on head temperature. This suggests that the fluoxetine treatment altered peripheral vasoconstriction of the legs specifically, with implications for thermoregulation.

The legs are vital for thermoregulation in birds, including starlings (Speakman and Ward, 1998, van Kampen, 1971, Ward et al., 1999, Martineau and Larochelle, 1988) and vasoconstriction in avian legs during cold weather allows heat to be conserved (van Kampen, 1971). Conversely, the bare, uninsulated skin of the legs allow heat to be dissipated during flight or high temperatures (Ward et al., 1999, Martineau and Larochelle, 1988). In the aforementioned Blue tit study, Jerem et al. chose to use the skin around the eye preferentially to the leg skin temperature to measure basal stress, since thermoregulatory heat transfer by the legs could obscure the relationship between body temperature and physiological state (Jerem et al., 2018). The failure of fluoxetine leg skin temperature to increase with air temperature, or to increase relative to head temperature with increasing air temperature, supports the conclusion that fluoxetine was causing vasoconstriction in the legs. This is suggestive of a treatment effect on thermoregulation.

Fluoxetine is known to affect both thermoregulation and the cardiovascular system in mammals (Roose et al., 1998, Maswood et al., 2006, Marcy and Britton, 2005), although exact mechanisms remain unclear and effects vary between species and dosages. In ovariectomised rats, a single injection of fluoxetine has been found to dose-dependently lower core body temperature at all administered doses (3, 10, 30 and 60 mg kg^{-1}), although tail skin temperature only decreased at the two highest doses (Maswood et al., 2006), indicating that peripheral vasoconstriction increased only at higher doses. Our administered dose was several orders of magnitude lower, possibly indicating that peripheral vasoconstriction in starlings is more sensitive to treatment with fluoxetine than in rats. However, it is difficult to draw comparisons, since our study involved oral chronic dosing rather than a single injection. A common side effect of treatment with fluoxetine in humans, estimated to occur in 8 % of patients, is antidepressant-induced sweating (Marcy and Britton, 2005). This is posited to be a thermoregulatory response, occurring due to increased serotonin levels in the hypothalamus, which has been shown to cause hyperthermia (Lin et al., 1998). Indeed, rats whose hypothalami were perfused with 5 µM fluoxetine exhibited a significant increase in in colonic (core) temperature, accompanied by a significant decrease in foot (peripheral) temperature (Lin et al., 1998). The latter of these effects on body temperature mirrors the effect on leg temperature observed in the present study. Contradictorily, 20 mg fluoxetine administered daily to human patients for six weeks as a trial treatment for Raynaud's phenomenon was found to increase hand rewarming in patients with primary Reynaud's phenomenon following a cold challenge (Coleiro et al., 2001), possibly because fluoxetine treatment depletes platelet serotonin (5-HT) (Pigott et al., 1990). The apparently variable and context dependant effects of fluoxetine on

vascular tone, body temperature and thermoregulation in *in vivo* studies probably reflects the complex plethora of actions that serotonin exerts on these body functions (Vanhoutte, 1987, Hodges et al., 2008). Regardless, our findings provide the first evidence that fluoxetine can alter avian peripheral body temperature and thermoregulation at a dose of fluoxetine orders of magnitude lower than doses employed in mammalian studies.

In the current study, we have shown that chronic exposure to an environmentally relevant concentration of fluoxetine can alter the responsiveness of peripheral body temperature to changes in air temperature during spring and thereby potentially affect thermoregulation in a songbird. In particularly hot or cold climes or weather, such effects may represent a serious challenge for birds, since they rely on controlling the temperature of exposed skin, especially leg skin, to regulate body temperature (Speakman and Ward, 1998, Steen and Steen, 1965). There is already existing evidence that exposure to an environmental concentration of fluoxetine can affect another trait important to survival, by causing sub-optimal foraging behaviour in Eurasian starlings during winter (Bean et al., 2014). Taken in combination with our findings, if such effects are observed in the wild they could have negative impacts on individual survival, particularly during harsh weather. Indeed, the number of starlings foraging on wastewater treatment plants has been shown to spike during cold weather and is highest over winter (Fuller and Glue, 1978). Thus, the exposure risk for starlings is likely to be highest in winter, when the adverse effects of fluoxetine may be particularly problematic. Since birds use their legs to dissipate heat during hot weather (Steen and Steen, 1965), further work in this area should investigate whether the vasoconstriction observed in our study persists at higher ambient temperatures. Furthermore, birds foraging at wastewater treatment plants would not encounter fluoxetine in isolation but would rather be exposed to a mixture of SSRI antidepressants, with potential for additive effects (Simmons et al., 2017, Christensen Anne et al., 2009). Therefore, assessing the effect of environmentally realistic mixtures of SSRIs on the avian cardiovascular system and thermoregulation would better reflect the antidepressant load in prey consumed by starlings and other birds at wastewater treatment plants. Investigating the effect of fluoxetine on stress-induced hyperthermia rather than basal skin temperature, following exposure to an acute stressor, would also enhance our understanding of effects on vasoconstriction. Finally, it is important to determine whether the effects observed in this study occur following a shorter exposure to fluoxetine, for example acute or subchronic exposure, as this would significantly increase the risk to exposed wilds birds. Overall, our findings add to evidence that antidepressants in the environment can disrupt traits that are important for individual survival and may thus have detrimental effects on individual fitness and possibly local populations.

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Chapter 4: Effects of an environmentally relevant concentration of fluoxetine on avian anxiety and stress responses

4.1 Abstract

Pharmaceuticals, emerging compounds of environmental concern, can alter behaviour and physiology in exposed wildlife. Wastewater treatment plants are point sources from which pharmaceuticals enter the wider environment, yet are also foraging grounds for certain bird species, including Eurasian starling (*Sturnus vulgaris*). Starlings and other insectivorous birds consume pharmaceutical-contaminated prey directly from trickling filter beds. We designed an aviary study to assess the effects of chronic exposure to an environmentally relevant concentration of the antidepressant fluoxetine (2.7 µg day^{-1}), a priority pharmaceutical pollutant, at two time points (after 6 and 27 weeks' exposure; during winter and summer respectively). Fluoxetine is an anxiolytic medication, so anxiety-related behaviours and aspects of stress physiology were used as endpoints. Treated individuals exhibited reduced corticosterone metabolites in faeces after 27 compared to 6 weeks' exposure, whilst concentrations in control bird faeces increased. Fluoxetine treatment also suppressed the reduction in boldness observed in control birds between 6 and 27 weeks' exposure. No treatment effects on neophobia, exploration or activity were observed. The reduction in feather quality observed in feathers regrown over the first few weeks of treatment (compared to the quality of feathers grown in the wild, before the experiment) was significantly greater in control compared to fluoxetine-treated birds. Our results indicate that chronic exposure to environmental concentrations of fluoxetine can elicit anxiolytic effects, which appear to vary according to environmental cues and/or the length of exposure; yet early perturbations to stress physiology (feather growth) can occur far more rapidly. Our findings have implications for individual fitness, as alterations to boldness and stress hormone levels can affect traits such as predator evasion, which are vital for survival.

4.2 Introduction

Pharmaceuticals are widespread contaminants, which enter the environment via a multitude of different routes (Arnold et al., 2014) such as hospital waste (Escher et al., 2011), landfill (Ramakrishnan et al., 2015), wastewater treatment (Jones et al., 2005) and due to veterinary medicines used to treat livestock (Boxall et al., 2004). Wastewater treatment plants (WWTPs) are the interface between pharmaceutical-contaminated wastewater and the wider environment (Jones et al., 2005), and are also important foraging sites for certain species of bird (Fuller and Glue, 1978, Fuller and Glue, 1980). Some pharmaceuticals, such as antidepressants, are specifically designed to be potent modulators of behaviour in humans, as well as causing a range of side effects. If changes to individual behaviour were also to occur in wildlife exposed to antidepressants in the environment, not only could individual fitness be affected but also population-level effects could occur (Brodin et al., 2014).

Fluoxetine, a widely prescribed selective serotonin reuptake inhibitor (SSRI) antidepressant, is one such environmentally significant antidepressant pollutant (Oakes et al., 2010). Sewage influents can contain fluoxetine, since approximately 24 % is excreted as the parent compound by human patients (Lienert et al., 2007). Effluents can also contain fluoxetine, since wastewater treatment processes can fail to remove it, as well as many other pharmaceuticals, from wastewater (Vasskog et al., 2006). For example, the mean percentage of fluoxetine removed from influent across five sewage treatment plants (STPs) in Canada was 47 % (Lajeunesse et al., 2012). In biosolids generated during the wastewater treatment process, fluoxetine has been detected at concentrations of up to 4700 µg kg^{-1} (Kinney et al., 2006b), although a UK survey of concentrations in sewage sludge across 28 WWTPs reported a mean concentration of 130 µg kg^{-1} (Jones et al., 2014).

Much research effort into the effects of fluoxetine on wildlife has focused on aquatic ecosystems, yet fluoxetine is also present in certain terrestrial habitats (Shore et al., 2014). The most obvious terrestrial sites that can be fluoxetine-contaminated are WWTPs themselves, yet fluoxetine may also be present in farmland habitats, due to the use of sewage-derived biosolids for crop fertilisation (Arnold et al., 2014) or treated wastewater for crop irrigation (Kinney et al., 2006a). Fluoxetine is bioavailable to invertebrates in contaminated soils (Carter et al., 2014) and also to various crop plants (Redshaw et al., 2008). Consequently, terrestrial vertebrates that exploit such food sources can be exposed.

Wastewater treatment plants are important foraging grounds for many species of bird throughout the year (Fuller and Glue, 1978). Ground-foraging birds can consume contaminated prey directly from trickling filter beds (Fuller and Glue, 1978, Markman et al., 2008, Fuller and Glue, 1980), whilst

birds that hunt on the wing may consume contaminated flying insects that developed in tanks of pharmaceutical-contaminated water (Park and Cristinacce, 2006). One of the most numerous ground-foraging species recorded at WWTPs in the UK is the Eurasian starling (*Sturnus vulgaris*), which visits most frequently during harsh winter weather and also makes use of such sites immediately after breeding and during periods of migration or dispersion (Fuller and Glue, 1978, Fuller and Glue, 1980). Starling have been observed foraging directly on trickling filter beds at WWTPs (Fuller and Glue, 1978, Markman et al., 2008), where concentrations in Earthworm (*Eisenia fetida*) samples can contain up to 53.8 ng g^{-1} fluoxetine (Bean et al., 2017).

In humans, fluoxetine (brand name Prozac®) is prescribed to treat illnesses such as depression, anxiety, obsessive-compulsive disorder and bulimia nervosa. Therapeutic outcomes are thought to mainly be effected via inhibition of serotonin (5-HT) reuptake, with serotonin transporter (SERT) playing a key role. This leads to increased levels of extracellular serotonin in the brain, which produces anxiolytic effects (Leonardo and Hen, 2006), since anxiety and fear are modulated by serotonin. Birds exposed to fluoxetine at WWTPs might be expected to experience similar effects on anxiety-related behaviours to those observed in humans, since birds are known to possess SERT (Lovell et al., 2015) and due to the high degree of conservation of the serotonergic system between vertebrates (Parent, 1981, Gunnarsson et al., 2008). Undesirable side effects, such as restlessness (Joint Formulary Committee), lethargy (Uphouse et al., 2006) and changes to personality (Du et al., 2002) can result from treatment. Consequently, alterations to activity levels and personality traits are predicted in fluoxetine-exposed wild birds.

To understand the effects of exposure to fluoxetine on anxiety-related behaviours in wild birds, it is crucial to select ecologically relevant endpoints. There are several behavioural trial protocols that have commonly been used to assess avian anxiety-related behaviours. Exploration trials are well-established methods used to measure anxiety-related behaviour (Huang et al., 2016), allowing exploration and also activity to be measured. Exploration has been shown to positively correlate with survival (Smith and Blumstein, 2008), whilst activity is a measure of locomotory performance and is also indicative of anxiety (Bailey and Crawley, 2009). Further, alterations to activity may have implications for predator evasion (Archer, 1973). An exploration trial has been developed previously for testing Eurasian starlings, the subject of this study (Minderman et al., 2009) and was subsequently used to demonstrate that the area of the novel environment explored was correlated with maximum home range size (Minderman et al., 2010). Neophobia, often measured in birds using a novel object trial (Greenberg, 1984), is another measure that has classically been viewed as a measure of anxiety-related behaviour. However, neophobia has more recently been suggested to indicate tendency to innovate in novel feeding situations (Webster and Lefebvre, 2001). Boldness

can also be measured during neophobia trials and has important consequences for fitness, as it has been shown to relate inversely to survival (Smith and Blumstein, 2008). Employing such ecologically relevant endpoints will enable more realistic interpretations of experimental results to be made.

There is existing evidence that environmentally relevant concentrations of fluoxetine can alter anxiety-related behaviours in aquatic animals, with some effects occurring at low concentrations and after even short exposure durations (Ford and Fong, 2016). However, the effects of exposure to low concentrations can be variable and sometimes contradictory (Sumpter et al., 2014). Amongst the effects of fluoxetine on anxiety-related behaviours that have been studied in vertebrates, alterations to anti-predator behaviour have been investigated with particular frequency, and effects have been demonstrated at low exposure concentrations. For example, Wild guppies (*Poecilia reticulata*) exposed to 16 ng L^{-1} fluoxetine for 28 days spent more time under plant cover than controls, indicating heightened anxiety, and concentrations of both 16 and 4 ng L^{-1} caused individuals to freeze for longer than control fish following a simulated bird strike (Saaristo et al., 2017). However, the opposite effect was observed in Mosquitofish (*Gambusia holbrooki*) exposed to slightly higher environmentally relevant concentrations (25 and 226 ng L^{-1} for 28 days), causing the exposed fish to freeze for less time relative to controls (Martin et al., 2017). The Mosquitofish study also found that fluoxetine exposure altered anti-predator behaviour in other ways, for example by reducing latency to enter the predator strike zone (Martin et al., 2017). In addition to variation in the direction of treatment effects between species, species sensitivity to anxiety-related effects can also differ. For example, Fathead minnow (*Pimephales promelas*) exposed to 1 µg L^{-1} fluoxetine, which is too high to be considered truly environmentally relevant, exhibited decreased startle reactions to a mock predator, yet this effect was not induced by exposure to a more realistic environmentally relevant concentration of 0.1 µg L^{-1} (Weinberger and Klaper, 2014). In fish, environmental levels of fluoxetine have additionally been reported to alter activity and exploration (Saaristo et al., 2017, Martin et al., 2017), both frequently employed as measures of anxiety-related behaviour (Bailey and Crawley, 2009). Once again, effects differed between studies, with one study on Mosquitofish reporting an increase in activity following exposure to 25 ng L^{-1} for 28 days (Martin et al., 2017) and another study on Wild guppies reporting a decrease in activity following exposure to 16 ng L^{-1} for 28 days (Saaristo et al., 2017). Conversely, in a terrestrial study with Eurasian starling (*Sturnus vulgaris*), there was no effect of 1.58 µg $weekday^{-1}$ administered chronically over 22 weeks on activity or exploration (Bean et al., 2014). Despite the variability in responses to fluoxetine exposure between species, there is clearly potential for environmentally relevant concentrations to elicit effects on anxiety-related behaviours, in one direction or another.

In contrast to mammals and fish, attempts to understand the effects of exposure to fluoxetine on behaviour and physiology in birds have so far been limited. To the authors' knowledge, only two papers to date have focused on the effects of environmentally relevant concentrations of fluoxetine on avian behaviour (Bean et al., 2014, Whitlock et al., 2018). Of these, one paper focused on anxiety-related behaviours and physiology. This study reported that Eurasian starlings exposed chronically to 1.58 µg weekday^{-1} habituated differently to isolation compared to control birds, in terms of the relationship between corticosterone metabolites in faeces and body mass loss (Bean et al., 2014). However, no corresponding effects on anxiety-related behaviour (boldness, exploration, activity) were uncovered. The study also found that diurnal feeding patterns in fluoxetine-exposed birds differed from control birds and were atypical for the time of year, indicating that fluoxetine can alter the behavioural responses of animals to challenges in their environment (Bean et al., 2014). Based on results in fish and the likelihood that wild birds would encounter mixtures of fluoxetine and other SSRIs with potentially additive effects, further investigation in birds using alternative methods and exposure concentrations is warranted.

The effects on stress physiology Bean et al. observed in their starling study (Bean et al., 2014) illustrate the importance of assessing the effects of fluoxetine exposure on avian stress physiology in addition to behavioural traits. The findings of Bean et al. indicate that body mass loss and faecal glucocorticoid metabolite concentrations may both be appropriate physiological endpoints for fluoxetine-exposure (Bean et al., 2014). Birds can rapidly alter their body mass under stress to allow for optimal flight, for example losing mass to facilitate escape from predators (Lima Steven, 1986), and can even lose body mass when a predator threat is perceived but a predator is not encountered (Veen Ineke T. Van, 2008). Glucocorticoid levels in faeces are indicative of physiological stress, since increased circulating glucocorticoids help birds to respond to stressors by mobilising energy stores (McEwen and Wingfield, 2003). Further, measuring glucocorticoid concentrations in faeces confers two particular advantages. Firstly, collecting faeces is a non-invasive procedure and secondly, concentrations of metabolites in faeces provide an integrated measure of corticosterone over a period of time of up to several hours in birds (Goymann, 2009). Another stress physiology endpoint of special relevance to birds is the quality of feathers. Physiological stress has been shown to retard feather growth and to decrease feather quality, with both decreasing as circulating corticosterone increases, presumably due to the catabolic effects of increased glucocorticoids (DesRochers et al., 2009, Strochlic and Romero, 2008). A particular benefit of assessing feather quality is that it captures the effect of treatment over a period of weeks, integrated over the feather growth period. Similarly to the behavioural endpoints described above, the use of physiological endpoints relevant to birds allows conclusions drawn in captive studies to be more easily extrapolated to wild birds.

The aim of our experiment was to investigate whether chronic exposure to an environmentally relevant concentration of fluoxetine affected anxiety-related behaviours or stress physiology in the Eurasian starling, a species known to forage at WWTPs (Fuller and Glue, 1978, Fuller and Glue, 1980). We investigated the impact of chronic exposure at two time points (6 and 27 weeks' exposure). These time points allowed us firstly to assess the development of treatment effects over time but also to contextualise our findings with the seasonal activity of the birds within their environment, as the first of these time points was during winter whilst the second fell during summer. These times of year are particularly relevant to starlings, since numbers recorded foraging at WWTPs are especially high during harsh winter weather and also in early June, immediately after the breeding period (Fuller and Glue, 1978). The measures assessed at time these points were: 1) anxiety-related behaviours (exploration, activity, boldness, neophobia), 2) body mass loss under stress, 3) faecal corticosterone metabolite concentration. To assess the early (sub chronic/early chronic) effects of exposure over a time period (5-6 weeks) during the winter, we also assessed two measures of feather quality (a measure of physiological stress) in feathers regrown from the first week of dosing onward: 1) feather length, 2) feather mass. This allowed us to assess whether early evidence of physiological perturbations caused by fluoxetine exposure were evident.

4.3 Methods

4.3.1 Capture and husbandry

Under licences from Natural England and the British Trust for Ornithology, 27 wild Eurasian starlings (*Sturnus vulgaris*) were caught using mist nets at a roost in North Yorkshire, UK. A depuration period of at least four weeks then commenced, pre- data collection, to allow the birds to acclimate to captivity and excrete non-persistent contaminants. The birds were housed in Home Office licensed outdoor aviaries in single sex groups (3-7 individuals per aviary) and had *ad libitum* access to food and water. Further details are provided in Appendix A1.

4.3.2 Experimental treatment

Randomisation to either the fluoxetine treatment or control group was performed using stratification by aviary, so that each of the five single sex home aviaries contained balanced numbers from each group. Each aviary began experimental treatment in a different week over five a week period, starting on 30th November 2015 for the first aviary and 28th December 2015 for the last. The birds were orally dosed for 28 weeks in total, to mimic dietary exposure over winter and through to early summer, due to the propensity of starling to forage at WWTPs during winter, early spring and early summer (Fuller and Glue, 1978, Fuller and Glue, 1980).

We administered a daily dose of 3.8 µg to each fluoxetine-treated individual, five days per week (each weekday; corresponds to a daily dose of 2.7 µg d^{-1} per bird over a week) by handfeeding a spiked waxworm (*Galleria mellonella;* UK Waxworms Ltd, Sheffield, UK) (Bean et al., 2014). This represented a maximal environmentally relevant concentration, calculated based on the highest concentration of fluoxetine found in invertebrate prey species (*Eisenia fetida*) at UK sewage works (53.8 ng g^{-1}, see (Bean et al., 2017)) and the assumption that such contaminated prey constituted 100 % of the daily diet, the weight of which was taken to be 50 g day^{-1} (Feare, 1984). The delivered dose was confirmed by LC-MS/MS analysis of a subset of spiked waxworms, containing a mean quantity of 3.71 µg per worm (see (Whitlock et al., 2018)). Control individuals were sham-dosed with waxworms injected with deionised water, to control for handling stress.

4.3.3 Behavioural trials

In order to assess the temporal changes in anxiety related behaviours of fluoxetine and control birds, we introduced the birds to novel objects and environments, i.e. applied a mild, standardised stressor. Individuals participated in behavioural trials both after 6 weeks exposure (in mid-winter) and 27 weeks exposure (during early summer). These are ecologically relevant time points, since starlings make particular use of wastewater treatment plants as foraging grounds in winter, especially during periods of cold weather, and also immediately after the breeding period in June (Fuller and Glue, 1978, Fuller and Glue, 1980). At each of time point, each individual participated in two replicates within the same week (one in each arena, in random order) and each aviary underwent trials in a different week during a five-week period, in line with the staggered onset of dosing between aviaries.

Four birds participated in assays each day. Two birds participated in individual behavioural trials first, whilst the other two were introduced to individual holding cages for the collections of faeces. Once the first trials were completed, the birds were swapped over, so that each individual participated in both a trial and the faecal collection protocol. The order in which an individual undertook the behavioural trial and faecal sample collection was randomised during the first replicate and reversed during the second.

Each individual behavioural trial took two hours. The bird was first weighed (to the nearest 0.1 g) before introduction to the start cage, where the bird remained for 30 minutes without food or water, to standardise for hunger. The bird was then released from the start cage by a hidden observer. Once the bird entered the arena, the eight-minute exploration trial began. Subsequently, the bird was left to settle for 22 minutes, before the neophobia trial began. There was no access to food and water during the exploration trial or subsequent settling period. At the end of the

neophobia trial, the focal individual was captured by hand net, weighed (to the nearest 0.1 g) and returned to its home aviary.

Test Arenas: Two identical test arenas (6.9 m L x 2.5 m W x 2 m H) were set up in outdoor aviaries. These were designed to create an environment that was novel to the test starlings, yet not so unfamiliar as to provoke excessive fear. Observations were taken through a small hole, at the uncovered end of the arena. A start cage (40 cm L x 25 cm W x 25 cm H) with covered walls, so that a bird within could not see the arena, was positioned on the floor in the left-hand corner. The exit hatch on the start cage was operated from outside the arena by pulling on a cord. Each arena had eleven perches: eight corner perches (one high and one low per corner) and three central swinging perches (Minderman et al., 2009). See Figures B1 and B2 in Appendix B.

Exploration trial: The eight minute exploration trial followed a method based on (Minderman et al., 2009), which was designed for Eurasian starlings. During this component, two measures of exploration (number of unique perches visited, time to reach last new perch) and one measure of activity (number of flights) were recorded. Number of unique perches visited can be considered to relate to the size of the area explored, whilst time to reach last new perch relates to speed of exploration.

Neophobia trial: The neophobia trial was based on methods in (Herborn et al., 2010) and consisted of two phases, each 14 minutes long. One phase, termed “disturbance phase”, measured boldness and the other phase, termed “novel object phase”, measured neophobia. The addition of a “disturbance phase”, in which the individual undergoes the trial with food but no novel object, is a recent development and serves to control for human disturbance (Herborn et al., 2010). During each phase, latency to approach the food bowl (in seconds) was recorded. To qualify, the bird had to approach to within one metre of the food dish and be on the ground. In the disturbance phase, only the food bowl was present, whilst the same bowl with a novel object placed in the centre was presented in the novel object phase. In between these phases, the bird was left to settle without food or water for 16 minutes. Each individual undertook the two phases in random order during the first replicate and then in reverse order during the second replicate (on a different day). If a bird came to feed, five seconds of feeding time were allowed before the dish was removed (Webster and Lefebvre, 2001). Otherwise, the food dish (with or without novel object) was removed at the end of the neophobia trial phase. Novel objects were selected to be variable, similarly sized and were items that wild starlings would be unlikely to encounter (see Appendix C1 for details).

To ensure that neophobia was induced by the novel objects, a Wilcoxon signed-rank test was performed to test the difference in latency to feed between the two phases (disturbance and novel

object). This showed that latency to feed differed between phases and was significantly longer during the novel object phase (p = 0.003; see Appendix C1), confirming that the novel object phase measured neophobia.

4.3.4 Body mass differential

A body mass differential was calculated to assess the effect of treatment on body mass regulation during stressful situations (i.e. the individual behavioural trial). Individual body mass was measured (to the nearest 0.1 g) twice: before introduction to the start cage and immediately after completion of the behavioural trial.

4.3.5 Faecal corticosterone metabolites

To compare the stress hormones of control and fluoxetine treated birds, we induced mild stress by moving each starling to a novel holding cage (0.60 m L x 0.45 m W x 0.60 m H) in social isolation. At each exposure length, samples of faeces were collected whilst birds were kept in the cages (either before or after behavioural data collection) for two hours, with *ad libitum* access to food and water. See Appendix B2 for full details of faecal collection and sample preparation.

Levels of faecal corticosterone metabolites were measured by ELISA, using a kit previously validated for faeces in a range of species, including Eurasian starling (Bean et al., 2014) (AC-14F1 Corticosterone EIA; IDS Limited, Tyne and Wear, UK). The assay was conducted according to kit instructions. The mean recovery was 78.7 %, mean intra-assay CV was 20.1 % and mean inter-assay CV was 21.0 %.

In total, samples from nineteen individuals after 6 weeks' exposure (winter) and fifteen individuals after 27 weeks exposure (summer) were analysed by ELISA. At each of these points, faeces were collected before the behavioural trials during one replicate and after the behavioural trials during the other. A mean of the concentration across the two replicates at each exposure length was computed, to provide an integrated measure of corticosterone for each individual at each time point. This also allowed inclusion of results from faecal samples that had to be pooled due to low sample volume, enhancing sample size.

Note that baseline faecal corticosterone metabolite data, collected to assess the stress-responsiveness of individuals pre-treatment, showed that individuals allocated to the fluoxetine-treatment group exhibited higher concentrations of faecal corticosterone metabolites pre-treatment than control group birds, by random chance (p = 0.043; see Appendix C2).

4.3.6 Feather quality

To assess whether exposure to fluoxetine altered the quality of feathers, and to assess the impact of treatment over an integrated period of time at the start of the dosing window, we plucked the same two tail feathers (rectrices R4 and R9) from each individual after one week of exposure, so that the feathers would regrow during the exposure period. Once the feathers had fully regrown (unchanging length over three consecutive weeks), they were harvested. For each feather, the full length (including the calamus) was measured to the nearest 0.1 mm and mass recorded to the nearest 0.0001 g.

4.3.7 Statistical methods

All statistical analyses were performed using software R, version 3.4.2 (R Core Team, 2016). R package 'lme4' was used to fit generalised linear mixed models and linear mixed models (Bates et al., 2015). In all these models, individual ID was specified as a random effect. We calculated body condition using Fulton's index (M/L^3 ; see Appendix C3 for details of calculation) for inclusion in certain models, based on the body mass of the bird at the start of a trial day (i.e. either before behavioural data collection or faeces collection). Our critical significance level for *p* values was $\alpha = 0.05$ throughout.

The effect of treatment on the count variable number of unique perches visited (measure of exploration, in relation to size of area explored) was investigated using a generalised linear mixed model (GLMM) with a Poisson error structure and a log link function. See Table 4.1 for the fixed effects included in the GLMM.

Linear mixed models (LMMs) were used to assess the effect of treatment on the following responses: time to reach last new perch (measure of exploration, related to exploration speed), number of flights (measure of activity), latency to approach during disturbance phase (measure of boldness), latency to approach during novel object phase (measure of neophobia), body mass differential, faecal corticosterone metabolite concentration, feather length and feather mass. Number of flights is a count variable but had a mean of 34.4, making a linear mixed model appropriate. Natural logarithm transformations were applied to the following responses to improve the spread of model residuals: faecal corticosterone metabolites, latency to approach (both disturbance and novel object phase data). Birds that did not approach during the relevant phase of the neophobia trial were excluded from analyses (Herborn et al., 2010). For the feather quality LMMs, any feathers that were damaged, missing or incomplete were excluded from analyses (10 control and 3 fluoxetine-treated regrown feathers). See Table 4.1 for the fixed effects included in each LMM.

Response variable(s)	Fixed effects included
No. unique perches visited (exploration), time to reach last new perch (exploration), number of flights (activity), body mass differential	Treatment*exposure length interaction, treatment, exposure length, sex, home aviary, body condition index, trial replicate, faecal collection order
Latency to approach in disturbance phase (boldness)	Treatment*exposure length interaction, treatment, exposure length, sex, home aviary, body condition index, neophobia trial order
Latency to approach in novel object phase (neophobia)	Treatment*exposure length interaction, treatment, exposure length, sex, home aviary, body condition index, neophobia trial order, novel object
Faecal corticosterone metabolites	Treatment*exposure length interaction, treatment, exposure length, sex, home aviary, body condition index
Feather length, feather mass	Treatment*feather type interaction, treatment, feather type, home aviary, sex

Table 4.1: Fixed effects included in each full model (i.e. before model reduction). All responses were modelled using LMMs, except for number of unique perches visited, which was modelled using a GLMM. Note that trial replicate was out of two, body condition index was divided by 1000 to ensure model convergence, faecal collection order corresponded to whether the individual participated in the behavioural trial or faecal sampling first, neophobia trial order corresponded to whether the individual participated in the novel object or disturbance phase first and feather type denoted whether the feather was grown in the wild or experimentally (i.e. during the exposure period).

Model selection was performed using backwards-stepwise selection for all models. For the GLMM, the least significant predictor was removed at each step using Wald's Z test, whilst likelihood ratio tests (R function 'drop1') were used for this purpose for LMMs. Corrected Aikake's Information Criterion (AIC_c), calculated using R package 'MuMIn' (Barton, 2017), was used to inform selection of the minimum adequate model. To ensure that no significant variable was incorrectly dropped during model reduction, each removed predictor was re-inserted back into the relevant minimum adequate model. This was done one removed predictor at a time, to check that each was still non-significant.

Since our home aviaries were single sex, the predictors sex and home aviary were conflated with each other. As a result, we could not include both predictors in any one model. To determine the most appropriate of these two predictors to include, we ran models including one of each of these predictors (sex, home aviary) independently and decided which to include based on significance under Wald's z (generalised linear mixed models) or likelihood ratio tests (linear mixed models). The predictor was selected based on AIC_c in the case that both sex and home aviary were significant

in a minimum adequate model (although note that it was not necessary to use this selection criterium; see Appendix C3).

R package 'DHARMa' was used to check that GLMM model assumptions were met, whilst the LMM's were checked for homogeneity of variance and normality of residuals. QQ plots for LMMs time to reach last new perch, body mass differential and feather length exhibited non-ideal behaviour at extreme values (high and/or low) but diagnostics for these models were otherwise acceptable.

Note that there was a significant relationship between sex and faecal corticosterone metabolite concentration, with the faeces of males containing higher corticosterone metabolite concentrations than females ($p = 0.023$). However, sex was removed from the final model, as mixed models should not contain >1 fixed effect per 10 observations for statistical inference to be reliable. AIC_c indicated that the model could be considered equivalent with or without sex included.

4.4 Results

4.4.1 Exploration

There was no effect of treatment on either of our measures of exploration: time to reach last new perch (measure of exploration speed; see Table 4.2a and Figure 4.1a) or number of unique perches visited (measure of size of area explored; see Table 4.3); although the effect of treatment on time to reach last new perch approached statistical significance ($p = 0.085$). There was a significant negative relationship between number of unique perches visited (a measure related to size of area explored) and exposure length (see Table 4.3) that was independent of treatment, with a decrease in this measure between 6 and 27 weeks' exposure.

4.4.2 Activity

We found no effect of treatment or any other predictor on number of flights, our measure of activity (see Table 4.2b and Figure 4.1b).

4.4.3 Boldness

In the disturbance phase (i.e. with food but no novel object), there was a significant interaction between exposure length and treatment on latency to approach (see Table 4.2c, Figure 4.1c). Fluoxetine-treated birds remained consistently bold between 6 and 27 weeks' exposure, whilst control birds became less bold over time.

4.4.4 Neophobia

In the novel object phase, there was no effect of treatment on latency to approach (see Table 4.2d and Figure 4.1d), although birds became more neophobic between 6 and 27 weeks' treatment and home aviary also influenced latency to approach in the presence of a novel object (see Table 4.2d).

4.4.5 Body mass differential

There was no effect of treatment on body mass differential (the difference in body mass before and after the behavioural data collection protocol). However, body mass loss was greater after 27 compared to 6 weeks' exposure and birds that were in better condition at the start of the trial tended to lose more body mass during the trial (see Table 4.2e). Body mass differential was also influenced by home aviary (see Table 4.2e).

4.4.6 Faecal corticosterone metabolite concentration

There was a significant interaction between treatment and exposure length on faecal corticosterone metabolite concentration (see Table 4.2f and Figure 4.2a). The concentration of corticosterone metabolites in faeces increased between 6 and 27 weeks' exposure in control birds but decreased in fluoxetine-treated birds.

4.4.7 Feather quality

There was a significant interaction between treatment and feather type (i.e. whether a feather was grown in the wild, before the current study, or was grown during the exposure window following plucking) on both feather length (see Table 4.2g and Figure 4.2b) and feather mass (see Table 4.2h and Figure 4.2c). Experimentally grown control bird feathers were shorter on average compared to feathers grown in the wild, whereas no such decrease in quality was evident in the feathers of fluoxetine-treated birds (see Figure 4.2b). Further, whilst experimentally regrown feathers had lower mass on average compared to wild-grown feathers in both treatment groups, the decrease in quality was markedly greater in the feathers of control compared to fluoxetine-treated birds (see Figure 4.2c). Males also had significantly longer feathers (Table 4.2g) with significantly greater mass (Table 4.2h) on average than females.

Fixed Effects	Coef. β	SE (β)	t	χ^2	p
a) Response: Exploration (time to reach last new perch)					
Intercept	232.99	18.88	12.34	-	-
Treatment	45.52	26.70	1.71	2.97	0.085
b) Response: Activity (number of flights)					
Intercept	34.81	3.80	9.16	-	-
Treatment	-8.52	5.37	-1.59	2.58	0.108
c) Response: Boldness (latency to approach in disturbance phase)					
Intercept	4.35	0.25	17.75	-	-
Treatment	0.14	0.35	0.40	5.58	0.061
Exposure length	0.69	0.23	2.98	13.11	<0.001
Treatment*exposure length	-0.75	0.33	-2.31	4.86	0.027
d) Response: Neophobia (latency to approach in novel object phase)					
Intercept	4.87	0.20	24.36	-	-
Exposure length	0.54	0.20	2.71	7.31	0.007
Home aviary A	0.03	0.45	0.06	11.13	0.025
Home aviary B	-0.15	0.46	-0.32		
Home aviary C	0.91	0.39	2.36		
Home aviary D	0.95	0.44	2.18		
e) Response: Body mass differential					
Intercept	-3.58	1.64	-2.19	-	-
Exposure length	0.86	0.16	5.45	27.79	<0.001
Home aviary A	0.29	0.29	1.00	13.67	0.008
Home aviary B	-0.87	0.29	-3.03		
Home aviary C	0.18	0.25	0.72		
Home aviary D	-0.15	0.24	-0.62		
Body condition	1.81	0.55	3.31		
f) Response: Faecal corticosterone metabolite concentration					
Intercept	6.30	0.08	74.45	-	-
Treatment	-0.19	0.12	-1.62	5.88	0.053
Exposure length	-0.02	0.10	-0.22	3.87	0.144
Treatment*exposure length	-0.28	0.15	-1.92	3.87	0.049
g) Response: Feather length					
Intercept	68.18	0.38	177.27	-	-
Treatment	1.18	0.43	2.73	18.54	<0.001
Feather type	-0.74	0.16	-4.69	12.56	<0.001
Feather type*treatment	0.83	0.22	3.74	24.66	<0.001
Sex	2.36	0.63	3.76	12.32	<0.001
h) Response: Feather mass					
Intercept	0.018	0.00041	46.40	-	-
Treatment	0.00049	0.00046	1.08	20.25	<0.001
Feather type	-0.0021	0.00017	-12.42	77.28	<0.001
Feather type*treatment	0.0012	0.00024	5.00	19.90	<0.001
Sex	0.0015	0.00066	2.22	5.06	0.025

Table 4.2: Minimum adequate model outputs for LMMs investigating the effect of treatment and confounding variables on the following responses: a) exploration (time to reach last new perch; a measure of speed of exploration), b) activity (number of flights), c) boldness (natural log transformed latency to approach in the disturbance phase; 60 observations from 12 control and 12 fluoxetine-treated birds), d) neophobia (natural log transformed latency to approach in the novel object phase; 61 observations from 11 control and 12 fluoxetine-treated birds), e) body mass differential, f) natural log transformed faecal corticosterone metabolite concentration (34 measurements from 11 control and 8 fluoxetine-treated birds), g) feather length (mm), h) feather mass (g). For a), b) and e) there are 102 observations/measurements from 13 control and 14 fluoxetine-treated birds. For g) and h) there are 83 observations from 12 control and 12 fluoxetine-treated birds. Table shows: coefficient estimate (β), standard error of β, t-statistic (t), Chi-Square statistic (χ^2) and significance level p.

Fixed Effects	Coef. β	SE (β)	*z*	*P*
Response: Exploration (number of unique perches visited)				
Intercept	1.47	0.07	20.79	<0.001
Exposure length	-0.17	0.07	-2.44	0.015

Table 4.3: Minimum adequate model output of GLMM with Poisson error distribution, testing the effect of treatment and confounding variables on exploration (number of unique perches visited; a measure relating to size of area explored). *n* = 102 observations from 27 birds (*n* = 13 control and *n* = 14 fluoxetine-treated). Table shows: coefficient estimates (β), standard errors (SE(β)), Wald's *z*-score (= β /SE(β)) and significance level *p*.

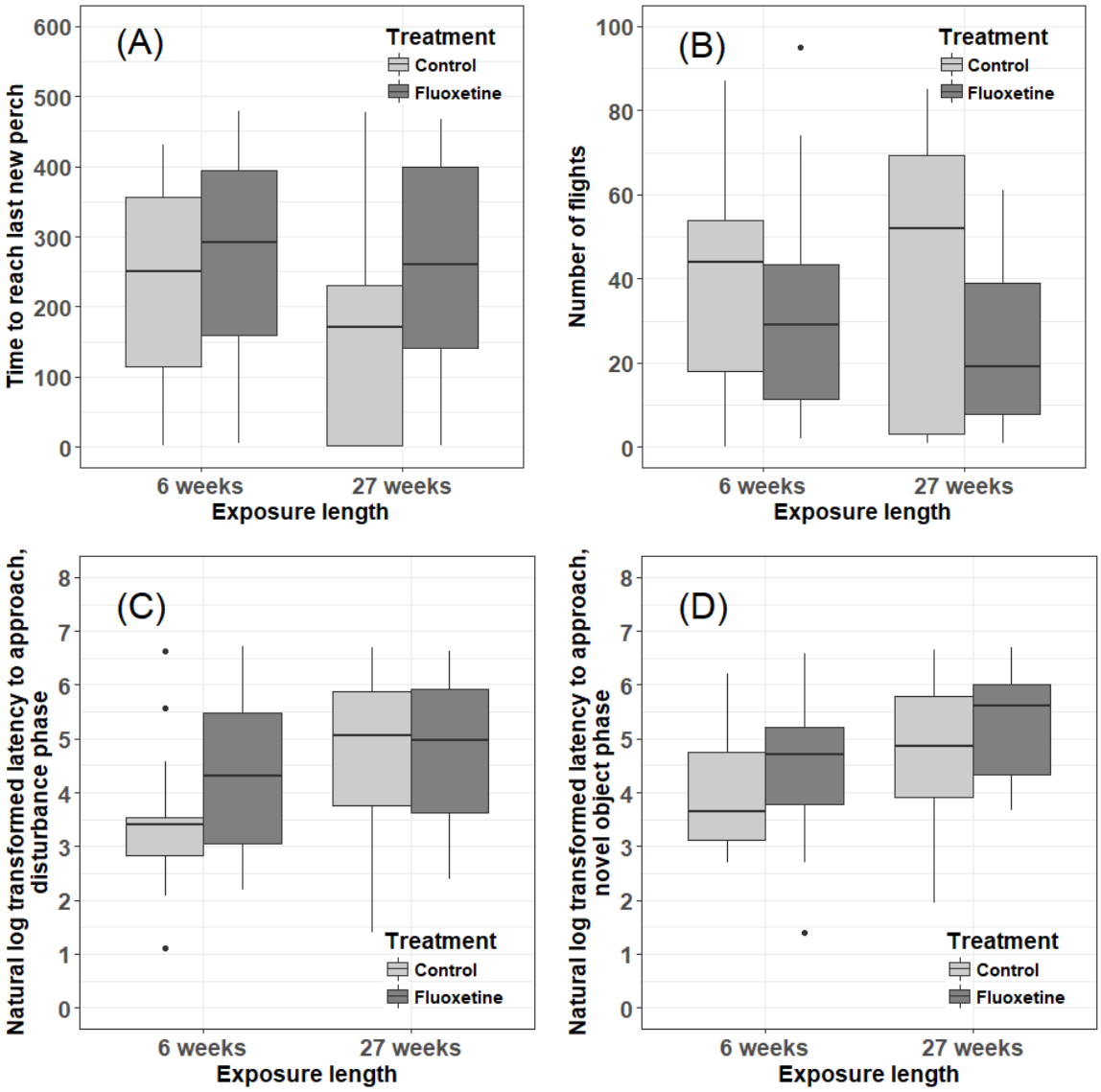

Figure 4.1: Plots showing the effect of treatment on: a) speed of exploration (time to reach last new perch), b) activity (number of flights), c) boldness (natural log transformed latency to approach during disturbance phase; 61 observations from 12 birds per group); d) neophobia (natural log transformed latency to approach during disturbance phase; 61 observations from 11 control and 12 fluoxetine-treated birds). For a) and b) there are 102 observations from 13 control and 14 fluoxetine-treated birds. Data collected after 6 and 27 weeks of treatment. Boxes correspond to upper and lower quartiles and points shown outside of whiskers are outliers (1.5*IQR, as defined by Tukey (Tukey, 1977)).

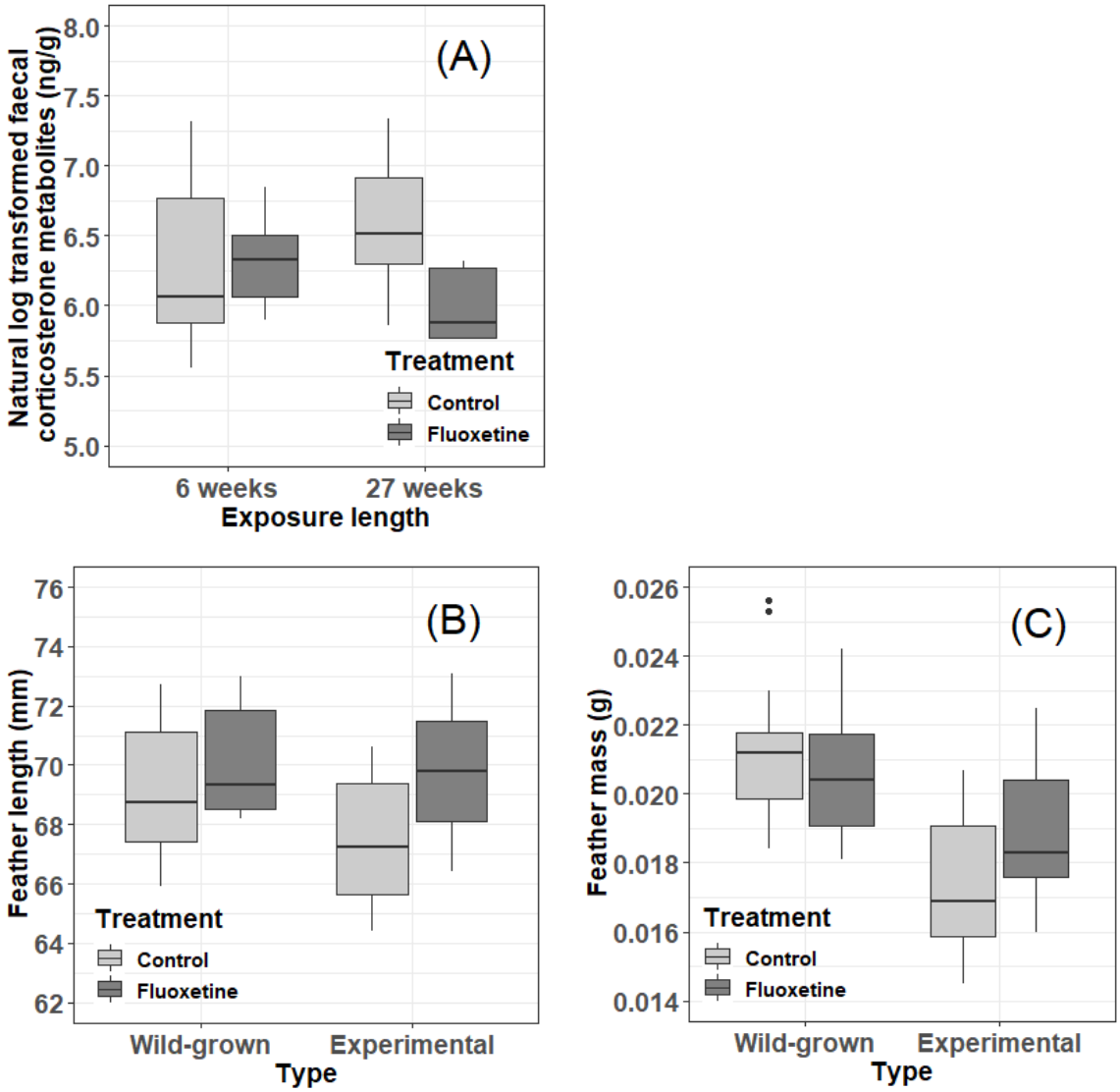

Figure 4.2: Plots showing the effect of treatment on: a) faecal corticosterone metabolite concentration (34 measurements from 11 control and 8 fluoxetine-treated birds), b) feather length (experimentally regrown compared to wild-grown), c) feather mass (experimentally regrown compared to wild-grown). For b) and c) there were 83 observations from 12 birds per group. For a), data were collected after 6 and 27 weeks of treatment. Experimental feathers typically regrew between weeks 2-7 of treatment. Boxes correspond to upper and lower quartiles and points shown outside of whiskers are outliers (1.5*IQR, as defined by Tukey (Tukey, 1977)).

4.5 Discussion

In our study, we found evidence that environmental concentrations of fluoxetine can alter certain anxiety-related behaviours and aspects of stress physiology in birds. Specifically, we found treatment effects on boldness, faecal corticosterone metabolite concentrations and feather quality. The effects of fluoxetine on both boldness and corticosterone metabolites were dependant on exposure length, becoming apparent after 27 weeks of exposure, whilst treatment effects on feather quality were evident during the first weeks of exposure.

Control birds became less bold between 6 and 27 weeks' exposure, whilst the boldness of fluoxetine-treated individuals was consistent. Control individuals took longer to feed (less bold) during the summer trials (27 weeks) compared to the winter trials (6 weeks). Thus, control birds exhibited more typical foraging behaviour, since birds are predicted to take higher risks when foraging during colder weather (Hilton et al., 1999), corresponding to reduced latency to feed during winter compared to summer in controls. This seems to indicate that exposure to fluoxetine produced context specific effects on boldness that depended on environmental and/or seasonal cues. Alternatively, the effect on boldness may have been related to exposure length. In this case, the treatment effect observed in fluoxetine-treated individuals would equate to a suppression of the reduction in boldness observed over time in controls, that did not become apparent until 27 weeks' exposure. Reversal of stress-induced suppression of another anxiety-related behaviour (exploration) has been reported in rats (Zhang et al., 2000). However, the rats were exposed to a far higher concentration (10 mg kg^{-1}) for just 14 days whereas we did not observe our effect until 27 weeks' exposure to a far lower concentration (0.034 mg kg^{-1} bodyweight). However, we have not been able to conclusively determine whether our observed effect on boldness varied according to the season or whether it related to the duration of treatment.

The result of a previous study that investigated the effects of chronic exposure to an environmentally relevant dose of fluoxetine (1.58 µg $weekday^{-1}$ for 22 weeks) in Eurasian starling (Bean et al., 2014) reported no treatment effect on boldness. However, this study did not compare between two time points, so any change or reduction in boldness over time would have been missed. Indeed, if our 27 week data were taken in isolation, an effect on boldness would not have been evident. Conversely, in Siamese fighting fish (*Betta splendens*), a high environmentally relevant concentration of 500 ng L^{-1} was found to reduce boldness overall (Dzieweczynski et al., 2016a, Dzieweczynski et al., 2016b). However, the fish were exposed subchronically for eight days, rather than chronically as in our study. The short exposure duration may explain the anxiogenic effect of fluoxetine observed in the fish, as effects on behaviour can vary according to exposure

length. For example, anxiogenic effects elicited in rats by acute treatment with fluoxetine have been shown to disappear following chronic treatment for 21 days (To et al., 1999). Further, the route of administration of fluoxetine in the fish studies was very different to ours, as the fish were continually immersed in fluoxetine-contaminated water, whereas we fed each bird a low dose of fluoxetine once each weekday.

Contrary to our observed effect on boldness, we observed no effect of treatment on neophobia during the novel object phase of the trial. This could be because novel object trials can be viewed as measuring tendency to innovate in novel situations (Webster and Lefebvre, 2001), rather than being strictly related to fear and anxiety. Indeed, this could be the case in the Eurasian starling, since one study previously found that neophobia was unrelated to circulating corticosterone (Apfelbeck and Raess, 2008). However, we did find that individuals from both groups were more neophobic on average during the trial after 6 compared to 27 weeks exposure, possibly because the 6 week trial was during winter, whilst the 27 week trial was in summer. Similarly to our observed effect on boldness in control birds, the colder conditions in winter compared to summer may have meant the birds were hungrier and therefore less neophobic and faster to feed. Our body mass loss data support this interpretation, as birds lost more mass in summer (27 weeks) than winter (6 weeks). Starlings would be predicted to lose less body mass in colder weather in order to preserve a higher body mass, since body mass varies negatively with air temperature (Peach et al., 1992). Alternatively, as with our control bird boldness result, it could instead be the case that the birds exhibited higher levels of anxiety-related behaviour after 27 compared to 6 weeks.

We found no clear effect of treatment on any of our measures of exploration or activity, although there was a borderline significant effect on exploration speed, indicating that fluoxetine-treated birds were slower to explore than controls on average. However, there was no indication of any treatment effect on number of unique perches visited, i.e. the size of the area explored, although individuals from both groups explored a smaller area of the arena after 27 weeks' (summer) compared to 6 weeks' (winter) exposure. As with our neophobia, boldness and body mass loss results, this may be related to increased motivation to take risks whilst foraging during colder weather (Hilton et al., 1999), leading to a larger area of the arena being explored, although it might instead relate to increasing anxiety-related behaviour over time. We could not relate the activity data collected during the exploration trial (number of flights) to any predictor. Our findings correspond well with a previous study in Eurasian starling, which reported no effect of fluoxetine on exploration or activity at a lower environmentally relevant concentration (1.58 µg $weekday^{-1}$), albeit utilising a different experimental set up (Bean et al., 2014). Although effects on activity and exploration have been reported in certain fish species exposed to low water concentrations (4-25

ng L^{-1}) for 28 days (Martin et al., 2017, Saaristo et al., 2017), based on our study and the work of Bean et al (Bean et al., 2014), the Eurasian starling appears to be less sensitive to such effects.

As for the observed effect on boldness, the effect of fluoxetine treatment on faecal corticosterone metabolite concentration also depended on exposure length. Indeed, a difference in faecal corticosterone metabolite concentration between the two treatment groups was evident after 27 but not 6 weeks' exposure. A lower environmentally relevant concentration (1.58 µg $weekday^{-1}$) administered chronically for 22 weeks has previously been shown to affect habituation to isolation, in terms of the relationship between faecal corticosterone metabolite levels and mass loss (Bean et al., 2014). However, this study did not report a direct treatment effect on the concentration of faecal corticosterone metabolites. In our study, we were able to observe a direct effect on faecal corticosterone metabolites, presumably due to our higher exposure concentration (3.71 µg $weekday^{-1}$). At the time the 27 week data were collected, the birds were beginning their annual prebasic moult. Lower circulating corticosterone is expected during this period compared to the rest of the year (Romero and Remage-Healey, 2000). Therefore, the reduction in fluoxetine-treated corticosterone between 6 weeks' (winter) and 27 weeks' (27) exposure suggests that the fluoxetine-treated individuals were exhibiting seasonally typical variation in corticosterone levels after 27 weeks, whereas control birds exhibited atypical variation, possibly due to increased stress. However, this does not explain the absence of a treatment effect on corticosterone after 6 weeks' exposure. Instead, the differences in corticosterone responses over time may relate to the duration of treatment but, as with our boldness result, our experimental design could not differentiate the influence of treatment duration from the influence of the seasonal context.

It is possible that the time-dependant anxiolytic action of fluoxetine we observed, on corticosterone levels and boldness, was associated with a delayed onset of such effects. In humans, it takes 2-6 weeks of treatment to establish therapeutic effects (Pejchal et al., 2002, Blier and de Montigny, 1994). This is posited to correspond to the time taken to progressively desensitise serotonin receptors (5-HT_{1A}), such that sufficient extracellular serotonin can accumulate at the synaptic cleft (Pejchal et al., 2002, Blier and de Montigny, 1994). During these first weeks of treatment, certain side effects may be more common and fluoxetine can even produce anxiogenic effects at this time (To et al., 1999). In this study, we collected data on faecal corticosterone metabolites and boldness after 6 weeks' exposure, corresponding to the far end of the 2-6 week delay in the onset of therapeutic effects reported in humans. Yet we also administered a very low dose, which was approximately 10 % of a typical human dose (controlling for body mass). One possibility is that such a low, environmentally relevant dose would be slower to desensitise serotonin receptors and by consequence, slower to produce therapeutic effects compared to a typical therapeutic dose. In this

case, it is possible that anxiolytic effects in our study were not produced after 6 weeks' treatment but could be observed by 27 weeks.

Though treatment effects on boldness and corticosterone became apparent only after 27 weeks' exposure, our feather quality data indicated that fluoxetine treatment was causing physiological changes during the first weeks of treatment. The fluoxetine-treated birds regrew higher quality feathers than control birds in terms of mass and length, yet no such difference in feather quality between the groups was evident in feathers grown in the wild. Higher feather quality indicates reduced physiological stress, since elevated corticosterone promotes catabolic pathways that can inhibit feather growth (Romero et al., 2005). Indeed, certain indices of feather quality, such as surface area (Strochlic and Romero, 2008) have been directly related to endogenous corticosterone, and both acute and chronic stress have been shown to increase inter-barb distances in rectrices (DesRochers et al., 2009). Although fluoxetine-treated feathers grown during the exposure window were not shorter on average than wild-grown feathers, they were lighter; although this reduction in feather mass was far greater on average in controls. This suggests that both groups were under more stressful conditions during the exposure period when the feathers regrew, compared to the conditions in which the feathers grew in the wild. This could have been due to the stress caused by capture and handling each weekday during the dosing procedure. If this was the case, then fluoxetine treatment reduced the magnitude of this stress response during the weeks of feather regrowth (typically weeks 2-7 of treatment), in such a way that feather quality was compromised to a lesser extent than in control birds. We may have observed an effect on feather quality, yet not any other early measure of stress physiology (faecal corticosterone metabolites or body mass loss after 6 weeks' exposure), since feathers regrow over a period of weeks. This provided a far longer, integrated time window during the early exposure period, over which to observe a treatment effect.

4.6 Conclusions

In this study, we have shown that exposure to an environmentally relevant concentration of fluoxetine can alter anxiety-related behaviours (boldness) and endogenous corticosterone levels; yet such effects developed over time and became apparent only when comparing results after 6 weeks' and 27 weeks' exposure respectively. Whilst there was no apparent anxiolytic action of our environmentally relevant dose after 6 weeks' treatment, there was early evidence of physiological perturbations in the quality of feathers grown during the first few weeks of exposure. This demonstrates the potential of environmental levels of fluoxetine to alter traits in wildlife in relatively short timeframes. Whilst it is unlikely that a starling would forage on contaminated prey

from a WWTP daily for 27 weeks, it is far more likely that a bird would visit a WWTP daily over a period of weeks. For example, during the coldest months of winter, whilst provisioning chicks or in the case of juveniles, in the period immediately after fledging (Fuller and Glue, 1978, Fuller and Glue, 1980).

In addition, wild birds foraging at WWTPs would be exposed to a mixture of SSRIs with potentially additive effects. For example, a field study in which male goldfish (*Carassius auratus*) were caged for 21-days downstream from a WWTP reported that the fish became bolder, more active and more exploratory, compared to fish at a control site (Simmons et al., 2017). The authors suggested that this was likely due to the presence of six serotonin reuptake inhibitors, including fluoxetine, in the water (Simmons et al., 2017). If our anxiolytic effects on boldness and corticosterone were dependant on treatment duration, then they developed quite slowly. However, exposure to additive SSRI mixtures could mean that that such effects appear far more rapidly or with a greater magnitude, in wild birds foraging on sewage-contaminated invertebrates. However, it is possible that our observed effects were context dependant; varying according to environmental or seasonal cues. Further work in this area should ensure that experimental designs can distinguish the effect of treatment duration from the attenuation of treatment effects by seasonal factors, and should aim to separate the contribution of each to observed effects.

To put our results in context, fluoxetine-induced alterations to boldness could translate to altered behaviour during encounters with predators (Wilson and Godin, 2009). Inappropriate responses to predators or altered risk-taking during foraging (Hilton et al., 1999) could have implications for individual survival. Further, individual behavioural alterations of this nature could in turn have implications for populations or even for food webs (Brodin et al., 2014, Saaristo et al., 2018). Meanwhile, an inappropriate stress response in terms of endogenous corticosterone could affect energy expenditure, predation risk and by consequence, individual survival (McEwen and Wingfield, 2003). Whilst improved feather quality might superficially appear to be a positive outcome of exposure, it is likely to occur concurrently with other effects on stress physiology that we did not measure, which may be detrimental to individual survival. Overall, our work illustrates the ability of psychotropic pharmaceuticals in the environment to alter traits that are vital for individual survival and adds to a growing body of evidence concerning the ability of environmentally relevant concentrations of fluoxetine to alter stress physiology and anxiety-related behaviour in free-living vertebrates (Abreu et al., 2014, Martin et al., 2017, Saaristo et al., 2017, Weinberger and Klaper, 2014, Barry, 2013, Dzieweczynski et al., 2016a, Dzieweczynski et al., 2016b, Bean et al., 2014).

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Chapter 5: Detecting fluoxetine and norfluoxetine in wild bird tissues and feathers

5.1 Abstract

The contamination of the environment with human pharmaceuticals is a widespread and growing issue. Wild vertebrates may be at particular risk from this threat, due to evolutionary conservation of drug targets. However, exposure of wildlife to pharmaceuticals is poorly characterised, partly due to challenges associated with detecting what are typically rapidly metabolised compounds. As part of a wider study on the behavioural effects of fluoxetine (Prozac®) on Eurasian starlings (*Sturnus vulgaris*), we aimed to determine which avian samples are best suited to detecting exposure in free-living birds. We analysed plasma, tissues and feathers (wild-grown and experimentally regrown) from starlings dosed chronically (0.035 mg kg^{-1} bodyweight, daily) for 28 weeks. In two out of 12 treated birds, we detected fluoxetine in plasma 30 minutes after the final dose but not subsequently. Median concentrations of fluoxetine/norfluoxetine in tissues taken two hours after the 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. Using decay curves, we estimated that fluoxetine would remain detectable in liver and kidney approximately 4.5 times longer than our estimated residence time in the brain (20 hours). In dosed birds, fluoxetine was detected in feathers regrown through the dosing period (median concentration = 11.39 ng g^{-1}) at concentrations significantly higher than in regrown feathers from control birds (thought to have arisen through cross-contamination between birds). 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 that liver and kidney can be used for detecting fluoxetine in avian carcasses and provide a first indication that feathers may be suitable for assessing wild bird exposure to fluoxetine.

5.2 Introduction

As analytical techniques have improved over the past few decades, the widespread occurrence of human pharmaceuticals in the environment has come to light (Daughton and Ternes, 1999). The burgeoning manufacture and consumption of pharmaceuticals (QuintilesIMS Institute, 2016) suggests that, assuming a "business as usual" scenario, the volume discharged to the environment will continue to increase over the coming years. Human pharmaceuticals have been detected in both aquatic and terrestrial ecosystems and biota (Hughes et al., 2013, Monteiro and Boxall, 2010). The concentrations detected are typically low relative to therapeutic doses, yet may still pose a threat to exposed biota, as pharmaceuticals 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 vertebrate wildlife, such as birds, are exposed to human pharmaceuticals. Potential exposure routes in wild birds 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).

One pharmaceutical that has received considerable attention in relation to its effects on non-target wildlife is the selective serotonin reuptake inhibitor (SSRI) fluoxetine (Prozac®), which is prescribed as an antidepressant. Its active desmethylated metabolite, norfluoxetine, is also a serotonin reuptake inhibitor and is similarly considered to pose an environmental risk, albeit to a lesser extent than the parent compound (Kumar and Xagoraraki, 2010). Both the parent compound and its metabolite have been detected in the tissues and plasma of freshwater fish, generally at micro- to nanogram per gram concentrations (Brooks et al., 2005, Chu and Metcalfe, 2007, Schultz et al., 2010). Relatively little is known about subsequent trophic transfer of fluoxetine through the food web, between fish and piscivorous birds although one study investigating pharmaceuticals in water, fish and ospreys (*Pandion haliaetus*) at contaminated riverine and estuarine sites detected fluoxetine in trace amounts (concentrations between the instrument detection limit and the higher method detection limit) in all water samples analysed and in some fish plasma samples, but not in osprey plasma (Bean et al., 2018).

Detecting exposure to pharmaceuticals in higher trophic terrestrial wildlife is likely to be more challenging than in fish. Fish in effluent-dominated waters can be continually living in dilute

concentrations of fluoxetine and norfluoxetine in freshwaters (Christensen et al., 2009). This ongoing, chronic exposure and uptake may result in the presence of detectable concentrations in plasma. In contrast, non-aquatic higher vertebrates are likely to be exposed to fluoxetine and other pharmaceuticals more sporadically, when they consume contaminated prey, water or vegetation. Ability to detect exposure of free-living individuals to compounds such as fluoxetine that have short plasma and tissue half-lives will depend 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 harvested 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 hours compared to 21 hours 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 occur rapidly in birds (Caccia et al., 1990). Thus, exposure to pharmaceuticals is difficult to identify from plasma measurements. 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*) two hours after they were given a sub-therapeutic dose (1.58 µg per bird) of fluoxetine (Bean et al., 2017). Faecal sampling is another non-destructive sample that can also be used to demonstrate exposure. However, faecal elimination of pharmaceuticals can also be rapid (Bean et al., 2017) and obtaining suitable faecal samples from wild-caught birds can be problematic.

Other methods for characterising exposure to contaminants in wild birds include the analysis of tissues from the carcasses of individuals found dead, an approach that is widely used to assess exposure to a range of contaminants (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 than that of many contaminants that are currently monitored (Gómez-Ramírez et al., 2014). For instance, concentrations have been found to decrease by 39 % (fluoxetine) and 23 % (norfluoxetine) respectively in canine liver 2- 12 hours after euthanasia, following a dose of 10 mg kg^{-1} fluoxetine administered three hours before death

(Pohland and Bernhard, 1997). Although the elimination half-lives of fluoxetine may be longer in tissues compared to plasma, information on how these vary between different body tissues is not well characterised and it is possible that residues may be relatively persistent in some tissues.

Feathers can also be used for detecting exposure to contaminants, although they reflect exposure that occurred in the past, at the time of feather growth. Various studies have shown that feathers can be used to demonstrate exposure to a range of other persistent contaminants (Espin et al., 2016), such as trace metals (Abdullah et al., 2015) and persistent organic pollutants; the latter encompassing compounds such as polyhalogenated compounds (Garcia-Fernandez et al., 2013) and organochlorine pesticides (Espin et al., 2012). Circulating plasma concentrations of contaminants can be stored within the feather as it grows and can remain stable therein over long periods of time (Garcia-Fernandez et al., 2013). Feathers can be collected from carcasses but also have the advantage that they can be sampled non-destructively from live birds through plucking, whilst moulted feathers can also be collected from nests (Garcia-Fernandez et al., 2013). We are unaware of any published data on transport of fluoxetine and norfluoxetine into feathers but is it known that fluoxetine is deposited into mammalian hair (Lefebvre et al., 1999, Fisichella et al., 2014), and is found in poultry feather meal, presumably a result of the treatment of poultry with veterinary feed additives (Love et al., 2012).

Detection of exposure is key for understanding whether pharmaceuticals are likely to 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 fluoxetine and norfluoxetine concentrations in a range of tissues (plasma, brain, kidney, liver and lung) taken from wild-captured Eurasian starlings held under experimental conditions (during which time they would have metabolised any existing plasma and internal tissue residues) and which were subsequently chronically dosed with an environmentally-relevant dose of fluoxetine. We used starlings as they are known to feed on invertebrates on trickling filter beds at waste water treatment plants (WWTPs) (Fuller and Glue, 1978) and so are at high risk of exposure to pharmaceuticals, such as fluoxetine, that are present in sewage (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 tissues residues were likely to be analytically detectable; such information is crucial for informing the design of biomonitoring programmes. We also quantified the levels of 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 evidence that starlings are currently exposed to fluoxetine in the wild in Britain.

5.3 Methods

5.3.1 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.

5.3.2 Aviary study

The tissues analysed were from birds subjected to an *in vivo* chronic effects experiment conducted with 27 wild-caught Eurasian starling, 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 (*n* =14) or control treatment (*n* = 13), 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 0.0343 (± 0.00144) mg 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 (Feare, 1984, Bean et al., 2017). We administered a 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 fluoxetine in a subset of spiked waxworms and the mean (± SE) concentration was 3.71 ± 0.18 µg $worm^{-1}$ (*n* = 10; see Appendix A7). Dosing of birds commenced in late November 2015 and continued for 28 weeks, at which point 24 individuals remained alive (*n* =12 per group). For details of the dose calculation, see Chapter 2.3.3, for details of animal capture and husbandry see Appendix A1.

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 treatment and one control group birds 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 or during acclimation to captivity. The plucked feathers, grown when the birds were free-living in the wild (hereafter termed wild-grown feathers) were harvested 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 fluoxetine and norfluoxetine concentrations. Euthanasia of each individual occurred two hours after individuals were given their final fluoxetine/control dose. Brain, kidney, liver and lung were immediately excised, frozen within 30 minutes using dry ice and subsequently stored at -80 ˚C until analysed. In between administration of the final dose and euthanasia, a blood sample was taken from each fluoxetine-treated bird for analysis of fluoxetine and norfluoxetine plasma residues. Guided by findings in (Bean et al., 2017) and to enhance the chance of detecting residues and allowing decay curves to be constructed, we blood sampled six individuals 30 minutes after dose administration, whilst the remaining six individuals were sampled after one hour. Assignment of birds to the 30 or 60 minute 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 minutes of collection at 16 000 *g* for 10 minutes. 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 the rectrices had not regrown (Appendix D, Table D4). 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 (Appendix D, Table D4). We included these birds in our assessment of whether fluoxetine was detectable in feather samples but excluded them from any statistical comparison of residue magnitude as concentrations for a single feather may not be directly comparable to that for a pooled pair (Bortolotti, 2010).

5.3.3 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 concentration 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 eleven of the twelve control bird liver samples, whilst the remaining bird had a concentration close to the limit of detection (concentration in sample: 0.34 ng g^{-1}, liver limit of detection (LOD): 0.27 ng g^{-1}). 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 feathers from both fluoxetine-treated and control birds. This was to assess: i) the influence of contamination in feathers, given that the birds were housed in mixed-treatment groups, ii) whether residues in wild-grown feathers were indicative of past exposure (during the time when the feather was grown and residues fixed in the feather matrix) in the wild.

Tissue samples for analysis were thawed, sub-sampled for analysis by cutting in half, homogenised and accurately weighed to the nearest 0.0001 g. The nominal mass (wet weight) analysed for each tissue was: brain 0.3 g, kidney 0.3 g, liver 0.3 g, lung 0.1 g. The samples were ground with sand in a glass pestle and mortar before being transferred to a Pyrex test tube. Feather samples were washed with diluted soap (Jenni-Eiermann et al., 2015), then HPLC grade water and allowed to dry for 24 hr before being cut into small pieces and ground in ceramic pestle and mortars with liquid N_2. After grinding, the feathers were transferred to Pyrex test tubes. 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 x 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 minutes and subsequently centrifuged for 10 minutes at 20 ˚C and 4696 *g*. 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 cc, 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 minutes, 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.

5.3.4 LC-MS/MS Analysis

The residues of fluoxetine and norfluoxetine 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 0-7.5 min, 0.25 ml min^{-1} from 7.5-12 min and 0.30 ml min^{-1} after 12 minutes. The gradient was performed as follows: 18 % mobile phase B from 0-7.5 minutes, 60 % B from 7.5-12 minutes, 100 % B from 12-15 minutes, 18 % B from 15-20 minutes.

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 Appendix D, Table D1 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 5.1 for method recoveries and detection limits, displayed for fluoxetine and norfluoxetine.

Sample matrix	LOD (ng g^{-1})	Recovery (%)
Liver	0.27	66.4
Kidney	0.07	62.3
Lung	0.07	60.6
Brain	0.26	62.3
Plasma	0.16	93.8
Feathers	0.84	108.0

Table 5.1. Method detection limits (LOD; ng g^{-1} wet weight) and mean recoveries (as percentages) are displayed for each type of sample matrix analysed by LC-MS/MS. Note that liver and feather samples came from both fluoxetine-treated and control birds (twelve samples per group for liver, 23 fluoxetine-treated and 20 control group for feathers), whilst other samples (kidney, lung, brain, plasma; twelve samples each) were only from fluoxetine-treated birds.

5.3.5 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 concentrations of: (i) fluoxetine; (ii) norfluoxetine; (iii) fluoxetine: norfluoxetine ratio. We examined the significance of associations between fluoxetine and norfluoxetine concentrations within and between different sample matrices (kidney, liver, lung, brain and feather) using Spearman's Rank correlation coefficients. Comparison of the 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 (parent and metabolite compound) 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.

5.3.6 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 in brain, kidney

and liver. The curves were derived from *in vivo* experimental data from Eurasian starlings given 1.58 µg fluoxetine per bird, 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 two hours (*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.

5.4 Results and discussion

5.4.1 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 minutes after administration of the final oral dose (0.20 and 0.42 ng ml^{-1} respectively) but was not detected any of the plasma samples (n = 6) taken 60 minutes 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 two hours (Bean et al., 2017) but our data indicate that a (>2 fold) higher dose is completely eliminated from plasma within one hour. 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 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.

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 tissue in mammals (Hiemke and Hartter, 2000, Caccia et al., 1990) and this also appears to be true in birds. In the present study, concentrations of parent compound and metabolite varied significantly between tissue types ($H_{(3)} \geq 32.5$, $p < 0.001$ in both cases). *Post-hoc* tests indicated that fluoxetine concentrations decreased in the order liver > kidney $\underline{\geq}$ lung ≥ brain (Figure 5.1a and Appendix D, Table D2) and norfluoxetine concentrations in the order liver > kidney ≥ lung > brain

(Figure 5.1b and Appendix D, Table D2). These results clearly indicate that fluoxetine and its metabolite distribute unevenly between tissues and that the highest concentrations are found in the liver. The latter had also been suggested by a limited previous study, [19] in which residues were measured in only three birds (dosed at half the rate to the present study). 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 5.2). On average, the equivalent of 8.59 % of the oral dose (given two hours earlier) was accounted for by the total burden in the four tissues, with most (7.40 % of the oral dose) in the liver (Table 5.2). We also examined the relationships between residue concentrations in different organs to determine whether concentrations in one tissue could be used to predict concentrations in others. For fluoxetine, there were positive relationships in all cases but only the correlation between brain and kidney fluoxetine was statistically significant (Figure 5.2 and Appendix D, Table D3). Thus, levels of fluoxetine in kidney tissues might best predict the concentration of fluoxetine in brain tissue and thus the risk of mode of action related adverse effects, although such estimates would be extremely crude.

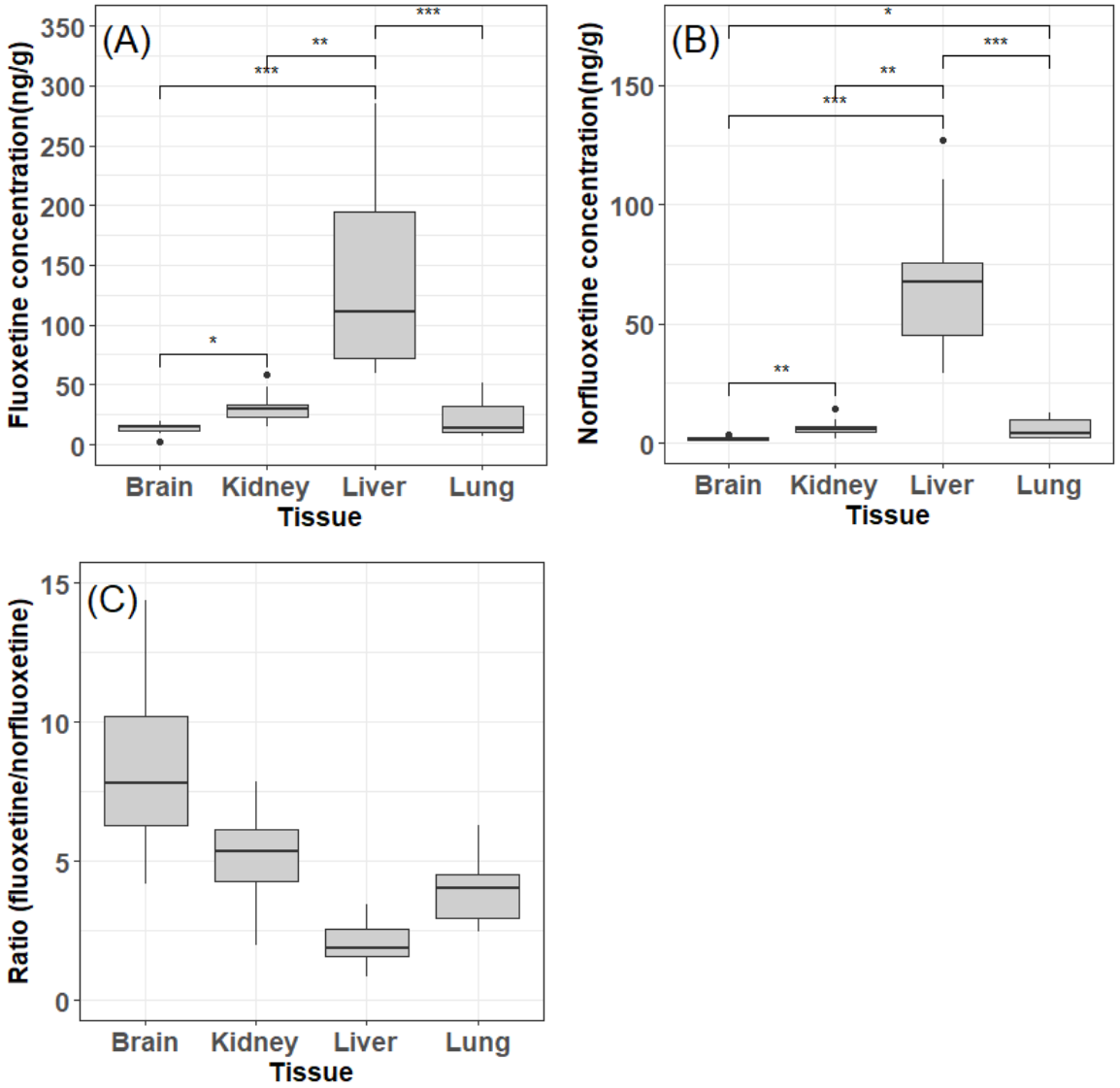

Figure 5.1. Median concentrations of (a) fluoxetine and (b) norfluoxetine in brain, kidney, liver and lung (*n* = 12 per organ). In (a) and (b), significant differences in concentrations between pairs of tissues are starred (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). See Appendix D, Table D2 for 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 two hours 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 (Tukey, 1977)).

Tissue	Median burden / IQR (ng)	Median percentage of dose / IQR (%)
Brain	14.5 / 3.55	0.390 / 0.0958
Kidney	18.9 / 15.6	0.509 / 0.428
Liver	274 / 180	7.40 / 4.86
Lung	10.9 / 10.3	0.293 / 0.278

Table 5.2. Median 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.

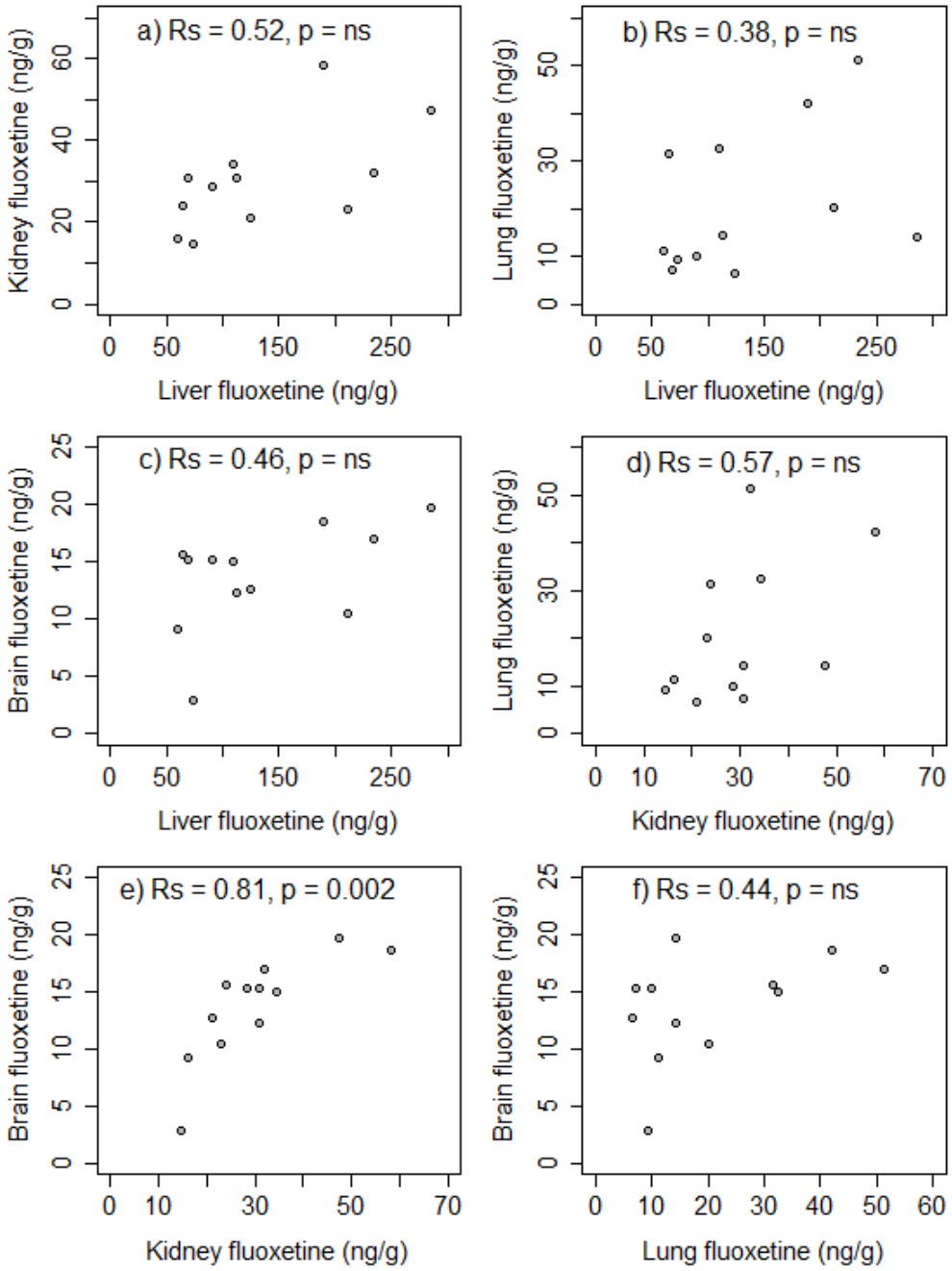

Figure 5.2. Scatter plots of fluoxetine concentrations between pairs of different tissues. Spearman correlation coefficients are shown, as are corresponding *p*-values for statistically significant correlations, whilst non-significant correlations are ascribed a *p*-value of "ns" (α = 0.05). Specifically, 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 Data collected at the end of a 28 week dosing period. Note that full results from Spearman's Rank-Order correlations are reported in Appendix D, Table D3.

On the basis of our concentration data and because of their 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. However, liver, and particularly kidney, may not be the best choice of organs for monitoring exposure in other wildlife, such as wild mammals. Mammalian studies have shown that fluoxetine is distributed extensively in liver, lung and brain tissues (Pohland and Bernhard, 1997, Caccia et al., 1990, 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 next examined the ratio of 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.*, $p < 0.001$; Figure 5.1c). However, within the kidney, liver and lung, fluoxetine and norfluoxetine concentrations were significantly correlated with each other (Figure 5.3 and Appendix D, Table D3). A similar relationship was apparent in brain but did not achieve statistical significance (Figure 5.3 and Appendix D, Table D3). 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 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 (calculated as the inverse of the 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 recorded in starlings in the present study (7.81). 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 hours (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 note that

the difference in activity between parent and active metabolite is not observed in the respective S-enantiomers (Fuller et al., 1992).

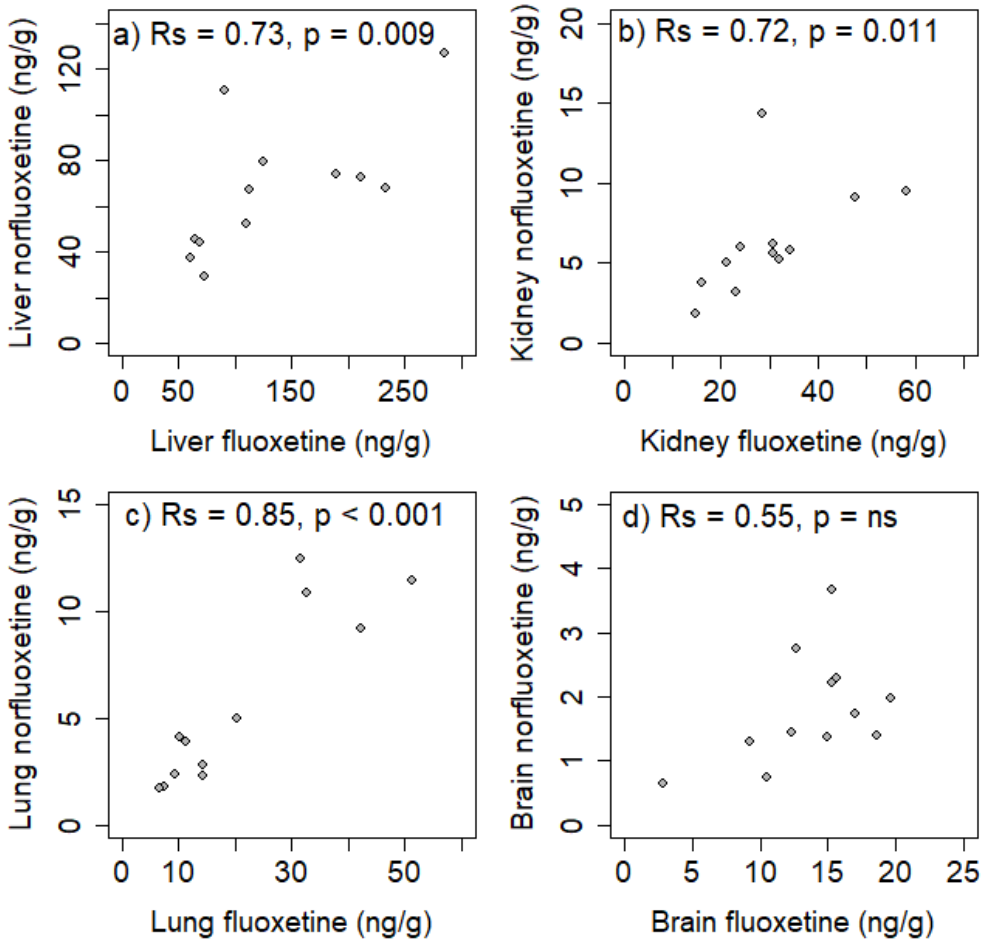

Figure 5.3. Scatter plots of fluoxetine and norfluoxetine concentrations (ng g^{-1}) within each tissue: a) liver, b) kidney, c) lung and d) brain. Data collected at the end of a 28 week dosing period. Spearman correlation coefficients are also displayed.

5.4.2 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 hours for brain, 90 h for kidney and 93.5h for liver (Figure 5.4). 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 residues 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 calculations suggest that fluoxetine persists in liver and kidney for more than 24 hours and therefore would have been bioaccumulated to some extent over the course of the dosing period. So, 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. As aforementioned, concentrations in kidney might allow some degree of rudimentary inference about previous concentrations in brain tissue.

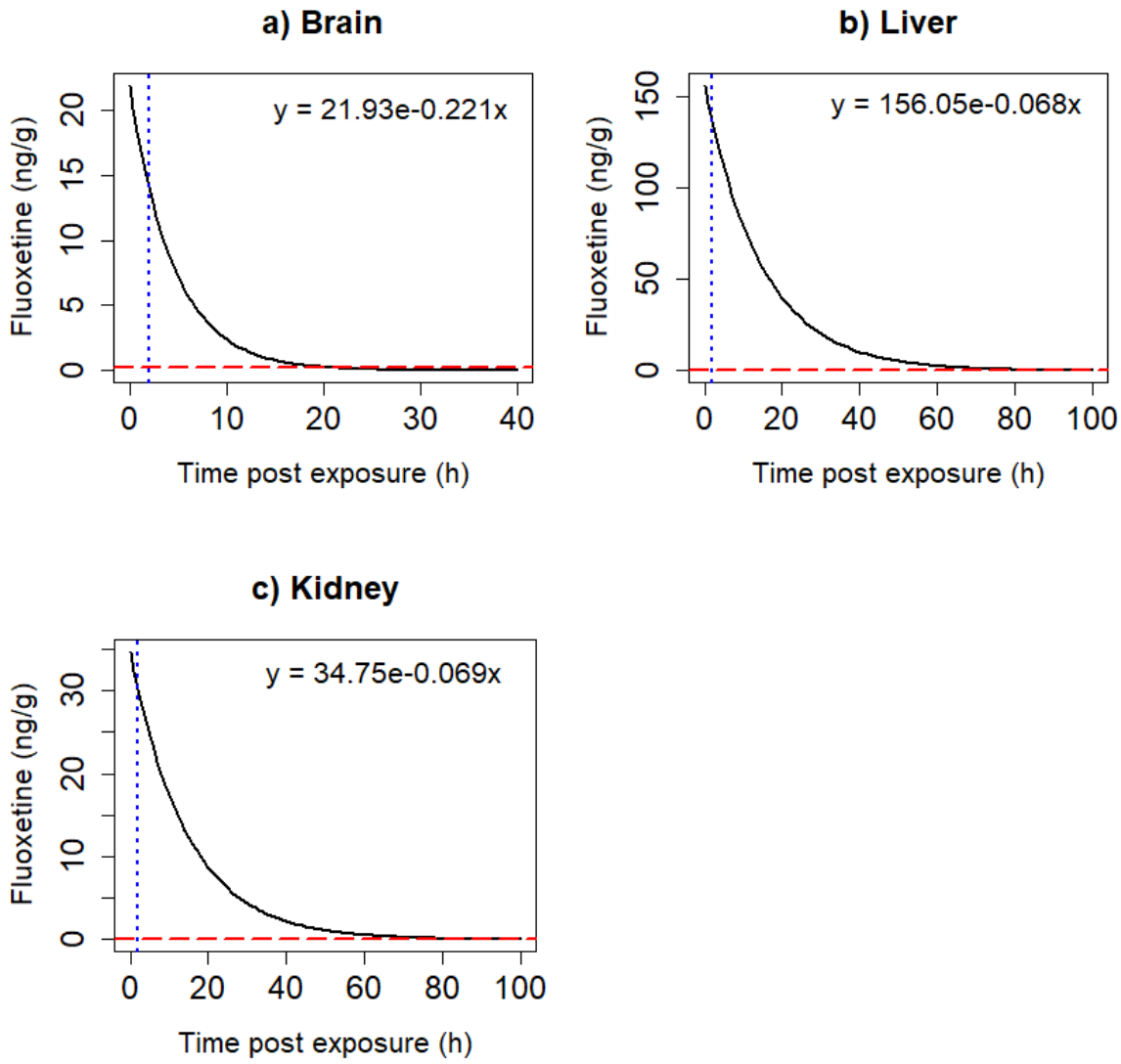

Figure 5.4. Decay curves that estimate the concentration of fluoxetine in various tissues (brain, liver, kidney) over time (hours) post exposure (*n* = 12). Data collected at the end of a 28 week dosing period. Curves generated using rate constants derived in another study, during an *in vivo* experiment (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.

5.4.3 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). Frequent detection of fluoxetine in the regrown feather samples 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 remove contaminant residues from feather surfaces (Cardiel et al., 2011, Garcia-Fernandez et al., 2013). 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 the wider study involved the collection of behavioural data, necessitating that the starlings be housed in mixed treatment groups.

While we were unable to determine whether residues in control 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 concentrations 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; Figure 5.5). The higher fluoxetine residues in regrown feathers in dosed birds may well be 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), although it is also possible that surface contamination was reinforced in dosed birds if the preen oil contained fluoxetine residues. There were no significant correlations between feather and organ fluoxetine concentrations (see Appendix D, Table D5). 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 compound from the plasma into feathers in fluoxetine-treated birds but it is unclear why norfluoxetine was not found in regrown feathers from all chronically dosed birds. Overall, our results suggest that feathers can be used to assess the occurrence of exposure to fluoxetine in wild birds, although further work is needed to elucidate the dynamics between exposure and deposition of residues into the feather.

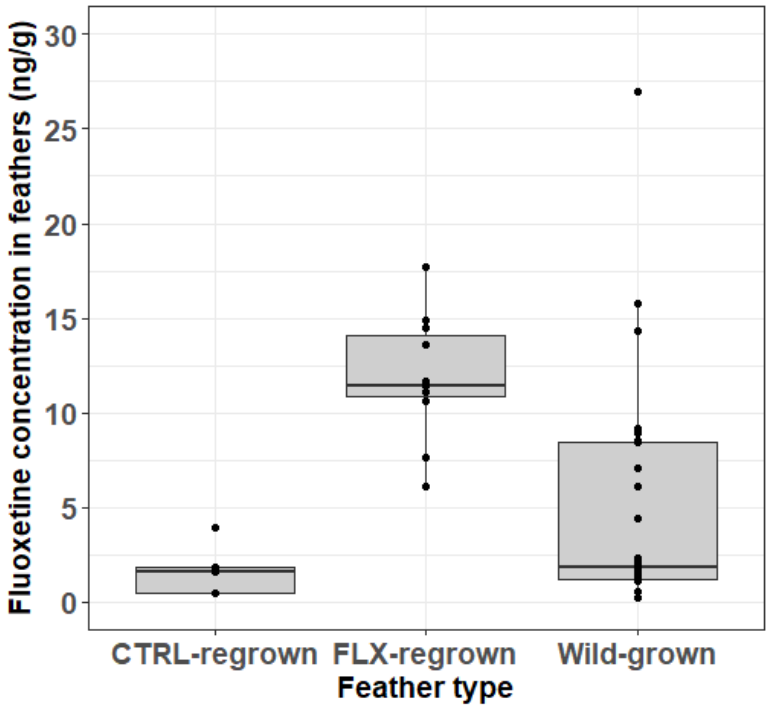

Figure 5.5. Concentration of fluoxetine in rectrice feathers (R4 and R9). Data shown correspond to regrown control (CTRL) group feathers (*n* = 5), regrown fluoxetine-treated (FLX) group feathers (*n* = 11) and wild-grown feathers (*n* = 25). Only samples with pooled R4 and R9 feathers are included. The boxes represent the upper and lower quartiles, whilst outliers are defined as 1.5*IQR (Tukey, 1977). The regrown feathers in the plot below grew during the dosing period (typically during weeks 2-7 of treatment), whereas the wild-grown feathers were fully grown before the onset of dosing and were harvested the week after dosing commenced.

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. However, it seems unlikely that external (surface) contamination could account for all the residues, especially given that two of the three highest concentrations were in birds from the 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.

5.5 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 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. Further, measured concentrations are likely to vary markedly with both magnitude of exposure and the period between last exposure event and death. Meanwhile, feather residues provide only an indication of exposure during the period when feathers are regrown [44] 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, as feathers grow over a period of weeks, analysis of sequential samples from the same feather may provide information on the variability of exposure over time within the feather growth period. Further work is needed to determine the extent to which feathers can be used to characterise the scale of exposure to fluoxetine, and indeed other pharmaceuticals, in wild birds, 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). The likelihood and significance of effects in free-living birds resulting from pharmaceutical exposures remains to be determined. Indeed, the wider challenge of implementing appropriate biomonitoring campaigns for detecting priority pharmaceuticals, that are often predicted to be transient within the tissues of free-living vertebrates, remains.

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Chapter 6: General Discussion

6.1 Summary of thesis aims and results

In this thesis, I investigated the consequences of exposure to environmental concentrations of antidepressants in wild birds by focussing on a priority contaminant, the antidepressant fluoxetine. My aims throughout this study were to determine whether chronic exposure to an environmentally relevant concentration of fluoxetine can alter avian behaviours and physiology. Given that fluoxetine affects the serotonergic system, which is known to influence multiple traits (Figure 6.1), I measured a number of endpoints that are associated with the known therapeutic and side effects in humans (Backström and Winberg, 2017). I also selected my endpoints to be ecologically relevant, to allow my results to be more readily extrapolated to free-living individuals. Finally, I assessed how best to monitor the presence of fluoxetine in wild populations.

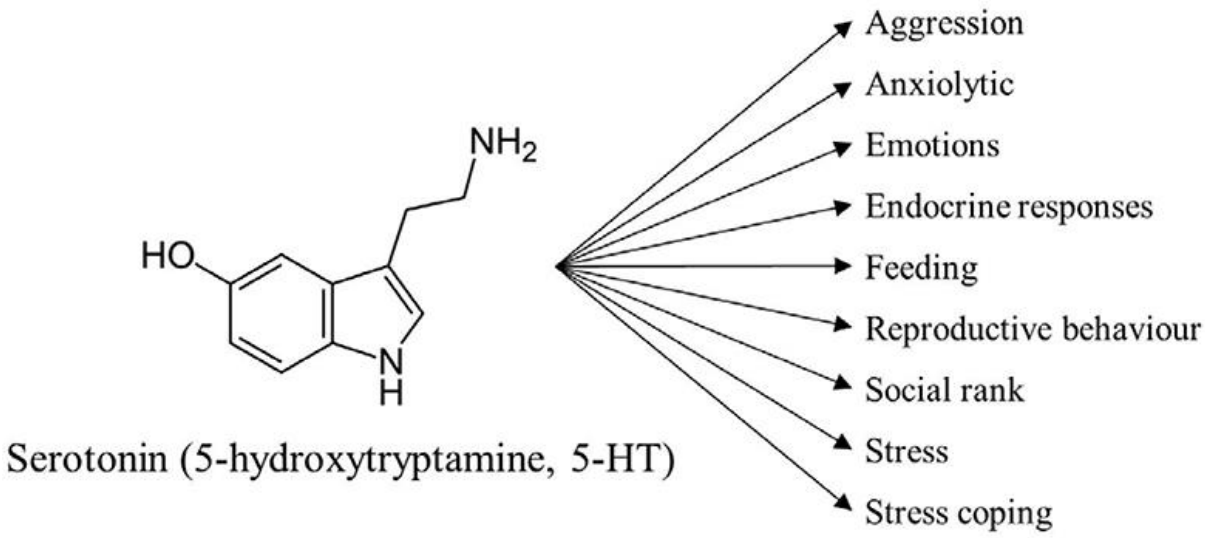

Figure 6.1. Shows selected processes that are under serotonergic control and are by consequence suitable endpoints for studying the effects of selective serotonin reuptake inhibitors such as fluoxetine. Reproduced from: https://doi.org/10.3389/fnins.2017.00595 (Backström and Winberg, 2017).

In Chapter 1, I highlighted the need for further research on the effects of psychotropic pharmaceuticals on terrestrial, as opposed to aquatic wildlife and in birds, compared to other vertebrates. I then proceeded to select a candidate psychotropic pharmaceutical for further empirical study. This led to the selection of fluoxetine, a selective serotonin reuptake inhibitor antidepressant, as the focal compound for my thesis. I then reviewed the effects of fluoxetine on vertebrates and identified endpoints for studying the impacts of environmental concentrations of fluoxetine on wild birds, based effects in other vertebrates.

In Chapter 2, I explored the effects of fluoxetine on avian courtship behaviour and reproductive physiology. These endpoints were selected to mirror sexual dysfunction side effects reported in mammals and also because they are important traits for individual fitness. During the breeding season, I paired each male starling with a control female during one trial and a fluoxetine-treated female during another identical trial, in order to assess male preference between the female treatments. I found that males sang less and were more aggressive towards fluoxetine-treated females than controls. However, this result could not be explained by any female trait measured (female courtship and agonistic behaviours, body condition, circulating sex hormone levels). My results demonstrated that environmental concentrations of antidepressants can alter behavioural traits that are crucial to individual fitness in birds, yet also highlighted the need to better understand the physiological and neural mechanisms that underlie such effects. My research also demonstrated the usefulness of song as a sensitive and non-invasive endpoint for assessing reproductive effects of pharmaceuticals in birds. I concluded that chronic exposure to environmental concentrations of antidepressants has the potential to disrupt songbird courtship and mate choice, with possible consequences for local population dynamics.

Chapter 3 aimed to test the effects of fluoxetine on stress physiology in starlings, given that fluoxetine is prescribed to treat anxiety disorders. I used another novel method in ecotoxicology, thermal imaging, as part of my investigation. In this chapter, I found that chronic exposure to an environmental concentration of fluoxetine altered peripheral skin temperature (temperature of exposed leg skin) but not as predicted. I originally hypothesised that fluoxetine-treated individuals would have higher peripheral skin temperature than control birds, since leg skin temperature has been shown to be inversely related to basal stress in another passerine species (Jerem et al., 2018). However, I found that fluoxetine-treated individuals had cooler leg skin on average than control birds. As none of my other measures of stress physiology or behaviour explained variation in leg skin temperature, I concluded that the effect was likely a treatment effect of fluoxetine on peripheral vasoconstriction, which was independent of stress. This effect may have been mediated by treatment effects on the serotonergic system, since serotonin is involved in thermoregulation (Lin et al., 1998). Moreover, the treatment effect was air temperature dependant: the leg skin temperature of control birds varied positively with air temperature as expected, whereas the leg skin temperature of fluoxetine-treated birds was unresponsive to changes in air temperature. This demonstrated how environmental concentrations of fluoxetine can interfere with the ability of birds to respond to their environment appropriately, as the regulation of leg skin temperature is essential for avian thermoregulation via heat dissipation (Ward et al., 1999). Building on the behavioural effects described in Chapter 2, Chapter 3 provided evidence of physiological effects

resulting from exposure to an environmentally relevant concentration. I concluded that such effects could have a negative influence on individual survival in fluoxetine-exposed individuals, especially since starlings and other species often visit sewage works to forage with particular frequency during periods of harsh weather (Fuller and Glue, 1980).

Chapter 4 related directly to Chapter 3, as it also aimed to assess the effect of fluoxetine on stress physiology and anxiety-related behaviour in starlings. However, in this chapter, I investigated how the effects exerted by fluoxetine varied with exposure duration and time of year. Firstly, I compared chronic treatment effects on certain endpoints after 6 weeks' exposure (during winter) to effects after 27 weeks' exposure (during summer). Further, I investigated short term effects during the first weeks of treatment on feather quality, a measure of stress physiology. My results highlighted the variability of effects exerted by exposure to fluoxetine with duration of exposure and across contexts. Firstly, I showed that exposure to fluoxetine can elicit physiological effects within the first few weeks of treatment, as fluoxetine-treated individuals regrew better quality feathers than controls (when comparing experimentally regrown feathers to feathers grown in the wild). Conversely, effects on anxiety-related behaviour (boldness) and on stress physiology (corticosterone) took longer to become manifest and were not observed until 27 weeks exposure. When I contextualised my findings with the time of year, I found that the boldness of fluoxetine-treated individuals was unchanging between summer and winter, whereas controls behaved more boldly during winter than summer, as would be expected owing to an increased motivation to feed during colder weather. No effect of treatment was observed on other measures of anxiety-related behaviour and physiology (activity, exploration, neophobia, body mass regulation). I concluded that exposure to environmental concentrations of fluoxetine could begin to alter certain stress-related traits within weeks but that clear effects on stress-related behaviour and corticosterone were both context specific and seemed to require a longer exposure duration to become apparent. However, further work is required to conclusively determine the contributions of exposure length and time of year respectively to effects on boldness and corticosterone levels.

Whilst Chapters 2-4 were concerned with the effects of fluoxetine on avian behaviour and physiology *in vivo*, Chapter 5 aimed to discover how best the presence of fluoxetine and its active metabolite norfluoxetine could be monitored in wild bird populations. My results highlighted the need to harvest the appropriate type of sample in order to maximise the chance of detecting fluoxetine. I found that environmental concentrations of fluoxetine were highly transient in avian plasma following exposure. Applying existing decay curves (Bean et al., 2017), I found that concentrations in tissues were unlikely to remain above analytical detection limits in tissues for

more than several days. Many existing biomonitoring schemes use tissues from birds that have been found dead to survey exposure to contaminants. My results indicate that monitoring fluoxetine within these is likely to be challenging and that carcasses collected as soon as possible after exposure are preferred. Of the tissues I analysed, kidney and liver were the most suitable for carcass-based biomonitoring. I also discovered that feathers have potential for monitoring exposure to fluoxetine in wild birds, as concentrations in feathers regrown during the exposure period were significantly higher in fluoxetine-treated than control birds. However, interpretation of the data was challenging as I could not conclusively differentiate between fluoxetine inside the feather and surface contamination. I concluded that feathers showed great promise for biomonitoring fluoxetine and other rapidly eliminated pharmaceuticals, as collection of feather samples is non-destructive, and contaminants can be sequestered within the feather matrix, where they should be stable over long time periods (Garcia-Fernandez et al., 2013).

6.2 Limitations and future research

My research has shown that chronic exposure to an environmental concentration of an antidepressant, fluoxetine, can alter both behavioural and physiological traits that are important for individual fitness in a species of passerine bird. Further, my work has explored how the prevalence of fluoxetine exposure in wild bird populations might be better assessed, by providing recommendations for biomonitoring. However, my study was subject to certain limitations and these have pointed me towards, in my opinion, the key topics in this area that require future work.

6.2.1 Sample size constraints

My conclusions were formed on the basis of an aviary study conducted with a small sample of wild birds in captivity, exposed to a maximally environmentally relevant concentration over a period of 28 weeks. Due to my low sample size (up to n = 28 birds depending on the endpoint), it is possible that I did not have sufficient statistical power to detect certain effects on behaviour and physiology. For example, the effects of treatment reported in Chapter 4 on speed of exploration (p = 0.085), and to some extent activity levels (p = 0.108), approached statistical significance. Measures of individual behaviour tend to be highly variable, making the detection of effects with low sample sizes challenging (Martin et al., 1993). Further, when studying the effects of fluoxetine and other antidepressants, statistical power issues related to low sample size can be exacerbated by individual resistance to drug effects. For example, in humans, some patients do not respond to treatment with fluoxetine and are termed “non-responders” (Nierenberg et al., 2000). Variability in responses to antidepressant treatment can be caused by individual differences in factors such as metabolism and endogenous hormone levels, or can be due to polymorphisms of the serotonin transporter

(SERT) gene (El-Hage et al., 2013). The presence of such non-responders in our sample of starlings could have reduced our effective statistical power. However, our sample was itself too small to investigate whether it contained such non-responders and indeed it is not known whether individual differences in avian responses to antidepressants are analogous to those reported in humans. Therefore, future work could investigate whether there is some percentage of a chosen model bird species that does not respond to the effects elicited by environmental concentrations of antidepressants. Such data would improve our understanding of exposure risks to wild individual and populations.

6.2.2 From laboratory to field

Perhaps the most glaring limitation of this and many other *in vivo* studies in the field of ecotoxicology is that the experimental work was carried out in a laboratory setting. Although many efforts were made to enhance the ecological relevance of my work, for example using wild-caught individuals and housing them in outdoor aviaries, it is unlikely that the birds, being wild-caught, would have fully habituated to the stress of being handled and dosed each weekday (Morgan and Tromborg, 2007), possibly attenuating certain experimental results (Barnett et al., 1994). Yet using wild-caught birds should have still delivered higher ecological relevance compared to using captive bred birds, as there can be substantial differences in certain traits between the two (Feenders et al., 2011). Another clear limitation of my work was the low sample size, yet ethical considerations and the associated drive towards reducing the use of animals in experiments demand that minimal numbers of birds be used in such captive studies (Burden et al., 2015). I therefore recommend that future work should study the effects of consuming antidepressant-contaminated invertebrates in wild birds living in their natural habitat, with minimal human contact. This would create real-world complexity and additionally enable mixtures of antidepressants and other pharmaceuticals with serotonin reuptake inhibiting activity to be assessed. This approach has been used in a recent study, where Goldfish were caged downstream from wastewater treatment plant (WWTP) discharge (Simmons et al., 2017). However, this would be extremely demanding in reality for several reasons. Firstly, wild birds are highly mobile. Whereas fish can be caged within the flow of a contaminated river (Simmons et al., 2017) or be contained within an effluent-contaminated lake (Klaminder et al., 2016), birds that forage at WWTPs can travel kilometres between different feeding grounds each day (Bruun and Smith, 2003) or might even feed at a WWTP whilst on passage, before moving on (Fuller and Glue, 1980). Secondly, it would be necessary to monitor focal contaminant levels in invertebrates living on the WWTP throughout the course of the experiment, necessitating a high level of collaboration between the research team and WWTP operators. Yet such an experiment could be feasible using a nest box study, if the focal birds occupied nest boxes suitably close to a

WWTP and were observed to forage there daily whilst they were incubating and/or provisioning chicks. However, note that nest box studies would be limited to studying the effects of exposure in breeding birds and nestlings, and only during the period that the nest boxes were occupied (typically several weeks). Tagging the parent birds with a radio or GPS transmitter could allow behavioural data to be collected, similarly to a recent study with fish (Klaminder et al., 2016). Further, investigating the effects of exposure to antidepressants on measures such as breeding success or survival could allow predictions of population-level effects to be made. Despite the challenges, investigating the effects of fluoxetine and other antidepressants on free-living birds exposed at WWTPs is key to furthering our understanding of the effects these contaminants can exert at the individual and potentially population-level in the wild.

6.2.3 Towards a better understanding of effects

The work presented in this thesis has identified certain physiological and behavioural effects of exposure to an environmental concentration of fluoxetine in birds, that typically reflect therapeutic and side effects of the drug in humans. Yet many questions regarding the nature of these effects remain unanswered. Firstly, I have focused primarily on the effects of chronic exposure in my thesis. This was partly because many of fluoxetine's effects are known to develop only after several weeks' exposure in humans (Blier and de Montigny, 1994), and partly because I wanted to conduct certain components of the experiment at specific times of year. For example, I started dosing the birds in early winter, as this is a key time during which starlings forage at WWTPs, yet I also wanted to investigate the effects of treatment on courtship, necessitating data collection during the natural breeding season, by which time the birds were chronically exposed. It is now important to ascertain whether the effects I have observed after chronic treatment (in Chapters 2 and 3 in particular) can occur after shorter exposure durations. There is evidence in rodents that certain effects can occur following subchronic or even acute exposure at clinical dosages (Sarkar et al., 2008). However, acute effects can differ from those that develop after chronic treatment (To et al., 1999). Nevertheless, future work should assess whether the behaviour of physiology of wild birds can be affected by foraging on antidepressant-contaminated prey over a period of days or 1-2 weeks, or even by just one visit. If effects on courtship or thermoregulation are elicited following these far shorter exposure periods, the risk to wild birds would be greatly enhanced.

In addition to the question of "when" the treatment effects detailed in this thesis occur following exposure, another key consideration is "how" they occur. An understanding of the mechanistic detail of the treatment effects I have observed is currently lacking. For example, I attempted to uncover whether fluoxetine-induced endocrine disruption underpinned the effects on courtship

behaviour observed in Chapter 2, yet found that they could not be explained by differences in circulating sex hormones. In order to better understand how such effects are mediated by physiological and neural mechanisms, the most obvious focus of future work would be to explore how exposure to environmental concentrations of fluoxetine alters the neurotransmission of serotonin in the avian brain. For example, future work could assess whether exposure to environmentally relevant concentrations of fluoxetine downregulates the serotonin transporter (SERT) (Benmansour et al., 2002) or blocks serotonin receptors (Ni and Miledi, 1997, Peng et al., 2014) in key areas of the avian brain (raphe nuclei, hypothalamus), leading to increased extracellular serotonin, which can in turn elicit behavioural and physiological effects. Other potential mechanisms that could underlie treatment effects, for example neurogenesis (Sachs and Caron, 2015), also warrant further investigation. In relation to this, when investigating treatment effects on hormones such as corticosterone or oestradiol, assessing the effects of fluoxetine on receptors in the avian brain might give a clearer picture of the endocrine effects that fluoxetine exerts (Leon-Olea et al., 2014). However, these mechanistic studies would require animals to be sacrificed in order to harvest brain tissue, therefore studying multiple mechanisms simultaneously would maximise the scientific insight generated by one experiment.

6.2.4 How prevalent is exposure in wild populations?

Whilst this thesis provided evidence that exposure to environmental concentrations of antidepressants can modulate avian physiology and behaviour, key information regarding the prevalence of exposure in wild populations is currently lacking. Such information is important for predicting population level effects (Kramer et al., 2010). I showed in Chapter 5 that feathers have promise as a way to monitor the presence/absence of exposure to fluoxetine in wild birds. Following on from this, I recommend that future work should further characterise deposition rates into feathers and develop analytical protocols that can differentiate between concentrations on the feather surface from those deposited within the feather matrix (Garcia-Fernandez et al., 2013). Further, if the relationship between exposure concentration and deposition into feathers over time could be thoroughly characterised, this might allow the quantity of antidepressant to which the bird was exposed whilst the feather grew to be estimated. This in turn could allow crude predictions about the likelihood of known adverse effects to be inferred. The benefit of feathers is that they can be harvested easily, either by plucking or by collection of fallen feathers from nest sites. However, the key needs, whether they are met by the analysis of feathers, tissue samples or other types of sample from wild birds, are to firstly ascertain how widespread exposure to antidepressants is and secondly to assess the magnitude of internal concentrations of antidepressants in wild birds that forage at WWTPs. This could be achieved by collecting and

subsequently analysing avian samples for concentrations of fluoxetine and other antidepressants, from individuals that forage at WWTPs. This would bridge the gap between existing concentrations reported in invertebrates collected from WWTPs (Bean et al., 2017) and the effects that I have observed experimentally, in birds consuming an environmentally realistic concentration of an antidepressant. Further, if invertebrate and avian samples were collected simultaneously during the same survey, the trophic transfer of antidepressants from invertebrate prey to avian predator could be determined. More broadly, concentrations of antidepressants in birds exposed by other routes should be surveyed. For example, measuring concentrations of antidepressants in farmland birds that forage in fields where sewage-derived biosolids are applied to crops, in order to establish whether such contaminants could be contributing to observed population declines (Donald et al., 2001).

6.3 Conclusions

In this thesis, I have demonstrated experimentally that an environmentally relevant concentration of an antidepressant can alter a plethora of behavioural and physiological traits, selected based on the therapeutic and side effects of fluoxetine in other vertebrates, that are crucial to avian individual fitness. Further, I have determined which types of sample are most suitable for monitoring fluoxetine in wild individuals, so that my observed effects can be extrapolated more effectively to free-living individuals.

In order to translate my findings into a format that can be more widely used for ecotoxicological risk assessment, for example by regulatory bodies, I can situate them within the framework of an adverse outcome pathway (AOP) (Ankley et al., 2009). My findings pertain primarily to the "organism response" category of the AOP framework (see Figure 6.2). In future, as previously discussed, further work is required to establish both a mechanistic understanding of the toxicity pathway and responses at the population level in birds.

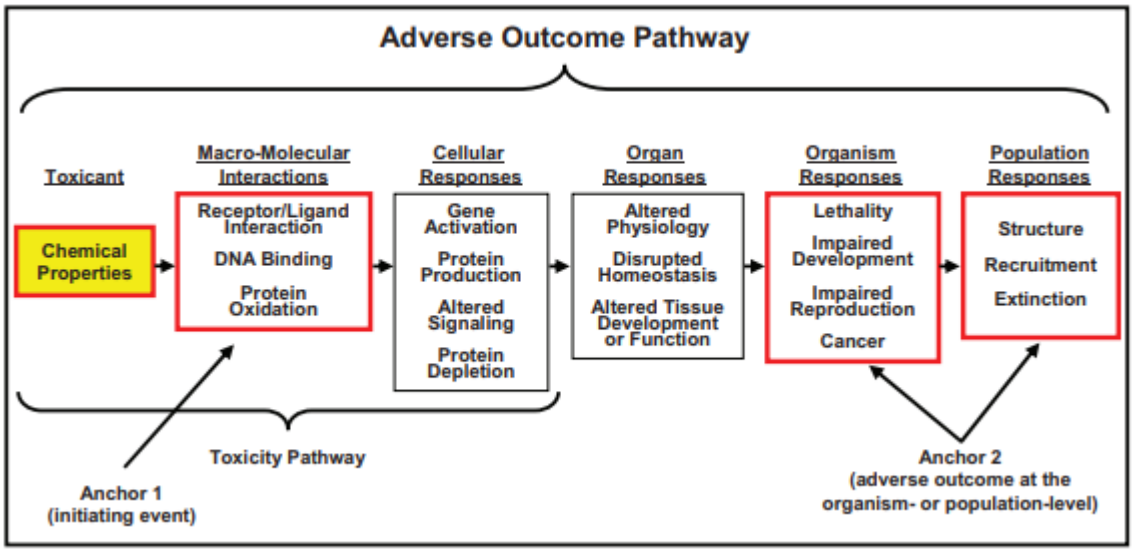

Figure 6.2. Adverse outcome pathway (AOP); a framework designed for ecological risk assessment. The findings from my thesis correspond primarily to the individual level responses category within the AOP. Reproduced from: https://doi.org/10.1002/etc.34 (Ankley et al., 2009)

Whilst the effects I have found may appear to be relatively benign, given that they are sublethal and reported at the individual level, they might have more serious consequences if they can impact on population dynamics. For example, my findings in Chapter 2 showed disruption to courtship behaviour, with predicted disruption of signalling of female quality. If males erroneously select lower quality females over higher quality females, this could lead to lower reproductive success, which could have implications for local population dynamics (Brodin et al., 2014). Further, even if exposure to an antidepressant alters behavioural traits in only one species within a food web, other species within that ecosystem may be affected due to indirect effects; for example, via altered predator-prey interactions (Saaristo et al., 2018). In this way, the population dynamics of multiple species in one ecosystem could be altered by exposure to environmental concentrations of antidepressants, even if the contaminants only elicit effects in certain species. Understanding such adverse outcomes at the population level in future could feed into adverse outcome pathways (AOPs), improving ecological risk assessments. However, understanding the effects of exposure to contaminants at the organism level, and at lower levels of the AOP, remains crucial, as effects at the population level are likely to be highly complex and thus challenging to predict.

Although I calculated my dose of fluoxetine to be maximal, based on concentrations in invertebrates from UK WWTPs (Bean et al., 2017), this exposure concentration is unlikely to be the upper limit that is possible in the UK or globally. Firstly, I have based my exposure scenario on birds foraging at WWTPs in the UK. However, concentrations of pharmaceuticals in the environment can be higher in other countries, particularly in developing nations with less rigorous wastewater

treatment processes (Larsson, 2014), increasing the risk of exposure to wild birds. Further, as living standards increase in developing countries, access to healthcare is likely to improve, resulting in increased prescriptions of pharmaceuticals (Arnold et al., 2014, Kookana et al., 2014), with concurrent increases in discharges to the environment predicted. Meanwhile in developed nations, where ageing populations are increasing the demand for pharmaceuticals, the quantity of antidepressants in the environment is predicted to rise over time (Kookana et al., 2014), unless removal efficiency from sewage is improved. Additionally, birds foraging at WWTPs are exposed to mixtures of serotonin reuptake inhibitors with potentially additive effects (Melvin et al., 2014, Simmons et al., 2017, Backhaus, 2014). All these factors mean that in certain areas of the world and in the future, wild birds could be exposed to far higher concentrations of antidepressants than I have studied, with the potential for stronger effects on behaviour and physiology to be elicited.

Another scenario in which effects of a greater magnitude could be observed is if some species of bird are more sensitive to fluoxetine than the Eurasian starling. This would be unsurprising, given that effect size and sensitivity appear to vary to such an extent in different species of fish (Sumpter et al., 2014). Indeed, a concentration of the anti-inflammatory pharmaceutical diclofenac that is lethal to *Gyps* vultures is non-toxic to other species of scavenging bird (Oaks et al., 2004, Naidoo et al., 2011). Differences in uptake may also be apparent between bird species, resulting in a higher percentage of the exposure concentration reaching the brain (site of drug action) in some cases. Predictions of exposure effects in different species of bird can be improved by taking into account the evolutionary conservation of drug targets (Brown et al., 2014) and differences in metabolism (Hutchinson et al., 2014) between species. Nevertheless, whilst this thesis has reported many new effects in a taxon that is generally underrepresented in ecotoxicological research, extrapolating these effects to other species of bird remains challenging.

Many of the effects I have observed are likely to act in synergy with other anthropogenic stressors. Firstly, human activity is driving climate change and whilst the climate is generally predicted to warm, extreme weather is also predicted to become more commonplace (Fischer and Knutti, 2015). Some of the effects I have reported in Chapters 3 and 4, for example atypical boldness during summer and impaired thermoregulation during early spring, could be exacerbated by increasingly extreme weather, becoming increasingly detrimental to individual survival as a result. During such periods, when food resources may be scarce and an individual is likely to be coping with physiological stress due to extreme temperatures, the consequences of exposure to fluoxetine are predicted to be greater. Further, as bird populations continue to shrink due to loss of suitable habitat, a growing global human population and increasing industrialisation of agricultural practices

(Reidsma et al., 2006, Butler et al., 2010), these smaller populations could become more vulnerable to additional stressors such as exposure to behaviour-modulating contaminants, particularly if these contaminants cause population-level effects (Brodin et al., 2014). Overall, fluoxetine and other antidepressants that exert sublethal effects at environmental concentrations add to the ever-increasing burden of anthropogenic stressors to which wild birds are exposed.

My work has clearly shown that a broad range of effects on individual birds can be elicited by an environmentally relevant concentration of an antidepressant and has identified key directions for future research. The work presented in this thesis is amongst the first studies that have documented the behavioural and physiological effects of exposure to a pharmaceutical within the terrestrial environment and in a species of bird. Moreover, I have successfully employed endpoints that are highly ecologically relevant to many species of bird (song, thermal imaging, feather quality). These endpoints could be easily employed in future *in vivo* assessments of sublethal effects of pharmaceuticals. Finally, my assessment of fluoxetine concentrations in feathers provides novel insight into the value of such samples for biomonitoring, helping to solve the detection-related problem of the transience of fluoxetine and many other pharmaceuticals in avian tissues. It is crucial that future work now determines the extent to which birds are exposed to antidepressants in the wild, as realistic mixtures, and how these effects impact on individuals and populations in the complexity of the real-world environment. This will allow us to better understand the contribution of antidepressants and other pharmaceuticals in the environment to bird population declines in the Anthropocene era.

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Appendix A

A1. Starling husbandry

Capture*:* In October 2015, we captured 24 wild Eurasian starlings (*Sturnus vulgaris*) from a roost site in North Yorkshire, UK and moved them to our experimental facility. All birds were captured by mist net over three nights within the same week in October, under licence from Natural England. After removal from the net, the birds were transported to Home Office Licensed outdoor aviaries.

Arrival and washout*:* On arrival, the starlings were weighed (to the nearest 0.1 g), given a visual health check, treated with pesticide spray (Frontline; Merial Animal Health, Woking, UK) and a pale blue, unique numbered leg ring (AC Hughes, Hampton Hill, UK) was applied to the right leg. Finally, five breast feathers were plucked for DNA sexing (Avian Biotech, Truro, UK), confirming that there were n = 16 females and n = 8 males. We initially housed birds in single sex groups and according to the day on which they were caught. We did not age the birds as the Eurasian starling undergoes a complete post-juvenile moult over summer, thus all first year birds had a full set of adult feathers at the time of capture. The birds were allowed to acclimate to captivity for four weeks which also ensured a "wash-out" period, to allow excretion of latent, non-persistent contaminants. For the first fortnight of the wash out period, the starlings were given a veterinary probiotic supplement (Avipro; Vetark, Winchester, UK) delivered in their drinking water, to help prevent infection.

Allocation to home aviaries*:* Two weeks before the onset of data collection, we allocated the birds to permanent outdoor home aviaries. The allocation was stratified by sex (single sex home aviaries) but otherwise random. This allowed two weeks for the birds to settle in their new groups before data collection started. At this time, we applied a combination of two leg rings to the left leg, in two of the following colours: white, black, red, yellow, green, blue (AC Hughes, Hampton Hill, UK). This combination of two coloured leg rings was unique within each aviary but not amongst all individuals, to allow for easy identification of individuals within each home aviary. The total weight of the three leg rings on each individual was < 1 % bodyweight.

Home aviaries*:* We housed the birds in single sex groups (3-7 individuals per pen) in five (two male, three female) outdoor aviaries (6.9 m L x 2.5 m W x 2 m H), which were covered by a roof at one end for shelter but otherwise open to the environment. The wall of the covered end contained a removable one-way glass window into a central corridor. This window was used to release birds back to their home aviary after dosing them in the central corridor. The birds were given *ad libitum* access to drinking water and food. In the outdoor home aviaries, food was provided via two ground feeder trays and one wall feeder, with a diet composed of an insectivorous mix (Uni patee; Orlux,

Deinze, Belgium) and a chick starter crumb. In addition to the feeders, each aviary contained one water bath (provided post-acclimation), one water hopper, six fixed perches, two swinging perches and four potted conifer trees (*Cupressus x leylandii*).

A2. Experimental Treatment

The onset of dosing was staggered over five consecutive weeks across the five aviaries, with each aviary starting treatment in a different week. We dosed the birds each weekday but not on weekends; therefore, the birds received a five day corrected dose of 3.8 µg d^{-1}. Each day, a stock solution of Prozep (fluoxetine solution 20 mg per 5 ml; Chemidex Pharma, Egham, UK) in distilled water was prepared, such that a 2.5 µl aliquot contained 3.8 µg of fluoxetine. The mean body mass of the 12 fluoxetine-treated starlings was 82.93 g and they were administered 3.8 µg d^{-1} over five days, equating to a 7-day mean daily dose (corresponding to 2.7 µg d^{-1}) of 0.033 mg kg^{-1} bodyweight.

In order to deliver the fluoxetine-spiked or control spiked waxworms, all birds were caught each weekday at approximately 14:30. The birds were caught one aviary at a time with padded hand nets and kept in bags in the adjacent viewing corridor. The birds were removed one at a time from the bags, in the order they had been caught (first caught => first to be dosed). The spiked waxworm of the appropriate treatment (fluoxetine or control) was then fed to each bird. Once the worm was consumed, the bird was immediately released back to the home aviary, through a window from the viewing corridor. This was repeated until dosing was completed for all individuals in the aviary, before moving on to the next aviary. They were then immediately released back to their aviary. The process of catching all individuals within one aviary typically took <5 minutes and the birds typically spent < 10 minutes in bird bags. Handling and dosing each individual took approximately 20 s.

A3. Courtship Experiment

Courtship arenas: Two arenas, mirror images of each other, were used during courtship trials. These arenas were contained in a separate block from the home aviaries, providing a degree of auditory occlusion and enabling us to test two pairs simultaneously (one pair per arena). Both straw and green nesting material were provided on the floor and the birds had *ad libitum* access to food and water. Two food trays were provided to ensure both birds could feed. Two potted conifer trees (*Cupressus x leylandii*) in the aviary provided shelter and enrichment. One third of the aviary was covered by a roof, with the remaining two thirds open to environmental conditions. Each aviary measured 6.9 m L x 2.5 m W x 2 m H. See Figure A1 for diagram and photograph of a test arena.

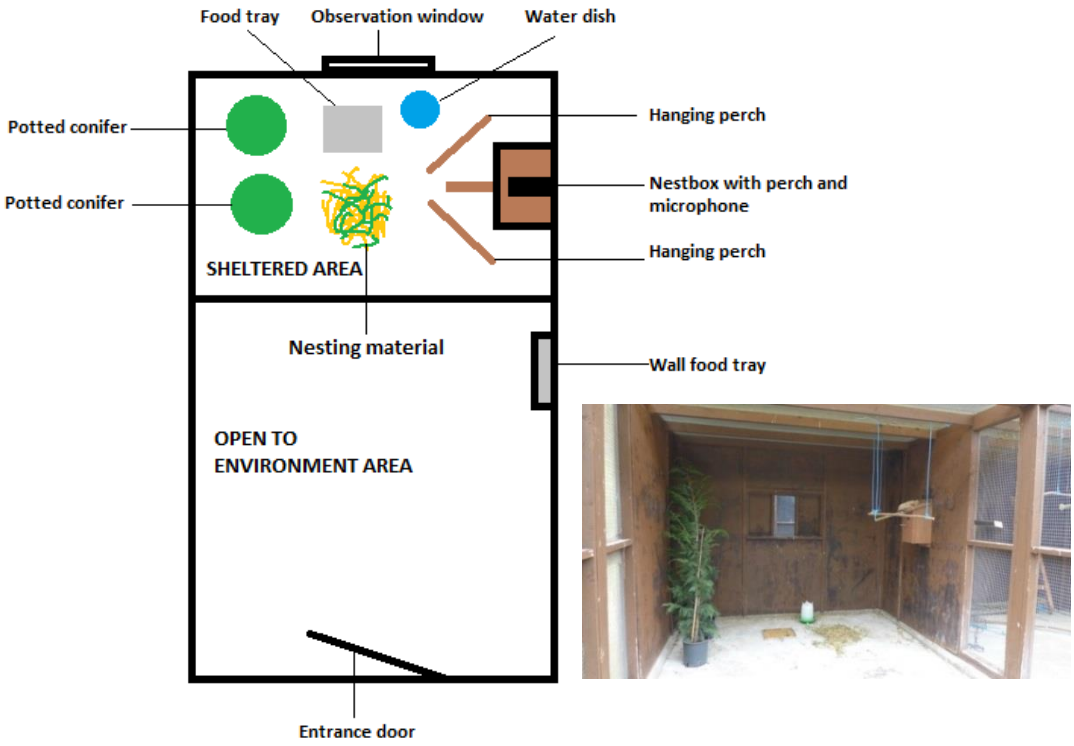

Figure A1: Plan view of courtship arena (left, not to scale); photograph of courtship arena (right)

Trial timings*:* Capture of the two pairs was staggered by 15 minutes, with the second pair introduced to their respective courtship aviary 15 minutes after the first pair. The order of introduction to the arena was randomised for the two pairs in the first week of testing and then switched (for each male) during the second week of testing. Each pair was observed for a total of 30 minutes, in two 15 minute blocks with a 15 minute break in between. Observations were conducted in the same order as the introduction of the two pairs to the courtship arenas, resulting in the 15 minute blocks being staggered between the two arenas.

Blood Sampling*:* A 500 µl blood sample was collected from each focal individual by jugular venepuncture, using a 1 mL syringe (BD, Wokingham, UK) and 23 gauge needle (AN-2316R; Terumo UK, Bagshot, UK). After collection, the blood was immediately transferred to a 3.5 ml lithium heparin container (BD, Wokingham, UK) and stored between frozen cool packs in a cool bag whilst remaining individuals were sampled and the focal birds were released to their home aviaries. Note that the males were only blood sampled after the first trial they participated in (out of two) but not after the second, due to ethical concerns. The blood samples were centrifuged at 16 000 *g* for 10 minutes. Centrifugation started within 30 minutes following blood collection. The plasma layer was pipetted into a 1.5 ml polypropylene Eppendorf tube (Eppendorf UK, Stevenage, UK). The samples were stored at -20 ˚C prior to analysis.

A4. Statistical Methods

For all count data (male aggression, female aggression, female courtship behaviour, number of male song bouts) a Poisson GLMM was constructed first but was replaced with a negative binomial if overdispersion was present. The variance should approximate the mean under the Poisson distribution whereas the negative binomial distribution allows the variance to be greater than the mean, meaning it is suitable for overdispersed count data. We used AIC_c instead of AIC for model selection, as it may be considered more appropriate for small sample sizes (Burnham and Anderson, 2004).

Since the minimum adequate models for female aggressive behaviour and total male time singing were overparameterised (>1 fixed effect per 10 observations), we removed the least significant predictor in each case. If models are overparametised, statistical inference may be less reliable. From the total male time singing model, we removed male body condition index ($p = 0.028$) and from the female aggressive behaviour model we also removed male body condition index ($p < 0.001$; less highly significant than the female treatment*trial phase interaction term included in the minimum adequate model).

Under the Poisson error distribution, male body condition was found to be significant in the model with response "count of female courtship behaviours", resulting in a reduced model containing predictors female oestradiol and male body condition. However, this model was potentially unreliable, as it was overdispersed. We could not include male body condition in an equivalent model with negative binomial error distribution, as the model would not converge. Therefore, we selected a negative binomial model with only female oestradiol as the minimum adequate model. AIC_C supported this decision and was almost 9 lower in the negative binomial model with only female oestradiol compared to the Poisson model with male condition index also included (a model is preferred if the AIC_c value is two or more lower than a comparable model AIC_c).

The R package DHARMa (Hartig, 2017) was used to carry out model diagnostic tests for GLMMs and LMMs. DHARMa simulates scaled residuals for mixed models, which can be easily interpreted. n = 10 000 simulations were used to produce the simulated scaled residuals, which were used in subsequent plots and tests within the DHARMa package. Residuals QQ and residuals versus predicted plots were produced for each GLMM/LMM produced in DHARMa. Note that the diagnostics for the number of male song bouts GLMM displayed a mild negative skew. For each model, the DHARMa simulated residuals were also plotted against each fixed effect in the model. We also used DHARMa to check for the presence of zero inflation in the data, whilst dispersion was tested using the overdisp_fun code available at (Bolker).

To investigate the effects of female aggressive behaviours, female courtship behaviours, female circulating testosterone, female circulating oestradiol and female body condition index on male behaviours, we used negative binomial GLMMs (number of male song bouts, count of male aggressive behaviours, count of male courtship behaviours) and an LMM (total male time singing). These models were reduced and minimum adequate models selected as described previously. The full model outputs are shown in Table A1 (GLMMs) and Table A2 (LMM).

Predictor	Coef. β	SE (β)	*z*	*p*
a) Number of male song bouts				
Intercept	2.76	0.36	7.66	<0.001
Female courtship behaviours	0.03	0.06	0.52	0.601
b) Count of male aggressive behaviours				
Intercept	0.37	0.47	0.79	0.432
Female aggressive behaviours	0.17	0.09	1.90	0.057
c) **Count of male courtship behaviours**				
Intercept	1.25	0.29	4.37	<0.001
Female courtship behaviours	0.14	0.05	2.98	0.003

Table A1: Models testing the effects of female behaviours and physiology on male behaviours. Summary of GLMM minimum adequate model outputs for the following response variables: a) number of male song bouts; b) count of male aggressive behaviours; c) count of male courtship behaviours. *n* = 32 observations in all models. Table shows: coefficient estimates (β), standard errors (SE(β)), Wald's *z*-score (= β /SE(β)) and significance level *p*. All GLMMs were of the negative binomial family.

Predictor	Coef. β	SE (β)	*t*	χ^2	*p*
Total male time singing					
Intercept	16.97	4.79	3.55	-	-
Fem. circulating testosterone	-1.03	1.98	-0.52	0.24	0.626

Table A2: Summary of LMM minimum adequate model output for the response variable total male time singing (square root transformed, *n* = 32 observations). Table shows: coefficient estimate (β), standard error (SE(β)), t-statistic, Chi-Square statistic (χ^2) and significance level *p*.

A5. Testosterone Radioimmunoassay

Method: Our method followed a protocol applied previously to the quantification of cortisol concentration (Pickering and Pottinger, 1983), which was also been applied previously to the quantification of testosterone (Pottinger and Pickering, 1985). The plasma samples were extracted using ethyl acetate (1:5, v/v). 180 µl of sample extract was placed in a polypropylene tube (55.535; Sarstedt, Nümbrecht, Germany) and evaporated to dryness in a vacuum oven at 40 ˚C. 200 µl of antibody solution (1:20000 dilution in assay buffer, optimised based on manufacturer's instructions; mouse monoclonal [14P1F9], AbCam, Cambridge, UK) and 25-50 µl tracer solution ([1,2,6,7-3H(N)]-Testosterone, Perkin Elmer, Seer Green, UK; diluted to give a count in the range 12 000 – 25 000 dpm) were added to all samples and left to incubate at 4 ˚C overnight. The samples were then placed on ice, 100 µl of activated charcoal solution was added to each and they were

then vortex mixed. The resulting mixture was left to incubate for 5 minutes, before being centrifuged for 5 minutes (3506 g, 4 ˚C). 150 µl of the resulting supernatant was transferred to a polyethylene snap-twist scintillation vial (720-0494; VWR, Lutterworth, UK) and 4 ml scintillation fluid was added (Ecoscint A; National Diagnostics, Atlanta, USA). The samples were inversion mixed and counted on the scintillation analyser (Tri-Carb 2910TR; Perkin Elmer, Seer Green, UK) for 10 minutes under standard tritium count conditions. For each sample run, two calibration curves of testosterone standard (≥ 98 % purity; Sigma Aldrich, Gillingham, UK) were performed. The standards were processed in the same way as the samples. The samples were analysed blind, with the order of analysis during runs randomly assigned. Two samples had concentrations below the detection limit and were assigned a value of half the detection limit.

Reagents*:* Assay buffer was prepared by dissolving 200 mg bovine serum albumin (A-7888, RIA grade; Sigma Aldrich, Gillingham, UK), one phosphate buffered saline tablet (P-4417; Sigma Aldrich, Gillingham, UK) and 0.04 g sodium azide in 200 ml distilled water. Activated charcoal solution was prepared by dissolving 200 mg dextran (D-4751, clinical grade; Sigma Aldrich, Gillingham, UK) , one phosphate buffered saline tablet and 0.04 g sodium azide in 200 ml distilled water, before adding 1.0 g charcoal (C-4386, activated charcoal, neutralised; Sigma Aldrich, Gillingham, UK). Assay buffer and charcoal solution were stored at -4 ˚C for up to two weeks. The testosterone tracer solution was prepared by making up radiolabelled testosterone tracer ([1,2,6,7-3H(N)]-Testosterone; Perkin Elmer, Seer Green, UK) to 5.0 ml with toluene and ethanol (9:1) on arrival and was stored at -20 ˚C. To prepare a working solution for the assay, a 25 µl aliquot of the tracer stock solution was dried down under an air stream at 40 ˚C and reconstituted with 5 ml assay buffer. The tracer working solution was prepared fresh for each assay run and was stored at 4 ˚C until use. The antibody solution was prepared by diluting anti-testosterone antibody (mouse monoclonal [14P1F9], AbCam, Cambridge, UK) in assay buffer solution (dilution 1: 20 000). The antibody solution was prepared fresh for each assay run and was stored at 4 ˚C until use.

Calibration curves*:* For each sample run, two calibration curves, each with the following concentrations of standard testosterone (≥ 98 % purity, Sigma Aldrich, Gillingham, UK) in ethyl acetate, were run: 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 0 pg tube^{-1}. The calibrants each had a volume of 100 µl and were processed in the same way as the samples.

Repeatability*:* For QA purposes, a subset of samples with sufficient volume (n = 8) were assayed in duplicate. To assess repeatability we calculated the intra-assay coefficient of variation, which was 8.96 %.

Limit of detection (LoD*)*: LoD was calculated using six replicates of standard calibrations. LoD was defined as the testosterone standard concentration for which the standard deviation did not overlap with the standard deviation of the blank.

A6. Oestradiol ELISA

The assay was conducted according to the kit instructions (DKO003 Estradiol ELISA; DiaMetra, Milan, Italy). 25 µl aliquots of plasma were used and samples were analysed in duplicate where possible (16/23). Note that we included assay results (*n* = 6) with concentrations below the stated analytical range (i.e. < 20 pg ml^{-1}) but above the sensitivity limit (8.7 pg ml^{-1}) of the kit, as the inclusion of a 0 pg ml^{-1} standard enables contains fairly robust extrapolation. Nevertheless, this means that our circulating oestradiol concentration data falling in the range 8.7 - 20 pg ml^{-1} should be regarded as estimates. Samples with concentrations below the kit sensitivity limit were given a value of half the kit sensitivity limit (0.5*8.7 pg ml^{-1}).

A7. LC-MS/MS analysis of spiked waxworms

In order to check the dose delivered to the starlings and to compare the amount of fluoxetine present in the spiked wax worms to the calculated dose of 3.8 µg d^{-1}, a subset of 10 wax worms was randomly selected and analysed by LC-MS/MS.

Sample extraction*:* The extraction method closely followed (Bean et al., 2017). The waxworm samples were defrosted and accurately weighed, with each whole spiked worm comprising one sample (weight range 0.181 – 0.286 g, *n* = 10). The samples were homogenised and spiked with fluoxetine-d5 internal standard (98 % purity; Sigma Aldrich, Gillingham, UK). The samples were extracted with 2 x 2 ml portions of 0.2 % formic acid in methanol/water (50:50 v/v) and vortex mixed for 20-30 s after the addition of the solvent. The samples were sonicated for 15 minutes at 20 ˚C and centrifuged at 4696 *g* and 20 ˚C for 10 minutes. 200 µl supernatant was transferred to a clean tube and 8 ml water was added. Solid phase extraction (SPE) was used to clean up the samples using Oasis HLB cartridges (6 cc, 200 mg sorbent, 30 µm particle size; Waters, Elstree, UK), based on the method in (Grabic et al., 2012). The cartridges were first conditioned with methanol and water, before the samples were run through at a flow rate of 1 ml min^{-1}. The samples were eluted with methanol followed by acetonitrile. The combined eluents were evaporated to dryness under a N_2 stream, in a water bath set to 40˚C. The samples were reconstituted in 0.2 % formic acid in water/acetonitrile (80:20 v/v) and vortex mixed for 20-30 s.

Analysis by LC-MS/MS*:* The samples were analysed by reverse phase LC-MS/MS, using a Thermo Scientific UltiMate 3000 HPLC coupled to a TSQ Quantum Ultra triple quadrupole mass

spectrometer, operated in positive ion mode with electrospray ionisation (ESI). The separation was performed with a Thermo Scientific Hypersil GOLD HPLC column (particle size 1.9 µm, length 100 mm, internal diameter 2.1 mm). The analytes were eluted at 30 ˚C using a ramp gradient method, with water 0.2 % formic acid as mobile phase A and acetonitrile 0.2 % formic acid as mobile phase B. The gradient was 0-7.5 min 18 % B, 7.5-12 min 60 % B, 12-15 min 100 % B, 15-20 min 18 % B and the mobile phase flow rate was 0.35 ml min^{-1} at 0-7.5 min, 0.25 ml min^{-1} at 7.5-12 min, 0.30 ml min^{-1} after 12 min. The MS/MS was performed in selected reaction monitoring (SRM) mode and two fragment ions were monitored.

Quality Control*:* We ran a reagent blank, a matrix blank and a spiked control in the batch. We used calibration curves of the target compounds (fluoxetine hydrochloride ≥ 98 % purity, norfluoxetine hydrochloride ≥ 97 % purity; Sigma Aldrich, Gillingham, UK). The method recovery was 76.4% and the concentrations were recovery corrected, using the internal standard method. The method LoD was 0.88 ng g^{-1} (wet weight) for both fluoxetine and norfluoxetine.

Results*:* The mean concentration and standard error of fluoxetine in the waxworm samples was 3.71 µg $worm^{-1}$ ± 0.18 (n = 10), with 15.4 % relative standard deviation (RSD). We also detected norfluoxetine in all of the waxworms analysed (28.5 ng $worm^{-1}$ ± 2.50 (*n* = 10), with 27.7 % RSD.

A8. References

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Appendix B

B1. Behavioural data collection

At the start of a trial day, all individuals from the relevant aviary were caught in the morning (at 08:30) by hand net and dosed, to avoid disturbing the home aviary twice in one day. The focal individuals (up to four per day) were held in cloth bags whilst non-focal individuals were released back to the aviary. Subsequently, two of the four focal individuals being tested were transferred to the two start cages in the two arenas to participate in behavioural trials (conducted simultaneously), whilst the other two were released into individual holding cages for the collection of faecal samples (see faecal corticosterone metabolite methods below). Once the behavioural trials were finished, the two participating individuals were captured and introduced to individual cages to collect faeces, whilst the other two individuals were moved from the individual holding cages to trial start cages, to participate in behavioural trials. Once these were completed, all four birds were released back to their home aviary. Focal birds were transported between arenas, cages and home aviaries in cloth bags throughout and spent <5 minutes in the bag during one transport.

During a trial week, each individual from the relevant home underwent two behavioural trial/faeces sample collection replicates on two separate days. The order in which the birds from a home aviary were tested was randomised. On each trial day, the relevant individual was first weighed to the nearest 0.1 g, before undergoing a behavioural trial and faeces collection protocol (2 hours), in random order. During the second trial (on a separate day), the order of behavioural data/faeces collection were reversed. Before data collection began during a behavioural trial, the focal individual was introduced into a start cage and remained there for 30 minutes, without food or water. This standardised for hunger between individuals and also provided a settling period for the focal individual following capture and handling. The walls of the start cage were covered so that the individual could not see into the arena. At the end of the 30 minutes, the focal individual was released from the start cage by the observer, who opened the door by pulling on a cord from the outside of the arena. The entry of the bird into the arena was considered to be the start of the behavioural data collection period.

The number of days between the two trials for each individual varied due to practical constraints. Therefore, we performed Spearman's rank correlations between the number of days between the two trials and either the exploration or activity score from the second trial. We found that neither exploration (R_s = -0.15, p = 0.44, n = 27) nor activity (R_s = -0.16, p = 0.42, n = 27) during the second trial were significantly related to the number of days between the trials. Therefore, this was not included as a factor in subsequent statistical analyses.

Each behavioural trial took place in one of two identically arranged arenas (see Figure B1 and Figure B2). The focal individual was randomly allocated to one of the arenas during the first trial replicate and underwent the second trial replicate in the other arena. Both were outdoor arenas, measuring 6.9 m L x 2.5 W x 2 m H and the walls of each arena were covered with hessian to provide visual occlusion. There was a roofed area at one end of the arena but otherwise the ceiling was open to the environment. In each arena, there were eight corner perches (two per corner: a high and a low perch) and three swinging perches in the middle of the arena (see Figure B1 and Figure B2), giving a total of 11 perches (Minderman et al., 2009). An observer recorded data through a small viewing hole at one end of the arena and there was a start cage in one corner at this end of the aviary (see Figure B1 and Figure B2).

Figure B1: Left image shows view of trial arena looking towards the observer end, whilst right image shows view of trial arena looking away from observer end.

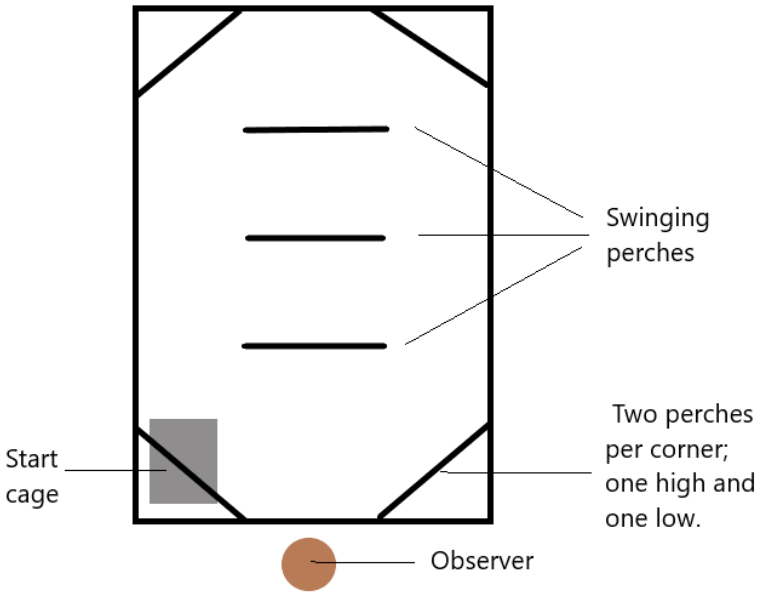

Figure B2: Birds eye view of arena layout.

B2. Faecal corticosterone metabolite data collection

During collection of faecal samples, the individual cage was lined with paper, to collect faeces. Once the bird had been removed from the cage, the faeces was collected in a 3.5 ml Eppendorf tube (Eppendorf UK, Stevenage, UK). On the same day, the faeces was weighed (to the nearest 0.001 g) and dried in an oven at 40 °C. The samples were weighed each day thereafter, until constant mass was achieved. The final weight was recorded and the dried faeces samples were stored in a 3.5 ml Eppendorf at room temperature prior to extraction.

Each sample was ground in a ceramic mortar and pestle. Two 0.05 g aliquots of each sample were weighed out into fresh 3.5ml Eppendorf tubes for extraction. A minority of samples had insufficient material for duplication, so a single 0.05 g portion was weighed instead. Extraction was performed by adding 1 ml 80:20 v/v methanol:water. The mixture was then briefly mixed by inversion, vortex mixed for 10 seconds and centrifuged for 30 minutes at room temperature and 16 000 *g.* The supernatant was transferred to a fresh 3.5ml Eppendorf and concentrated to dryness under a N_2 stream at 75 °C. At this point, the extracts were stored at room temperature before analysis by ELISA, for no more than a week.

Some samples were too small to provide a single portion of 0.05 g. Therefore, 0.025 g was weighed and pooled with 0.025 g of sample from the same individual, taken during the other trial replicate in the same week. These pooled samples provided a mean faecal corticosterone metabolite concentration across the week (across the two trial replicates in that week) for that individual.

B3. Body condition

So that we could include a measure of body condition in our models as a predictor, each individual was weighed to the nearest 0.1 g on the same day that they participated in behavioural trials/faecal sampling protocol, and also at the end of each week following thermal imaging data collection. We used the individual body mass measurements (kg) and also tarsus length (m) to calculate individual body condition based on Fulton's index: M/L^3 (M = mass in kg, L = length in m). Fulton's index has previously been used to score body condition in various bird species (Saino and Møller, 1996, Moller and Erritzoe, 2003) and was selected as regressing body mass against tarsus, the basis of a preferred method for scoring body condition in passerines, did not result in a significant relationship .

B4. Thermal imaging

We used the temperature of the skin of the right leg for our analyses, this was because the left leg of each bird had two plastic leg rings, whereas the right leg had only one. The leg skin measurements taken from four individuals were excluded from further analysis, as exploratory

thermal imaging data analysis showed that the temperature difference between the right and left legs was particularly pronounced for these birds (one fluoxetine-treated female, two control females, one fluoxetine-treated male), possibly indicating injury or some other abnormality.

Details of confounding factors recorded for each individual during thermal imaging data collection are as follows: distance from the camera (whether at the front, middle, rear of the feeder, or behind the feeder, relative to the thermal camera), head orientation to the camera (which side of the face, whether the side of the face was tilted toward/away from camera and head angle relative to vertical plane), body position (leg angle and whether head was above or below shoulders), behaviour (whether still, feeding or walking), latency to approach the feeder (measure of risk sensitivity), number of other birds at the feeder (measure of risk sensitivity).

B5. Statistical analyses

Generalised linear mixed models (GLMMs) were reduced using backwards-stepwise model selection, with the least significant predictor under Wald's Z test removed at each step. The minimum adequate model was selected using corrected Aikake's Information Criterion (calculated using R package 'MuMIn') (Barton, 2017) and R package 'DHARMa' (Hartig, 2017) was used to check that model assumptions were met.

Linear mixed models (LMMs) were also reduced using backwards-stepwise selection. At each step, the significance of each predictor in the model was computed using likelihood ratio tests (R function 'drop1'). This allowed the least significant predictor to be identified and dropped. Aikake's Information Criterion (AIC) was used to inform the selection of each minimum adequate model. The choice of which to remove was based on which led to a lower AIC. Each model was checked for homogeneity of variance and normality of residuals. The residuals for all models for response variable right leg temperature departed from normality for extreme values (according to QQ plots) but diagnostic tests were otherwise acceptable.

To check that no significant variable had been erroneously dropped during model reduction, each removed predictor was inserted back into each minimum adequate model (GLMMs and LMMs), one at a time, to ensure that each was still non-significant. Since sex and home aviary were conflated, only one of these could be included in the minimum adequate model. However, note that in all cases, home aviary was a significant predictor under likelihood ratio tests (LRTs), whereas sex was non-significant under LRTs.

For response variables leg temperature and head-leg temperature differential, leg angle (relative to camera, factor levels: back, front, left, right) was included as a fixed effect in the LMM, whilst for response variables head temperature and head-leg temperature differential, the following fixed effects were included: head position (factor levels: above or below shoulder), head angle (relative to vertical plane, factor levels: 50°, 90°, 120°), head tilt (head angle towards camera, factor levels: towards, flat or away) and head side (left or right).

Fixed Effects	**Coef. β**	**SE (β)**	***z***	***p***
a) Exploration				
Intercept	1.39	0.08	18.10	<0.001
Trial replicate	-0.16	0.10	-1.68	0.093
b) **Activity**				
Intercept	8.33	2.08	4.01	<0.001
Body condition	-2.64	0.65	--2.52	0.012

Table B1: Models testing the effect of treatment on individual behaviours. Summary of GLMM minimum adequate model outputs for the following response variables: a) exploration (number of unique perches visited); b) activity (number of flights). *n* = 54 observations from 27 birds (*n* = 13 control and *n* = 14 fluoxetine-treated) in both models. Model a) had a Poisson error distribution whilst b) had negative binomial error distribution. Table shows: coefficient estimates (β), standard errors (SE(β)), Wald's *z*-score (= β /SE(β)) and significance level *p*.

Fixed Effects	Coef. β	SE (β)	t	χ^2	p
a) Response: Right leg temperature					
Intercept	-1.79	2.75	-0.65	-	-
Air temperature	0.88	0.28	3.17	10.35	0.001
Minutes since sunrise	0.03	0.01	4.90	23.62	<0.001
Behaviour (still)	-0.94	0.58	-1.60	8.65	0.013
Behaviour (walking)	0.74	0.58	1.26		
Aviary (E)	9.26	2.17	4.28	22.65	<0.001
Aviary (C)	-3.09	2.11	-1.47		
Aviary (B)	-0.72	1.77	-0.41		
Aviary (A)	-2.11	2.11	-1.00		
b) Response: Head-leg temperature differential					
Intercept	21.00	2.82	7.44	-	-
Air temperature	-0.78	0.26	-2.95	9.56	0.002
Behaviour (still)	-0.19	0.69	-0.28	26.98	<0.001
Behaviour (walking)	-3.11	0.68	-4.57		
Aviary (E)	-3.81	1.70	-2.24	9.52	0.049
Aviary (C)	2.02	1.80	1.12		
Aviary (B)	-0.95	1.41	-0.67		
Aviary (A)	0.43	1.68	0.25		
Head tilt	-2.87	0.79	-3.64	13.57	<0.001
Head position	2.34	1.05	2.24	5.01	0.025

Table B2: Minimum adequate model outputs for LMMs investigating the effects of individual activity, exploration, and confounding variables, on the following responses: a) right leg temperature (*n* = 167 observations from 8 control and 11 fluoxetine-treated birds), b) head-leg temperature differential (*n* = 167 observations from 8 control and 11 fluoxetine-treated birds). Note that the head temperature minimum adequate model output was identical to that shown in table 1b. Table shows: coefficient estimate (β), standard error of β, t-statistic, Chi-Square statistic (χ^2) and significance level *p*.

Fixed Effects	Coef. β	SE (β)	t	χ^2	p
a) Response: Right leg temperature					
Intercept	-5.95	3.42	-1.74	-	-
Air temperature	1.09	0.34	3.22	9.86	0.002
Minutes since sunrise	0.04	0.01	4.18	18.24	<0.001
Position at feeder (behind)	2.65	0.86	3.07	9.41	0.024
Position at feeder (at front)	0.09	1.06	0.08		
Position at feeder (at middle)	-0.18	0.71	-0.26		
Aviary (E)	13.19	2.43	5.43	24.39	<0.001
Aviary (C)	-3.74	3.11	-1.21		
Aviary (B)	0.15	2.03	0.07		
Aviary (A)	-1.93	2.48	-0.78		
b) Response: Head temperature					
Intercept	21.73	1.22	17.84	-	-
Minutes since sunrise	0.03	<0.01	9.66	82.00	<0.001
Behaviour (still)	-0.75	0.28	-2.70	49.62	<0.001
Behaviour (walking)	-2.07	0.29	-7.24		
Aviary (E)	6.91	1.08	6.41	35.08	<0.001
Aviary (C)	-1.07	1.43	-0.74		
Aviary (B)	-2.45	0.83	-2.94		
Aviary (A)	-1.86	0.83	-2.24		
Head tilt	-1.29	0.36	-3.54	12.13	<0.001
Head angle (90°)	-1.54	0.39	-4.00	24.07	<0.001
Head angle (120°)	-0.39	0.24	-1.66		
c) Response: Head-leg temperature differential					
Intercept	25.11	3.22	7.79	-	-
Air temperature	-0.95	0.30	-3.18	10.63	0.001
Behaviour (still)	0.02	0.78	0.02	26.07	<0.001
Behaviour (walking)	-3.54	0.77	-4.60		
Aviary (E)	-5.49	1.94	-2.82	11.31	0.023
Aviary (C)	2.59	2.74	0.94		
Aviary (B)	-1.65	1.67	-0.99		
Aviary (A)	-0.20	2.07	-0.10		
Head tilt	-2.35	0.87	-2.69	7.96	0.005

Table B3: Minimum adequate model outputs for LMMs investigating the effect of faecal glucocorticoid metabolite concentration and confounding variables on the following responses: a) right leg temperature (n = 125 observations from 7 control and 5 fluoxetine-treated birds), b) head temperature (n = 259 observations from 9 control and 7 fluoxetine-treated birds), c) head-leg temperature differential (n = 125 observations from 7 control and 5 fluoxetine-treated birds). Table shows: coefficient estimate (β), standard error of β, t-statistic, Chi-Square statistic (χ^2) and significance level p.

B6. References

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Appendix C

C1. Neophobia trial

Each phase proceeded as follows: the food dish (with or without novel object) was placed in the same position on the floor of the arena by the observer. Once the observer had left the arena, the 14 minutes of data collection began. Two distinct novel objects were used at each time point (i.e. after 6 and 27 weeks' exposure respectively). Each novel object was presented only once to each individual, in random order between the trial replicates.

During the novel object phase, the object was placed in the centre of the food dish, in the same orientation each time. Any objects that did not possess eyes had two sticky-backed googly eyes attached to create a "head", with the objective of mimicking a predator gaze to enhance the likelihood of eliciting a neophobic response. Example novel objects are shown in Figure C1.

Figure C1. Example novel objects

In order to verify that the novel object phase of the trial truly measured neophobia, we performed a paired test (Wilcoxon signed-rank) to check that latency to feed was significantly longer during the novel object compared to the disturbance phase. We found that it was (W = 133, $P = 0.003$, $n = 35$; figure C2). We pooled the data from both 6 weeks and 27 weeks exposure for this test and included only individuals that participated in both phases of the trial.

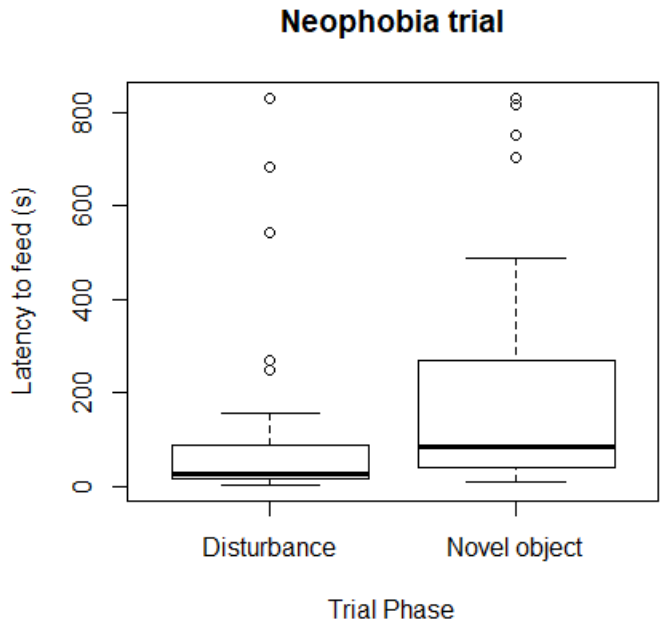

Figure C2: Latency to feed in the disturbance and novel object phases, pooled across 6 and 27 week treatment data.

C2. Pre-treatment corticosterone

To facilitate better understanding of the results obtained during the fluoxetine exposure period, we collected pre-treatment faecal samples from both groups for baseline corticosterone metabolite analysis. This was done in the week before experimental treatment began. When we later analysed these by ELISA, after the aviary experiment was completed, we found that by chance (allocation was block randomised) the birds in the fluoxetine-treated group exhibited higher concentrations of corticosterone metabolites than controls in faeces. This indicated that the fluoxetine group birds were more stressed than controls naturally, before treatment began (Mann-Whitney U-test, U = 20, p = 0.043, n = 10 control group, n =9 fluoxetine group; figure C3).

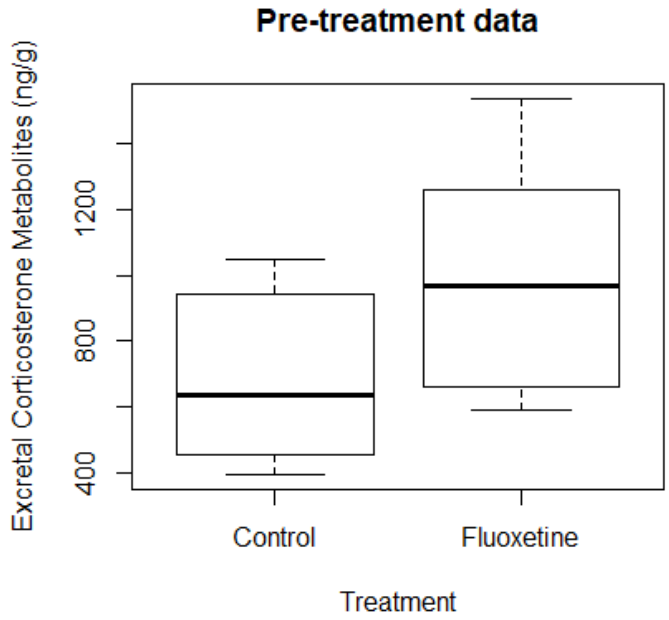

Figure C3. Faecal corticosterone metabolites in each group in the week before experimental treatment began.

C3. Statistical Methods

We calculated a measure of body condition to include in some of the mixed models. We used Fulton's index instead of a more widely employed method based on the residuals of a regression of body mass against structural length (Peig and Green, 2010). This was because our regression of body mass against tarsus was non-significant. Fulton's index (K) is given by: $K = M/L^3$, where M = body mass (in kg) and L = structural length (in m). We used tarsus length for L. Fulton's index has been previously applied to birds (Saino and Møller, 1996, Møller and Erritzøe, 2003) and performs reasonably compared to calculation methods based on a regression of mass against length (Peig and Green, 2010).

Univariate tests were performed before modelling to identify problematic correlations between pairs of predictors. Variance inflation factors (VIFs) were also calculated for the sets of predictors in each full model to identify multicollinearity. No issues were found. In the generalised linear mixed model, we tested for overdisperion using the overdisp_fun code at (Bolker). For model selection, AIC_c was used instead of AIC, as it is preferred for datasets with low samples size (Burnham and Anderson, 2004). Note that sex and home aviary were conflated, therefore only one of these could be included in any one minimum adequate model. However, for all minimum adequate models with sex included as a predictor, home aviary was found to be non-significant under likelihood ratio tests when exchanged for sex. Likewise, for all minimum adequate models with home aviary included as

a predictor, sex was found to be non-significant under likelihood ratio tests when exchanged for home aviary.

The following fixed effects were specified as ordered factors: treatment (0 = control, 1= fluoxetine), feather type (0 = wild-grown, 1 = experimentally regrown), exposure length, home aviary, trial replicate (out of two, at particular exposure length), order of neophobia trials (whether novel object phase came first or second).

C4. References

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Appendix D

Compound	Parent ion (*m/z*)	SRM product ions (*m/z*)	Collision Energy	Tube lens	Retention time (min)
Fluoxetine	310.2	44.2	15	46	9.4
Fluoxetine	310.2	148.2	5	54	9.4
Fluoxetine-d5	315.2	44.2	15	46	9.4
Fluoxetine-d5	315.2	153.2	5	54	9.4
Norfluoxetine	296.2	30.3	13	31	9.2
Norfluoxetine	296.2	134.2	5	35	9.2

Table D1. Fragmentation parameters for LC-MS/MS analysis of fluoxetine and norfluoxetine.

Organ Pair	Pairwise Comparisons	
i) Fluoxetine concentration		
	Difference (ng g^{-1})	***P***
Kidney-Brain	2.377	0.013*
Liver-Brain	5.205	< 0.001***
Lung-Brain	0.642	0.261
Liver-Kidney	2.829	0.005**
Kidney-Lung	1.735	0.050
Liver-Lung	4.564	< 0.001***
ii) Norfluoxetine concentration		
	Difference (ng g^{-1})	***P***
Kidney-Brain	2.974	0.002**
Liver-Brain	5.963	< 0.001***
Lung-Brain	2.318	0.012*
Liver-Kidney	2.989	0.003**
Kidney-Lung	0.656	0.256
Liver-Lung	3.645	< 0.001***
iii) Fluoxetine:norfluoxetine ratio		
	Difference (ng g^{-1})	***P***
Brain-Kidney	1.604	0.065
Brain-Liver	5.453	< 0.001***
Brain-Lung	3.091	0.002**
Kidney-Liver	3.849	< 0.001***
Kidney-Lung	1.487	0.069
Lung-Liver	2.362	0.014*

Table D2. Post hoc tests of differences in residue concentration for i) fluoxetine concentration, ii) norfluoxetine concentration and iii) ratio of fluoxetine:norfluoxetine between different tissues (pairwise comparison), with endpoint adjustment applied to *p* values. Computed using Dunn's test (Dinno, 2017), with False Discovery Rate (Benjamini-Hochberg) as endpoint adjustment method. Statistically significant pairs are starred (p < 0.05*, p < 0.01, p < 0.001***).**

	Kidney FLX	Liver FLX	Lung FLX	Brain NFLX	Kidney NFLX	Liver NFLX	Lung NFLX
Brain FLX	$r_s = 0.811$ $p = 0.002$	$r_s = 0.455$ $p = 0.140$	$r_s = 0.441$ $p = 0.154$	$r_s = 0.552$ $p = 0.067$			
Kidney FLX		$r_s = 0.524$ $p = 0.084$	$r_s = 0.566$ $p = 0.059$		$r_s = 0.720$ $p = 0.011$		
Liver FLX			$r_s = 0.378$ $p = 0.227$			$r_s = 0.734$ $p = 0.009$	
Lung FLX							$r_s = 0.853$ $p < 0.001$

Table D3. Spearman's Rank-Order correlations of fluoxetine and norfluoxetine concentrations in brain, kidney, liver and lung. Spearman correlation coefficients and corresponding *p*-values are shown. FLX denotes fluoxetine, NFLX denotes norfluoxetine. Note that correlations between fluoxetine and norfluoxetine concentrations in different tissues and correlations of norfluoxetine between different tissues are not displayed.

Fluoxetine-treated group feathers			Control group feathers		
Rectrices	Fluoxetine (ng g^{-1})	Norfluoxetine (ng g^{-1})	Rectrices	Fluoxetine (ng g^{-1})	Norfluoxetine (ng g^{-1})
4, 9	14.90	<LOD	4*	1.84	<LOD
4, 9	17.73	<LOD	9**	2.03	<LOD
4, 9	14.51	1.25	4, 9	0.42 (0.5*LOD)	<LOD
4, 9	13.59	1.27	4, 9	3.89	<LOD
4, 9	11.39	<LOD	4, 9	1.60	<LOD
4, 9	11.63	<LOD	4, 9	1.80	<LOD
4, 9	10.63	0.74	4, 9	0.42 (0.5*LOD)	<LOD
4, 9	11.07	0.85	9**	10.71	<LOD
4, 9	7.61	<LOD			
4, 9	11.13	<LOD			
4, 9	6.08	<LOD			

Table D4. Concentrations of fluoxetine and norfluoxetine displayed for all regrown (i.e. grown during the dosing period) tail feather (rectrices R4 and R9) samples analysed (n = 11 fluoxetine-treated, n = 8 control). The LOD for fluoxetine and norfluoxetine was 0.84 ng g^{-1}. Note that only one feather of the rectrice pair was successfully harvested in three of the control birds: * denotes that the missing feather was damaged or lost before harvest, ** denotes that the feather never regrew.

Organ	Tissue fluoxetine conc.	Tissue norfluoxetine conc.
Brain	r_s = 0.25, *p* = 0.521	r_s = 0.500, *p* = 0.178
Kidney	r_s =0, *p* = 1	r_s = 0.067, *p* = 0.880
Liver	r_s = 0.300, *p* = 0.437	r_s = 0.133, *p* = 0.744
Lung	r_s = 0.033, *p* = 0.948	r_s = -0.200, *p* = 0.613

Table D5. Results of Spearman's rank correlations between concentrations of fluoxetine in feather samples versus concentration of fluoxetine and norfluoxetine in various tissues (*n* = 11 in each case). Table shows correlation coefficient (r_s) and associated *p* value for each correlation.

Fluoxetine (ng g^{-1}) in fluoxetine treatment group wild-grown feathers	Fluoxetine (ng g^{1}) in control group wild-grown feathers
7.09	1.13
8.48	1.71
8.94	1.41
2.04	1.54
14.31	1.51
2.28	nd
6.07	nd
4.39	nd
nd	26.98
1.66	15.77
9.17	1.19
nd	1.8
	8.43

Table D6. Concentrations of fluoxetine present in wild-grown rectrice feathers (feathers grown whilst the birds were living in the wild; *n* = 12 fluoxetine-treated and *n* = 13 control birds). Two wild-grown rectrices were harvested from each individual in the week following the onset of dosing with fluoxetine (during the dosing period, a dose of 3.71 µg fluoxetine was administered five days per week, each week). All feathers were fully regrown before dosing started, thereby preventing deposition of the fluoxetine dose in feathers by the blood supply. Samples with concentration below LOD (0.84 ng g^{-1}) are marked as nd for non-detect.

D1. References

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