# An assessment of the risks posed by human pharmaceuticals in the environment to wild birds

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## Abstract

When a pharmaceutical is ingested, a proportion of it is excreted unchanged, ending up in sewage. Birds foraging at sewage treatment works can take up these pharmaceuticals by ingesting invertebrates living in the sewage. There is concern that low concentrations of human pharmaceuticals can alter behaviour and physiology in wildlife species because of the evolutionary conservation of drug targets. In this thesis I focus on a heavily prescribed and environmentally persistent antidepressant, fluoxetine, which is used by humans to treat anxiety, but also causes side effects e.g. changes in appetite, libido and activity. To assess the risks posed by fluoxetine in contaminated invertebrates to foraging birds, wild-caught starlings (12 fluoxetine-treated and 12 controls) were fed with an environmentally relevant dose (928 ng/day) over winter and into the breeding season. After 22 weeks of treatment, liquid chromatography coupled with a triple quadrupole mass spectrometer revealed that fluoxetine accumulation in the starling brain (3.1 ng/g) was approximately 30 times lower than in humans (corrected for differences in dose and body mass) and elimination from starling tissues was 2.2 to 31 times faster than is found in human plasma. After 16-weeks of fluoxetine treatment, relative to controls, the fluoxetine-treated birds fed 24% less, and at times of nutritional stress, they fed 41% less. In mate choice experiments, fluoxetine-treated females showed less interest in males, spending 49% more time in the 'no-choice' zone than the control females did. However, no effects on anxiety related behaviour or activity levels were found. In a separate *in-vitro* experiment, the bioaccessibility of fluoxetine in invertebrate prey only varied by 13% between humans and birds and so could not explain all the inter-species variability in tissue accumulation. The observed changes to behaviour and physiology in starlings may have been caused by neuro-adaptations occurring over time, as are observed in humans possibly due to greater receptor sensitivity than humans. It is now important to assess how the effects of fluoxetine on captive individuals could translate to free living individuals; how these impacts interact with other stressors and whether there could be consequences for population dynamics and ecosystem functioning.

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Data from Chapter 2 has been published as a paper in an internationally peer reviewed journal. All chapters have been written as stand-alone papers but, are presented in a consistent style and format in this thesis. Publications from the data presented in Chapters 3-5 are currently being prepared. The current status of papers is presented in Table 0.1.

**Table 0.1: The publication status of the papers presented in this thesis**

| Title | Journal | Status | Chapter |
|---|---|---|---|
| Internal concentrations of fluoxetine in starlings (*Sturnus vulgaris*) following chronic oral uptake of an environmentally relevant dose | Target: Environmental Science and Technology | in preparation | 2 |
| Behavioural and physiological responses of birds to an environmentally relevant concentration of an antidepressant | Philosophical Transactions of the Royal Society B | Published online October 2014 Volume 369 (1656) | 3 |
| The effects of fluoxetine on female mate choice in starlings (*Sturnus vulgaris*) | Target: Journal of Applied Ecology | in preparation | 4 |
| An *in-vitro* assessment of differences in bioaccessibility of an antidepressant between humans and birds | Target: Environmental Science and Technology | in preparation | 5 |

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# Chapter 1:

# Introduction

## 1.1 Background

Pharmaceuticals have brought large benefits to human populations enabling individuals to have better quality of life for longer than was previously possible (MEA 2005). In developed countries, the ageing, growing and increasingly obese human population, has led to increasing use of a diverse range of pharmaceuticals (Calow 2009). With around 3,000 pharmaceuticals licenced for use in Europe (Ternes et al. 2009) and recent advances in analytical chemistry improving the sensitivity of instruments for the detection of micro-pollutants such as pharmaceuticals (Boxall et al. 2012), there is growing interest in assessing the risks pharmaceuticals in the environment pose to humans, non-target species and ecosystems in general (Arnold et al. 2014; Boxall 2009).

The main source of human pharmaceuticals to the environment is believed to be patient excretion of active pharmaceutical ingredients (APIs) and active metabolites in urine and faeces (Daughton and Ternes 1999). Figure 1.1 shows some of the main sources, flows and sinks of pharmaceuticals in the environment. Patient-excreted APIs end up in the wastewater system (Williams 2005). For logistical reasons, it is almost impossible to measure the contribution of patient excretion to the wastewater system although modelling estimates such as Ort et al. (2010) estimate it to be around 70% of total pharmaceutical contamination in the environment. Other, less significant sources of pharmaceuticals to the environment (in Figure 1.1) include residues released directly to surface waters from hospitals and manufacturers, disposal of products from the supply chain to wastewater or in solid waste, disposal of unused medicines by patients and health care facilities in solid waste or to wastewater by flushing down the toilet (Ayscough et al. 2000; Daughton and Ternes 1999; Monteiro and Boxall 2010). The emission of pharmaceuticals to air during the manufacturing process is believed to be a very minor source of total pharmaceuticals in the environment as pharmaceuticals typically are not very volatile molecules (Williams 2005) (Figure 1.1).

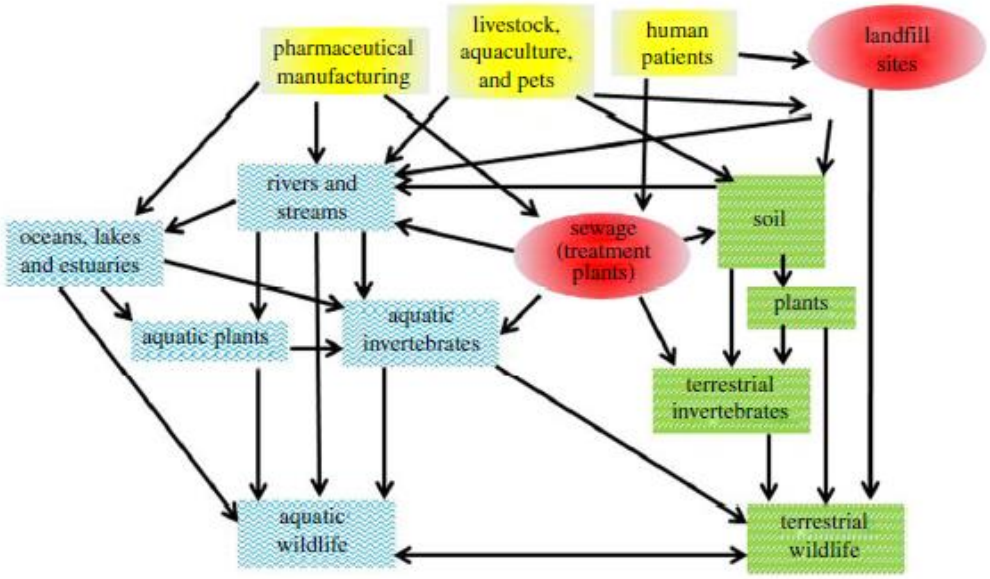



**Figure 1.1: Sources (yellow boxes), pathways (red ovals) and fate of pharmaceuticals in the terrestrial (green boxes) and aquatic environment (blue boxes). Terrestrial wildlife foraging on sewage treatment plants, landfill and aquatic organisms can be exposed to pharmaceuticals which have been released to the environment via patient and animal excretion, disposal of unused product to landfill and via the manufacturing process (Arnold et al. 2014).**

Pharmaceuticals contained in urine and faeces go down the toilet and are transported via the sewerage system to the wastewater treatment plant (WWTP, red oval labelled sewage treatment plant in Figure 1.1). As the release of many pharmaceuticals to the environment is relatively constant even if the degradation rate of a particular pharmaceutical within the WWTP is high, it can remain at high levels through *pseudo*-persistence (Boxall 2004). Additionally seasonal peaks in drugs such as antihistamines in the summer months when pollen levels rise may occur (Monteiro and Boxall 2010).

Terrestrial wildlife species such as birds and bats are attracted to forage on food sources contaminated with pharmaceuticals such as WWTPs and fields spread with sewage sludge by the abundance of invertebrates that develop therein (green boxes in Figure 1.1). Human population growth, urbanisation, climate change and intensive farming methods have reduced the number and quality of natural wildlife foraging habitats. This makes habitats potentially contaminated with pharmaceuticals both a common feature on the landscape and a valuable source of nutrition for wildlife (Fuller and Glue 1978; Fuller and Glue 1980; Markman et al. 2011; Park and Cristinacce 2006, Barron et al. 2010; Laturnus et al. 2007; McClellan and Halden 2010; Walters et al. 2010; Ausden 2014). To date little is known about the levels of pharmaceutical contamination in prey items that could be ingested by wildlife species, the extent to which wildlife feed on these contaminated

sources and what (if any) are the risks posed to wildlife by ingestion of pharmaceuticals at the levels of uptake that would be expected in the environment.

The large number of pharmaceuticals in use, in addition to the diversity of wild bird species feeding on potentially contaminated sources, makes filling this knowledge gap an important task. This thesis aims to start addressing this knowledge deficit by identifying pharmaceuticals of potential concern and investigating whether one case study compound could pose any risks to wild birds at an environmentally relevant level of uptake. The novel data generated by this thesis enables recommendations for future research effort to be made that will ensure the number of *in-vivo* effects studies required to perform these risk assessments are minimised.

In this Chapter, the broad concepts underpinning the thesis are introduced.

1) Environmental Risk Assessment and Read-across
2) Patient excretion of pharmaceuticals to the environment
3) Uptake of pharmaceuticals from the environment by terrestrial species
3) Wildlife potentially exposed to pharmaceuticals in the UK
5) Examples of pharmaceuticals causing adverse effects in wildlife
6) The model scenario

## 1.2 Environmental Risk Assessments and Read-across

Given that pharmaceuticals are detected in the environment and are likely to be taken up by non-target wildlife species, it is important to improve our understanding of the risks they pose at environmentally relevant levels. An understanding of risk is gained through conducting environmental risk assessments. Traditionally environmental risk assessments for human pharmaceuticals follow a series of test procedures set out by the Organisation for Economic Co-operation and Development (OECD) (EMEA 2005; 2006). These tests measure standard endpoints (such as growth, survival and reproduction) in model species (e.g. *Daphnia magna* and *Pimephales promelas*) and are generally focussed on the aquatic environment. While these Environmental Risk Assessments (ERA) may provide an indication of the level at which an adverse effect will occur in a model species, it is never going to be an all-encompassing risk assessment given the diversity of wildlife species.

As pharmaceuticals are designed for use in humans, they also undergo stringent tests in model mammals such as rats, mice, dogs and primates prior to clinical drugs trials in human subjects and eventually registration e.g. (EMEA 2005). The rigorous *in-vivo* testing of pharmaceuticals in model

mammals means a great deal is known about their pharmacology (Caldwell et al. 2014). There is increasing interest in tapping into the pool of mammalian data to read-across to non-target wildlife species (just as is currently done from model mammal to human) (Rand-Weaver et al. 2013) to complete Environmental Risk Assessments e.g. (Boxall et al. 2012). The read-across hypothesis was first suggested by Huggett et al. (2003) whereby a fish plasma concentration of a pharmaceutical was related to the human therapeutic plasma concentration to predict the likelihood of mode of action effects. Studies by Owen et al. (2007; 2009) and Lazarus et al. (2014) have demonstrated the potential application of read-across in the risk assessment process. Normally, read-across compares pharmacology data between model and non-target species to assess the likelihood of an effect observed in the model species occurring in the non-target species. To test the applicability of the read-across approach to a new non-target species, ecotoxicology data should also be collected in the non-target wildlife species that are expected to be exposed to pharmaceuticals. In reality, effects data in the non-target species are not often collected and so there are limited numbers of studies which have tested the applicability of the read-across hypothesis. By collecting relevant pharmacology data in which includes information on Absorption (Accumulation), Distribution, Metabolism and Elimination (ADME), some taxonomically diverse non-target species, it is possible to compare and test the broader applicability of read-across (Oh 2002). Below are the OECD accepted definitions of ADME (OECD 2008):

- Absorption is the accumulation of a compound from the blood stream into a tissue. Absorption is affected by many factors such as the route of administration (e.g. oral or intravenous) and the bioavailability of the drug (Hiemke and Hartter 2000).

- Distribution is the reversible transfer of a drug from one compartment, e.g. blood plasma or other bodily fluids to another, such as a tissue. Chemical properties, such as log Kow (which affects how lipophilic the pharmaceutical is) has a strong influence on distribution. Lipophilic drugs, with a high log Kow, tend to distribute more into tissues than bodily fluids and are said to have a high volume of distribution (Oh 2002).

- Metabolism is an enzyme catalysed reaction that converts the parent compound into new compounds known as metabolites. Metabolism can be categorised as Phase I, II or III. Phase I metabolism involves enzymatic reactions which add or expose reactive functional groups (-OH, -SH, $NH_2$ or COOH). Phase I metabolism is carried out by microsomal monooxygenases (Hutchinson et al. 2014) which are found in the endoplasmic reticulum of the liver and other organs. Cytochrome P450 (CYPs) are a superfamily of hemo-proteins which have a diverse

range of functions and provide the active centre for these enzymes. To date, 37 different CYP families have been identified in a diverse range of species of animal (reviewed in Arnold et al. 2013). Phase II metabolism involves conjugation of the pharmaceutical with the addition of large water-soluble polar molecules such as glucuronic acid, sulphate, acetic acid or amino acid (Ayscough et al. 2000; Hutchinson et al. 2014). By making the metabolite more polar, and therefore more hydrophilic, than the parent compound, metabolites can be excreted in urine and faeces more readily. Phase III metabolism involves cellular efflux processes where specific transporter proteins are used to pump lipophilic parent compounds or metabolites out of cells (Hutchinson et al. 2014). Metabolites usually have reduced or insignificant pharmacological activity, although active metabolites are known to exist for some pharmaceuticals (Monteiro and Boxall 2010), which can have equivalent or more potent activity than their parent compound e.g. norfluoxetine (Moraes et al. 1999). The metabolic processes that occur within a species can affect the rate of absorption, distribution and elimination.

- Elimination is the removal rate from a particular compartment. Elimination usually takes place via excretion of the parent compound and metabolites from the body of the organism. When absorption exceeds the rate of elimination, accumulation occurs.

- Bioaccumulation refers to absorption (or uptake) of a chemical from the environment. Bioaccumulation can include uptake via ingestion, inhalation, dermal absorption and transfer from mother to unborn foetus or embryo (Oh 2002).

- Bioconcentration is a specific type of bioaccumulation where the only source of the chemical which has accumulated in the organism was water.

Once ADME has been established for a compound in a particular species, there are a number of issues that need to be considered when applying the read-across hypothesis between species. Firstly, read-across is based on the idea that a drug target (a particular enzyme or receptor) has been evolutionarily conserved in the non-target species. Thus, in the non-target species the same mode of action related effects are to be initiated from a molecular interaction between drug and receptor, as occurs in the target-organism (usually human) (Rand-Weaver et al. 2013). Of course, the presence of an identical or analogous drug receptor in different species needs to be identified, but this has only been done for a limited number of species and pharmaceuticals (e.g. Brown et al. 2014) Secondly,

organisms differ widely in terms of digestive tract physiology and diet. A consideration of bioaccessibility, the proportion of the ingested contaminant that is mobilised from the food, may be an important factor as only bioaccessible drugs are available for uptake through the intestinal wall into systemic circulation (Laird et al. 2009; Ollson et al. 2009; Ruby et al. 1993). Bioaccessibility is distinct from bioavailability, which is the proportion of the dose that reaches circulation (Ruby et al. 1999). *In-silico* and *in-vitro* approaches (Hutchinson et al. 2014; Kaufman et al. 2007; Martinez-Haro et al. 2009) can be used to help fill in these knowledge gaps to improve the accuracy of read-across. Clearly there is great value in exploring the use of read-across in ERA. What is lacking at present is data on the effects pharmaceuticals can have in non-target wildlife species at the low concentrations typical of environmental exposure which can be coupled with ADME (and ideally bioaccessibilty and receptor sensitivity) data. Effects and ADME data for wildlife could then be compared and contrasted with effects and ADME data collected previously in model mammals to test the read-across hypothesis. This thesis contains data from experiments designed to do just that.

## 1.3 Patient excretion of pharmaceuticals to the environment

When humans take pharmaceuticals, most of the drug is well absorbed and extensively metabolised by Phase I, Phase II or Phase I followed by Phase II metabolism (a small proportion is metabolised by Phase III). The pharmacokinetics of some drugs results in low levels of patient metabolism and 50-90% of the parent compound and or pharmacologically active metabolites can be excreted to wastewater (Lienert et al. 2007). Even if only a small percentage of the parent compound is excreted unchanged, the large volumes of some drugs that are used (e.g. Ibuprofen, Paracetamol and Aspirin see NHS, 2010) could still result in a large volume of pharmacologically active material entering the WWTP. Additionally, there is also some evidence to suggest that WWTP bacteria can hydrolyse conjugated metabolites back to the parent compound (Hirsch *et al.,* 1999; Andersen *et al.,* 2003). Potentially WWTPs provide a concentrated environmental source for a whole cocktail of pharmacologically active compounds. Although antagonistic interactions are most common in mixtures, the pharmaceutical mixture potentially has greater toxicity than that of the individual compounds in the mixture through additive and synergistic interactions (Backhaus 2014).

## 1.4 Uptake routes of human pharmaceuticals from the environment into terrestrial species: Wastewater Treatment Plants

Pharmaceuticals and their metabolites which are excreted or are disposed of to the sewerage system will eventually end up in a WWTP e.g. (Kasprzyk-Hordern et al. 2008). In the WWTP, the wastewater undergoes a series of physical and biological, and possibly chemical treatment,

processes, unless the WWTP's capacity has been exceeded, in which case raw sewage is released to the environment (Koivunen et al. 2003). WWTPs provide attractive foraging grounds for many species of birds (Fuller and Glue 1978). Thus any species that forages on sewage contaminated prey items is potentially at risk from ingested pharmaceuticals.

Wastewater treatment aims to convert waste materials present in the water into stable oxidised end products which can be disposed of to landfill or applied to arable land as a fertiliser. In the WWTP a series of physical, biological and sometimes chemical processes are used to remove organic matter, solids and contaminants from the wastewater, so that the effluent is of sufficient quality to be discharged to the receiving water (Gray 1989). Wastewater treatment may consist of up to five stages: Preliminary treatment, Primary (sedimentation) treatment, Secondary (biological) treatment, Tertiary treatment and Sludge treatment (Figure 1.2). The infrastructure used by different stages of the treatment process provides different habitats for animals, with the biological treatment stage being the most valuable (Fuller and Glue 1980, Gough et al. 2003). Biological treatment offers a relatively cost effective means of removing pharmaceuticals from wastewater, although removal is usually less than 100% (Kasprzyk-Hordern et al. 2009). Advanced tertiary treatment processes such as granular activated carbon and reverse osmosis are some of the most efficient means of removing small soluble organics, such as pharmaceuticals, from the final effluent; however, their implementation is expensive (US EPA 2000, Gray 1989). The exact number of stages and methods used depends upon the nature of the influent, the amount of water available to dilute the influent in and the water quality objectives of the receiving water (Gray 1989).

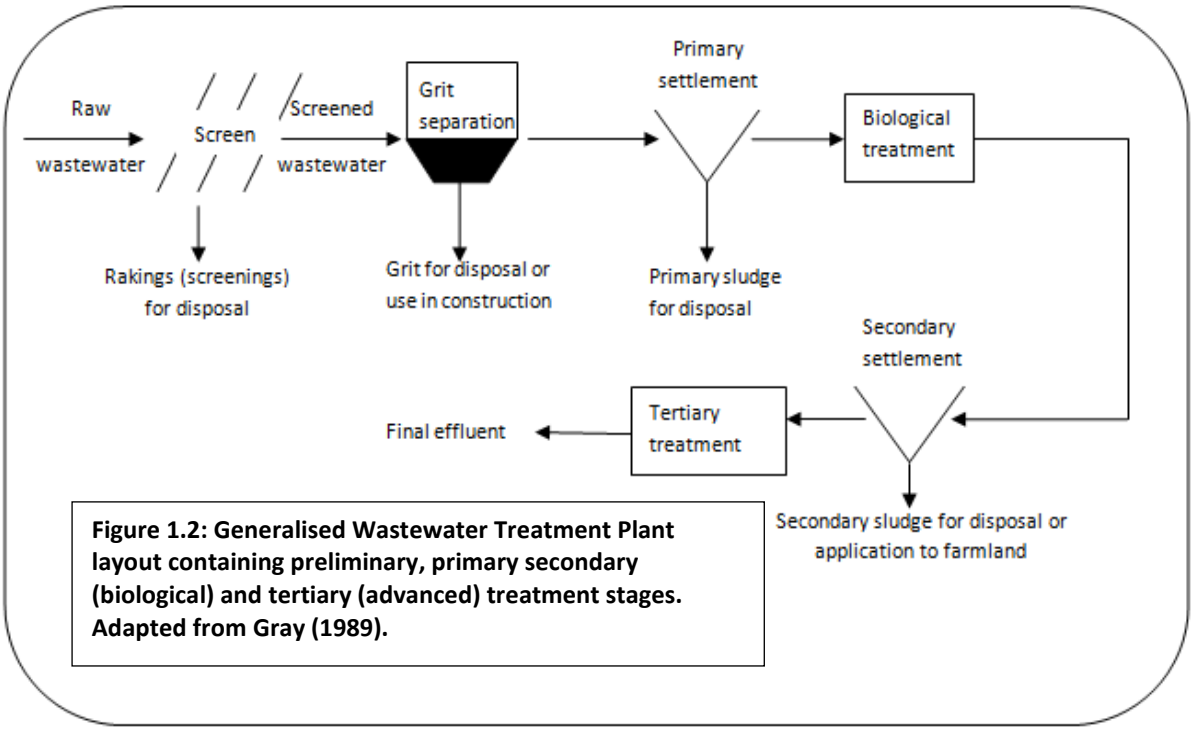



**Figure 1.2: Generalised Wastewater Treatment Plant layout containing preliminary, primary secondary (biological) and tertiary (advanced) treatment stages. Adapted from Gray (1989).**

### 1.4.1 Biological treatment of wastewater

At a WWTP, birds are most likely to be exposed to pharmaceuticals from foraging on the biological treatment phase (Markman et al. 2011; Gough et al. 2003). Wastewater received by the biological treatment phase is effectively untreated wastewater as no biological or advanced/chemical, which is what is required to remove pharmaceuticals, is included in the primary treatment phase (physical sedimentation) (Gray 1989). Therefore the concentrations of pharmaceuticals found on a trickling filter are likely to be much higher than in the final effluent (the wastewater that has received biological/chemical treatment) and in the receiving water (essentially diluted effluent) (Daughton and Ternes 1999). The biological treatment phase uses microbially mediated biodegradation processes to improve the effluent quality (Smith 2009). Biodegradation is performed by a biofilm consisting of microorganisms, predominantly bacteria, but also including fungi, algae, protozoans, nematodes and rotifers (Gray 1989). In the UK, one of two systems is used to provide biological treatment: activated sludge or a fixed film reactor such as trickling filters. Activated sludge is used in preference to trickling filters mainly because less space is required (Gray 1989). However, activated sludge is more expensive to set up than trickling filters, meaning that many trickling filters remain in use today, presenting an exposure route by which pharmaceuticals can get into wild birds. Pharmaceuticals can also be degraded by hydrolysis, oxidation or may be adsorbed to solids and become isolated in sewage sludge (biosolids) (Versteeg et al. 2005) (see section 1.4.1a).

### 1.4.1a Trickling filters

The food chain of a trickling filter (Figure 1.3) is more complex than it is for activated sludge, and subsequently results in the presence of an invertebrate grazing fauna which is what attracts foraging birds (Fuller and Glue 1978; Markman et al. 2011) and bats (Park and Cristinacce 2006). The biofilm has a porous structure, which is made even more so by the burrowing invertebrates that graze the film and bioaccumulate the pharmaceuticals from the film. Pharmaceuticals in the wastewater bioconcentrate into the cells of the microorganisms (Daughton and Ternes 1999) which are consumed by grazing invertebrates, predominantly worms (*Lumbricids* and *Enchytraeids*) and insects (springtails, beetles and dipteran flies). The pharmaceuticals contained within the microorganism's cells are transferred to the invertebrates, where they can bioaccumulate in the lipid if sufficiently hydrophobic. Bioaccumulation rates in invertebrates are largely unknown, although chemical properties such as log Kow and the acid dissociation 'pKa' constant are important factors, whilst there is also variability between species and individuals (Bringolf et al. 2010; Dussault et al. 2009; Liebig et al. 2005; Markman et al. 2007; Park et al. 2009). The invertebrates are then preyed upon by birds and bats (Fuller and Glue 1978). What remains uncertain are the levels of pharmaceuticals that birds are ingesting on a daily basis and whether these levels are sufficient to cause adverse effects. There is seasonal variability in the invertebrate fauna available for birds, which affects the species foraging there (Figure 1.4).

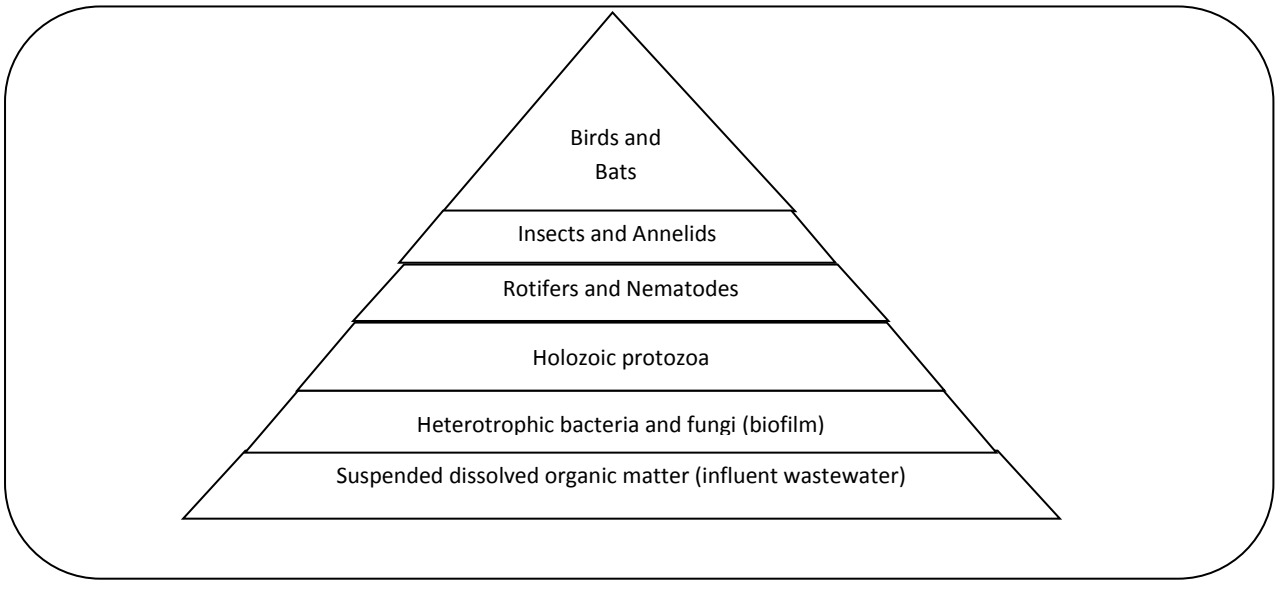



**Figure 1.3: Simplified trickling filter food pyramid (Adapted from (Gray 1989). A biofilm develops on the filter bed media (e.g. gravel or rocks) as a result of the nutrition provided by the suspended dissolved organic matter. A food pyramid develops with various trophic levels, at the top of which are birds and bats that forage on the grazing invertebrate communties of the lower trophic levels.**

### 1.4.2 Sewage sludge

Another source of human pharmaceuticals in the environment to which birds are exposed is the invertebrates that live in soils spread with sewage sludge. Since the EU ban on the disposal of biosolids at sea in 1998 (EU directive 91/271/EEC Article 14 paragraph 3, EU 1991), biosolids have been increasingly applied to farmland as a fertiliser in both Europe and the USA (McClellan and Halden 2010; Walters et al. 2010).

A large proportion of the pharmaceuticals (particularly those with high soil organic carbon-water partitioning coefficients (KOC) values e.g. carbamazepine) that are removed from suspension in the water are likely to end up in biosolids (Barron et al. 2010; Laturnus et al. 2007; McClellan and Halden 2010). Wildlife foraging on farmland spread with biosolids is likely to be exposed to the pharmaceuticals contained within. In the EU the use of sewage sludge in agriculture is regulated by Directive 86/278/EEC (EU 1986), (amended by 91/692/EEC, EU 1991). The aim of both 86/278/EEC (EU 1986) and 91/692/EEC (EU 1991) was to prevent harmful effects on soil, vegetation, animals and humans. However, neither Directive mentions pharmaceuticals with the entire focus of the directive being on heavy metals. The absence of any mention of pharmaceuticals in 86/278/EEC (EU 1986) and 91/692/EEC (EU 1991) reflects the fact that pharmaceuticals are an emerging pollutant category that was not in the thoughts of policy makers twenty years ago.

There has been no research specifically into the uptake of pharmaceuticals by livestock from pasture spread with biosolids. Rhind (2005) and Rhind *et al.,* (2010; 2007) investigated the effects of endocrine disrupting chemicals (EDCs) by sheep from pasture spread with sewage sludge. Although, none of the EDCs Rhind *et al.* (2005; 2010; 2007) investigated were pharmaceuticals, the flows of other bioaccumulative organics, such as Polychlorinated biphenols, can provide an idea of the potential fate of bioaccumulative pharmaceuticals contained in sludge. Rhind (2005; 2007) found that the EDCs from the sludge adhered to the grass and were ingested by grazing sheep, accumulating in tissues to levels which had the potential to affect immune and reproductive functioning. Additionally, Rhind's (2010) research also found that there is the potential for EDCs to be secreted in the milk of ewes and for it to reach levels that would potentially be of concern to animal and human health. A lag time between application of sludge and the initiation of grazing was recommended (Rhind et al. 2013). Of course, wildlife cannot be excluded from fields spread with sewage sludge, so would be ingesting contaminated vegetation and invertebrates directly.

The Environment Agency's Code of practice for agricultural use of sewage sludge does not include the requirement for pharmaceutical concentrations to be measured in sludge before it is applied to farmland, although heavy metals and ions have standards listed along with other parameters (EA 1996). Interestingly, the section on sludge quality in the Environment Agency Code of practice (EA 1996) mentions that sludge from abattoirs and other animal processing plants may be a significant source of pathogens, but fail to mention that pharmaceutical residues would provide an equal cause for concern. In light of work highlighting the problems presented by pharmaceuticals in the environment e.g. (Arnold et al. 2013; Ayscough et al. 2000; Boxall et al. 2012; Boxall 2004; Daughton and Ternes 1999), it is perhaps time for new legislation to promote monitoring that will establish the extent of the problem posed by spreading sewage sludge on agricultural land. However such legislation would not be without major logistical (sample collection), analytical (methods are often expensive or not well developed and contain errors) and financial (if the polluter pays, who is the polluter?) problems.

Although it is known that fields are commonly spread with sewage sludge likely containing pharmaceuticals, it is difficult to measure exposure for wild birds, as the area covered by contamination is much more vast (and therefore potentially concentrations are more variable and more dilute) than a WWTP trickling filter. As WWTP trickling filters are common structures in the environment and provide a point source for pollution, it is easier to model and assess the effects of foraging on them than it is for fields spread with sewage sludge.

### 1.4.3 Wildlife species foraging on pharmaceutically contaminated sources

Although, modern WWTPs are much smaller than the old fashioned sewage farms, and support lower populations of wetland bird species, trickling filters still provide important bird habitats. This is particularly true for passerines in winter when alternative food sources are at a premium (Fuller and Glue 1978; Gough et al. 2003) (Table 1.1, Figure 1.4). The most common birds observed foraging on trickling filters include Pied and Yellow wagtail (*Motacilla alba, Motacilla flava*) and European starling (*Sturnus vulgaris*). Other species that are known to forage on trickling filters are shown in Table 1.1. The importance of the trickling filters during different seasons is shown in Figure 1.3. One of the only studies to look at bird use on trickling filters observed 27 species foraging on WWTP (Fuller and Glue 1978), but for many of these the trickling filter was only of negligible value (Fuller and Glue 1978) (Figure 1.4).

Trickling filters also provide important sources of nutrition and habitats for birds in the breeding season. In Figure 1.3 pump houses and outbuildings are seen to be used for nesting sites by swallows, martins and thrushes. Starlings are known to routinely forage on trickling filter invertebrates in the breeding season (Markman et al. 2011). Pied wagtails (*Motacilla alba*) and Wrens (*Troglodytes troglodytes*) have also been found to nest in the walls of drying trickling filters. In the summer birds of prey, especially owls and Kestrel (*Falco tinnunculus*) (Table 1.1), have been observed hunting on WWTPs with trickling filters. Large numbers of swifts and starlings are attracted in summer (Parr 1963) whilst in spring and autumn the diversity of passerines is highest. WWTPs provide stopover points during migration. Fuller and Glue (1978) found that the highest density of birds on the Aston Clinton trickling filter (Buckinghamshire) was highest between November and March although diversity was lowest during this period with pied wagtails and starlings making up 92% of all birds. The remaining 8% of species was mainly composed of meadow pipit, grey wagtail and reed bunting. Trickling filters are also of high value to finches and sparrows in winter, while Gulls may also be seen on trickling filters at this time of year (Figure 1.4) (Fuller and Glue 1980). Despite technological advancements that make modern wastewater treatment plants less valuable habitats than old fashioned sewage farms, they remain an important foraging ground for diverse range of species particularly at times of nutritional stress. Although, it is known that trickling filter beds represent important foraging sites for birds, there remains limited data on which invertebrates they consume `and what proportion of their diet comes from the filter beds. It has been estimated by Markman et al. (2008) that a starling will take approximately half of their invertebrate food from WWTPs at times of nutritional stress such as over winter and the breeding season.

**Table 1.1: The diversity of UK bird species of bird found foraging on or around trickling filters (Fuller and Glue, 1980; Plant 1978).**

| **Family** | **Common name** | **Latin name** |
|---|---|---|
| **Swifts (*Apodidae*)** | Common swift | *Apus apus* |
| **Swallows and martins (*Hirundinidae*)** | Swallow | *Hirrundo rustica* |
| | House martin | *Delichon urbica* |
| | Sand martin | *Riparia riparia* |
| **Pipits and wagtails (*Motacillidae*)** | Meadow pipit | *Anthus pratensis* |
| | Pied wagtail | *Motacilla alba* |
| | Grey wagtail | *Motacilla cinerea* |
| | Yellow wagtail | *Motacilla flava* |
| **Starlings (*Sturnidae*)** | European starling | *Sturnus vulgaris* |
| **Finches (*Fringilidae*)** | Green finch | *Carduelis chloris* |
| | European chaffinch | *Fringilla coelebs* |
| | Eurasian linnet | *Carduelis cannabina* |
| **Sparrows (*Passeridae*)** | House sparrow | *Passer domesticus* |
| | Tree sparrow | *Passer montanus* |
| **Chats (*Turdidae*)** | Redwing | *Turdus iliacus* |
| | Fieldfare | *Turdus pilaris* |
| **Larks (*Alaudidae*)** | Skylark | *alauda arvensis* |
| **Buntings (*Emberizidae*)** | Reed bunting | *Emberiza shoeniclus* |
| **Warblers (*Sylviidae*)** | Reed warbler | *Acrocephalus scirpaceus* |
| | Sedge warbler | *Acrocephalus schoenobaenus* |
| **Rails (*Rallidae)*** | Moorhen | *Gallinula chloropus* |
| **Swans, ducks and geese (*Anatidae*)** | Mallard | *Anas platyrhynchos* |
| **Wrens** (*Troglodytidae*) | Wren | *Troglodytes troglodytes* |
| **Owls (*Tytonidae*)** | Barn owl | *Tyto alba* |
| **Falcons and allies (*Falconidae*)** | Kestrel | *Falco tinnunculus* |
| **Gulls (*Laridae*)** | Black-headed gull | *Larus ridibundus* |
| | Herring gull | *Larus argentatus* |

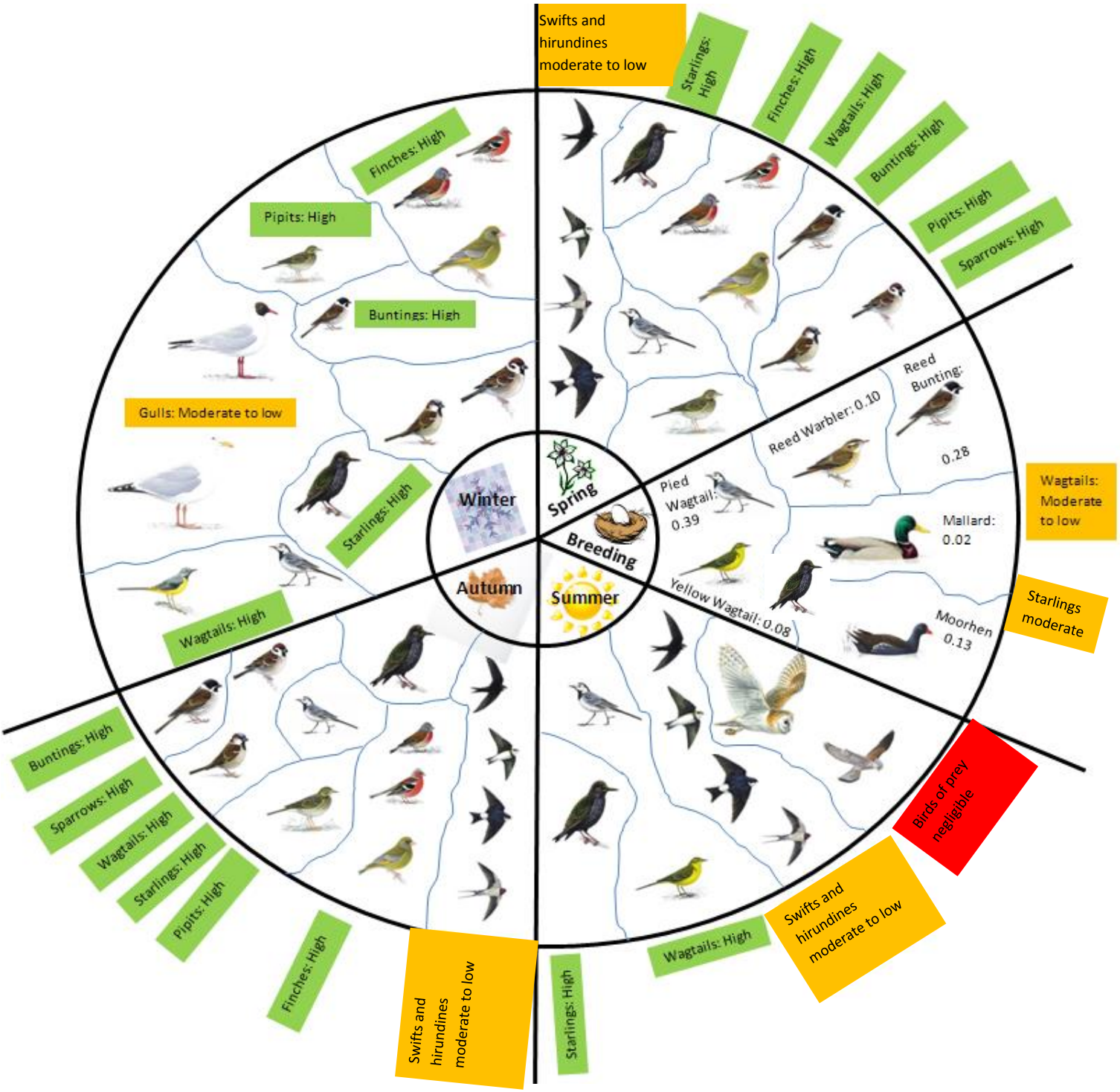



**Figure 1.4: The importance of trickling filters to species of birds observed at WWTPs at different times of the year (Fuller and Glue 1980). The numbers presented alongside the illustrations in the breeding section are the average number of territories or pairs per hectare from four trickling filters (Aston Clinton surveyed in 1975; Budds Farm (1974-5); Epping (1975-6) and Letchworth (1968). The illustrations are taken from www.rspb.org.uk and the colours of the boxes represent the importance of food from WWTPs to that species or group, green = high, yellow = moderate to low and red = negligible.**

### 1.4.4 Other terrestrial taxa foraging on WWTPs

Park and Cristinacce (2006) identified that bats commonly feed on WWTPs but that the type of secondary treatment used significantly influenced the number of aerial insects emerging and subsequently the quality of the WWTP as a foraging habitat for bats too. *Pipistrellus* species were found to have similar foraging levels on trickling filters to adjacent good quality foraging habitats but foraging activity was significantly lower on WWTPs using activated sludge than the adjacent good quality foraging habitats. The foraging activity of *Pipistrellus* species was also significantly lower on activated sludge than on trickling filters. The current preference of UK wastewater treatment service providers to use activated sludge for secondary (biological) treatment is reducing the value of WWTPs as foraging sites for bats. The Park and Cristinacce (2006) study is the only known work on bats foraging on sewage works, although it is likely that other species of small mammals will forage on trickling filters, there is currently a lack of published data in this area.

## 1.5 Effects of pharmaceuticals in wildlife species

### 1.5.1 Ethinylestradiol and the feminisation of male fish

There are only a few examples where pharmaceuticals in the environment have been clearly linked to adverse effects in free-living animals. Perhaps the most well-known case is an aquatic example; the feminisation of male fish. Male fish downstream of wastewater treatment plant (WWTP) effluent discharges containing the synthetic estrogen 17α-ethinylestradiol have been found to have elevated levels of vitellogenin and the presence of eggs in their testes (Brion et al. 2004; Desbrow et al. 1998; Gross-Sorokin et al. 2006; Kidd et al. 2007). 17α-ethinylestradiol is the active ingredient in the (human) female contraceptive pill. It is estimated that 16% of women in the UK are taking (and therefore excreting residues of) 17α-ethinylestradiol (Johnson et al. 2000). At present, there is no legislation in place to limit the levels of 17α-ethinylestradiol in surface wasters, although the EU proposed a level of 0.035 ng $L^{-1}$ (Gilbert 2012). It is estimated that it would cost the UK water companies £26-30 billion to clean-up wastewater to the 0.035 ng $L^{-1}$ target level (Gilbert 2012).

Kidd et al. (2014) recently reported findings on the direct and indirect effects of 17α-ethinylestradiol on a freshwater food web from a seven year study in one of the Canadian experimental lakes. At concentrations as low as 5-6 ng $L^{-1}$ direct effects were observed on fathead minnow (decreased recruitment), in the body condition of male lake trout (*Salvelinus namaycush*) and white sucker (*Catostomus commersonii*) of both sexes. No effects were observed in the algal, zooplankton, microbial or invertebrate community. However, after two to three years, the decline in small bodied fish resulted in a major increase in zooplankton and emerging insects while the biomass of top

predatory species of fish declined by 23-42%. This experimental study demonstrates that food-web interactions mean a species does not have to be directly affected by pharmaceuticals to suffer declines at the population level.

### 1.5.2 Diclofenac and the Asian (*gyps*) vulture crisis

In the terrestrial environment, the Indian and Pakistani vulture crisis is the most notable example of pharmaceuticals being directly related to an adverse effect in wildlife. The populations of three species of vulture, the oriental white backed vulture (*Gyps bengalensis*), the slender billed vulture (*Gyps tenuirostris*) and long-billed vultures (*Gyps indicus*) declined by over 95% from the early 1990s to the early 2000s (Oaks et al. 2004; Shultz et al. 2004; Swan et al. 2006a; 2006b). It has been demonstrated that livestock treated with the Non-steroidal anti-inflammatory drug (NSAID) diclofenac to treat symptoms of fever, inflammation or pain associated with disease, was the cause of the drastic population declines (Oaks et al. 2004). Vultures were exposed to diclofenac when they ingested meat from the carcasses of diclofenac-treated livestock fatalities that were left out in the fields. Diclofenac residues remain if the drug was administered to the livestock shortly prior to death. Vultures do not possess the necessary enzymes to detoxify diclofenac (Swan et al. 2006a) and subsequently they bioaccumulate diclofenac in the kidney where it has been shown to cause renal damage, elevated concentrations of uric acid in serum, visceral gout and eventually lead to renal failure and death. The use of diclofenac in veterinary medicine was banned by the Indian Government in 2006 (Cuthbert el al. 2014). Meloxicam has been advocated as a safe alternative; however, despite the ban on diclofenac, analysis of residues in the livers of vulture carcasses collected in 2009 found that meloxicam usage had only replaced diclofenac by around 50% based on 2004 levels (Cuthbert et al. 2014). The damage at the population level on *Gyps* vulture populations has already been done. The goal now is to reduce the use of diclofenac further by improved implementation of the diclofenac ban (Cuthbert et al. 2014).

The decline in vulture populations in India has led to an increase in the population of stray dogs, as they have come to occupy the position in the food web vacated by the vultures (Markandya et al. 2008; Sharp 2006). Just as Kidd et al. (2014) demonstrated the potential for pharmaceuticals to cause alterations to food web dynamics in the aquatic environment, the increased incidence of rabies cases in humans in India from dog bites demonstrates the potential for pharmaceuticals in the environment to have negative indirect effects.

Recently diclofenac has been licenced for veterinary use in Europe which is potentially a concern for species of scavengers EMEA (2014). Interestingly, a laboratory study found diclofenac not to be toxic to turkey vultures (*Cathartes aura*) (Rattner et al. 2008) when experimentally administered

with a dose that was up to two orders of magnitude higher than the lethal oral dose for the *Gyps* vultures. Similar results were found in the pied-crow (*Corvus albus*) at environmentally relevant concentrations (Naidoo et al. 2011). Even though these two species have not been found to suffer adverse effects from diclofenac, assessing the toxicity of diclofenac (and other non-steroidal anti-inflammatories) to other species of scavenging and predatory birds remains an important area for future research.

### 1.5.3 Other examples of pharmaceuticals adversely impacting upon wildlife

The toxicity of other NSAIDs was demonstrated by a recent forensic examination of a wild Eurasian Griffon Vulture (*Gyps fulvus*) carcass by Zorrilla *et al.* (2014). The bird was found to have severe visceral gout which is believed to have been caused by the high concentrations of the NSAID Flunixin and is believed to be the first example of a NSAID killing a vulture outside of Asia.

There have been other episodes of pharmaceutical poisonings in the USA and Canada in recent decades. Langelier (1993), Thomas (1999) and the US Fisheries and Wildlife Service (USFWS No date) have found that avian predators and scavengers (bald eagle, golden eagle, raven and magpie) have suffered mortalities as a result of ingesting carcasses from livestock and domestic animals that had been euthanized with sodium pentobarbital. The carcasses were either left out on the field, or inappropriately buried/dumped to landfill, leading to exposure of predatory and scavenging species of birds leading to acute intoxication and comatose.

In terms of exposure pathways to pharmaceuticals for species of bird other than avian scavengers are exposed, recent work in the USA detected the calcium channel blocker diltiazem at all levels of a water-fish-osprey food web but did not measure effects (Lazarus et al. 2014). The aim of this thesis is to identify a pharmaceutical that could cause adverse effects in wild-birds in the UK. As there are no species of vulture in the UK and livestock carcasses are not left in fields for avian scavengers, NSAIDs and avian scavengers are not necessarily going to be the top priority.

The likelihood that pharmaceuticals other than 17α-ethinylestradiol and NSAIDs could have adverse effects on wildlife is made all the more plausible by the number and variety of pharmaceutical compounds licensed for use, approximately 4,000 globally (3,000 in Europe) (Boxall et al. 2012; Ternes et al. 2009). Additionally, as pharmaceuticals are designed to affect specific protein targets at low doses and evolution has conserved human drug targets across animal taxa (Boxall 2004), it is possible for pharmaceuticals to affect wildlife through interactions with a specific conserved receptor rather than via a general toxic effect (Gunnarson et al. 2008), even at the low concentrations typical of environmental exposure.

## 1.6 Biomarkers used in Avian Ecotoxicology

Mortality and reproductive failure resulting from exposure to pharmaceuticals in the environment have already been observed in wildlife including birds e.g. (Gross-Sorokin et al. 2006; Oaks et al. 2004). Avian ecotoxicology studies use a range of biomarkers and diagnostic tools to assess severe toxicological effects of chemical contaminants in birds. The measurements taken are specific to the expected effects. For example, if endocrine disruption is expected then levels of circulating thyroid hormones may be measured (e.g. Cesh et al. 2010). If embryo-toxicity is expected, then eggshell injections can be used as a method to assess effects on survival, growth, reproduction, molecular, biochemical, and endocrine endpoints (Rattner et al. 2013) provides an efficient way to assess potential toxicity in birds. If immune function is expected to be impaired, then measures of oxidative stress (Rainio et al. 2013) or measures of cell mediated response, such as T-cell and B-cell activity may be taken (Smits and Nain 2013). If impacts on skeletal structure are expected, then a measurement of bone density or mineralisation can be used as a biomarker (Alvarez-Lloret et al. 2014). For a contaminant that may affect blood clotting and coagulation, such as a rodenticide, then measures of clotting time (e.g. Prothrombin or Russell's Viper venom time) are used (Rattner et al. 2014b). When toxicity is expected to result from failure to metabolise the contaminant, *ex-vivo* techniques, such as hepatocyte cultures, have been used to determine toxicity at the cellular level (Naidoo et al. 2011). Oxidative stress is a general response to many stressors, as is an up-regulation of liver function (e.g. Larcombe et al 2010; Arnold et al; 2010; Rodriguez-Estival et al. 2010). Clearly there are a wide range of techniques used in avian ecotoxicology to assess toxicity of chemical contaminants.

Severe effects on individuals that result in mortality and population declines as a result of pharmaceuticals in the environment are probably going to be rare. Beyond these severe endpoints, pharmaceuticals may act indirectly on fitness. There is increasing interest in how sub-lethal/subtle endpoints could impact on individual fitness and possibly alter population dynamics or even ecosystem functioning. For example, it has recently been demonstrated in the laboratory that 'environmentally relevant' concentrations of the anxiolytic drug, oxazepam, caused changes in activity, feeding rate and social behaviours of wild European Perch (*Perca fluviatilis*) (Brodin et al. 2013). Wild starling (*Sturnus vulgaris)* nestlings fed endocrine disrupting chemicals (EDC), including 17α-ethinylestradiol from the contraceptive pill, mimicking exposure via invertebrates from WWTPs, grew more slowly and showed poorer immune function than controls (Markman et al. 2011). Interestingly, basal stress hormone levels, an index of chronic stress, were unaffected by the treatment. Changes in behaviour or physiology could be just as important in terms of fitness, and

consequently population dynamics, as direct effects on mortality and reproduction (Gross-Sorokin et al. 2006; Oaks et al. 2004) also see Brodin et al. (2014).

Behavioural effects are notoriously difficult to measure in the field, not only must a dose response be measured but it must also be demonstrated that the behavioural effect could have significant consequences for the individual or population (Walker 2003). Measurement of behaviour in the field, for example using a remote electronic tagging system to record foraging behaviours, should be coupled with other biomarkers e.g. using non-invasive measures of exposure such as feather, fur or faeces to confirm the dose response relationship (Walker 2003).

In terms of the relevance of the behavioural biomarkers in the field, personality traits measured in captive passerines have been shown to be a good predictor of how an animal will behave in the wild (e.g. Herborn et al. 2010, Minderman et al. 2010). Chemically induced changes to animal behaviour can have important consequences at the individual, population and ecosystem level, for example changes to boldness can directly impact risk perception, which indirectly affects foraging and mass balance with consequences for predator avoidance and the ability to combat starvation risk (Brodin et al. 2014). Altered foraging behaviours can have indirect effects at other trophic levels through changes in food web dynamics which can disturb ecosystem functioning, (see Kidd et al. 2014) for an example from an aquatic system.

The biomarkers and tools used to assess effects are context dependent, and as most of the 'traditional' measures used in avian ecotoxicology are designed to investigate cases where acute toxicity is expected, their use in a sub-lethal context needs to be determined.

## 1.7 The selected pharmaceutical: Why fluoxetine?

The selective serotonin reuptake inhibitor antidepressant fluoxetine was selected as the case study compound. Fluoxetine is used in the treatment of anxiety related conditions such as depression. In addition to altering anxiety related behaviours the connectivity between the serotonergic system upon which fluoxetine acts and the neuroendocrine system through the hypothalamic-Pituitary-Adrenal (HPA) axis mean that a range of side effects are also observed e.g. fluoxetine is also known to reduce libido and cause changes to activity levels, sociality and appetite (Eli Lilly 2009). Fluoxetine has been detected at ng/g level in the tissues of fish collected from effluent dominated streams (Berninger and Brooks 2010; Brooks 2014; Brooks et al. 2005; Chu and Metcalfe 2007). A number of experiments have demonstrated effects on aquatic organisms at low concentrations typical of

environmental exposure (Bossus et al. 2014; Di Poi et al. 2013; Guler and Ford 2010; Mennigen et al. 2010; Weinberger and Klaper 2014) (Table 1.2). To date, no study has looked at the effects of an environmentally relevant concentration of fluoxetine in a terrestrial vertebrate (Table 1.2).

### 1.7.1 Uptake of fluoxetine into invertebrate prey

Several studies have detected fluoxetine and norfluoxetine in WWTP effluent and effluent dominated surface waters with concentrations up to the μg $L^{-1}$ (reviewed in Calisto and Esteves (2009) but also see (Kasprzyk-Hordern et al. 2009; Grabic et al. 2012; Monteiro and Boxall 2010) level in effluent and surface water. Although some studies have failed to detect it (Monteiro and Boxall 2010), so presumably levels are spatially very variable.

As a secondary amine, the uptake of fluoxetine is strongly affected by the pH of the environmental matrices close to its pKa of 10.06 (Brooks et al. 2003). At a basic pH, the Log Dow (Octanol water distribution coefficient) of fluoxetine reduces as it is increasingly present in the ionised form (AH+ is more easily dissolved in water than the non-ionised A species). Thus the accumulation and toxicity of amines such as fluoxetine varies considerably at pH values just below the pKa due to the reduction in hydrophobic non-ionised species with decreasing pH (Nakamura et al. 2008). For example, Paterson and Metcalfe (2008) found the bioconcentration factor (BCF) in Japanese medaka increases by an order of magnitude from pH 7 to pH 9 for both fluoxetine and norfluoxetine. Bringolf *et al.* (2010) have found Bioaccumulation factors (BAFs) in freshwater mussel to vary spatially, it is possible that this difference was due to differences in pH between sampling locations. Fluoxetine and norfluoxetine have been found to accumulate in the tissues of fish from effluent dominated surface waters up to the ng $g^{-1}$ level (Brooks et al. 2005; Chu and Metcalfe 2007) with BCFs of 74 and 117 established experimentally for parent compound and active metabolite respectively in Japanese medaka (*Oryzias latipes*).

It is clear that the extent to which fluoxetine accumulates in the tissues of fish and invertebrates is affected by environmental variables such as the pH of the water body. Sewage treatment works are typically in the region of pH 7-8 (Gray 1989); slight variations within this narrow range could lead to large differences in uptake into the invertebrates that attract foraging birds. Additionally, it is important to consider that uptake from aquatic systems by both bioconcentration and bioaccumulation. Recently Brodin *et al*. (2014) and Du *et al*. (2014) have demonstrated that bioaccumulation (via ingestion) is an important route by which pharmaceuticals are taken up by aquatic organisms in addition to bioconcentration (e.g. through the gills). Therefore, if estimates of uptake into aquatic vertebrates do not include bioaccumulation, then there may be an

underestimation. The best way to assess the accuracy of a predicted environmental concentration is to collect field data (see Chapter 2 and Appendix A1).

## 1.8 Priority Species: The European starling

To study the effects of fluoxetine in wild birds, a model organism needed to be selected. The species was chosen based on the following criteria: 1) the species must feed on the contaminated source throughout the year or intensively at one time of the year, 2) the species must be robust in captivity, 3) the species need to be of sufficient size for sampling a variety of matrices (e.g. faeces, blood or tissue) so that the limits of detection of analytical methods are exceeded, 4) the behaviour and or physiology of the species should be relatively well characterised.

Based on these criteria, the European starling (*Sturnus vulgaris)* was selected as the model species for this thesis. Starlings were ideal for this project because they are known to feed on WWTP trickling filters throughout the year (Fuller and Glue 1978; Gough et al. 2003), intensively so in winter and the breeding season (Markman et al. 2008; 2011) and the species are robust in captivity e.g. (Boogert et al. 2006; 2008). Adult starlings weigh over 70 g (Feare 1984), which was sufficient mass to allow a blood sample large enough for the analyses planned. Additionally, their behaviour (Boogert et al. 2008; Brilot et al. 2010; Minderman et al. 2009; 2010), physiology (Apfelbeck and Raess 2008; Cyr and Romero 2007; Romero and Remage-Healey 2000; Witter and Goldsmith 1997), diet (Feare and McGinnity 1986; Tinbergen 1981) and life history (Feare 1984) are all well characterised. Additionally, starlings have suffered declines of 84% since 1979 (RSPB 2014) resulting in them being placed on the UK red list as a species of high conservation concern (RSPB 2012; 2014), making population level effects a potential cause for conservation.

## 1.9 An Adverse Outcome Pathway for wild birds ingesting fluoxetine contaminated invertebrates

Having identified that there is a potential pathway for fluoxetine to be taken up by birds and that the levels of uptake could be sufficient to cause effects, I decided to present the existing pharmacology and toxicology information for fluoxetine as an Adverse Outcome Pathway (AOP) for wild birds (following the approach of Ankley et al. 2010). An AOP is used to relate the molecular interaction of the pharmaceutical through various levels of biological organisation that are relevant to risk assessment (Ankley et al. 2010; Rattner et al. 2014a). An AOP contains six levels of biological organisation: The toxicant initially induces a response at the macromolecular level. The macromolecular response is followed by cellular, tissue/whole organ, whole organism and eventually population level responses. AOPs are becoming increasingly accepted as an approach

that can extend mammalian toxicology data to an ecological application such as wildlife risk assessment (Caldwell et al. 2014). In the AOP for fluoxetine and wild-birds presented in Figure 1.5, the conceptual framework is used in order to justify the experiments presented in Chapters 2-5.

### 1.9.1 Toxicant

In this AOP, the toxicant is fluoxetine (Figure 1.5). Fluoxetine is an antidepressant drug in the selective serotonin re-uptake inhibitor category. Fluoxetine is a racemic mixture of R-fluoxetine and S-fluoxetine. S-fluoxetine is eliminated more slowly than R-fluoxetine and so is the dominant enantiomer present in plasma at a steady state (Lesch et al. 1991).

### 1.9.2 Macro-molecular level responses

Fluoxetine prevents the reuptake of the neurotransmitter serotonin (associated with feelings of wellbeing) by the pre-synaptic cell in the brain thus increasing the neurotransmission of serotonin (Lesch et al. 1991). Fluoxetine works by immediately desensitizing the autoreceptors in the pre-synaptic cell (Newman et al. 2004) and forming a blockade to prevent serotonin re-uptake resulting in elevated levels of serotonin in the synapse. Fluoxetine also increases affinity for binding on the post-synaptic autoreceptor (Lesch et al. 1991). The increased functional serotonin in the synapse (Figure 1.5) sets in motion a series of neuroadaptive mechanisms. The abundance of serotonin in the synapse over activates the post-synaptic serotonin receptors, which work harder to remove the extra serotonin in the synapse, leading to eventual desensitization of the post synaptic cell serotonin receptors. The mammalian serotonin system, upon which fluoxetine acts to initiate therapeutic effects on behaviour and physiology (Lesch et al. 1991), is well conserved in birds (Challet et al. 1996). Additionally Gunnarsson et al. (2008) found 70% similarity between the orthologs of chickens (*Gallus gallus*) and the orthologs of human drug targets. As the read-across hypothesis is based on the assumption that drugs will have effects in non-target organisms only if molecular targets such as receptors and enzymes have been conserved a comparison of the pharmacological and toxicological effects between mammals and birds is valid (Huggett et al. 2003; Rand-Weaver et al. 2013).

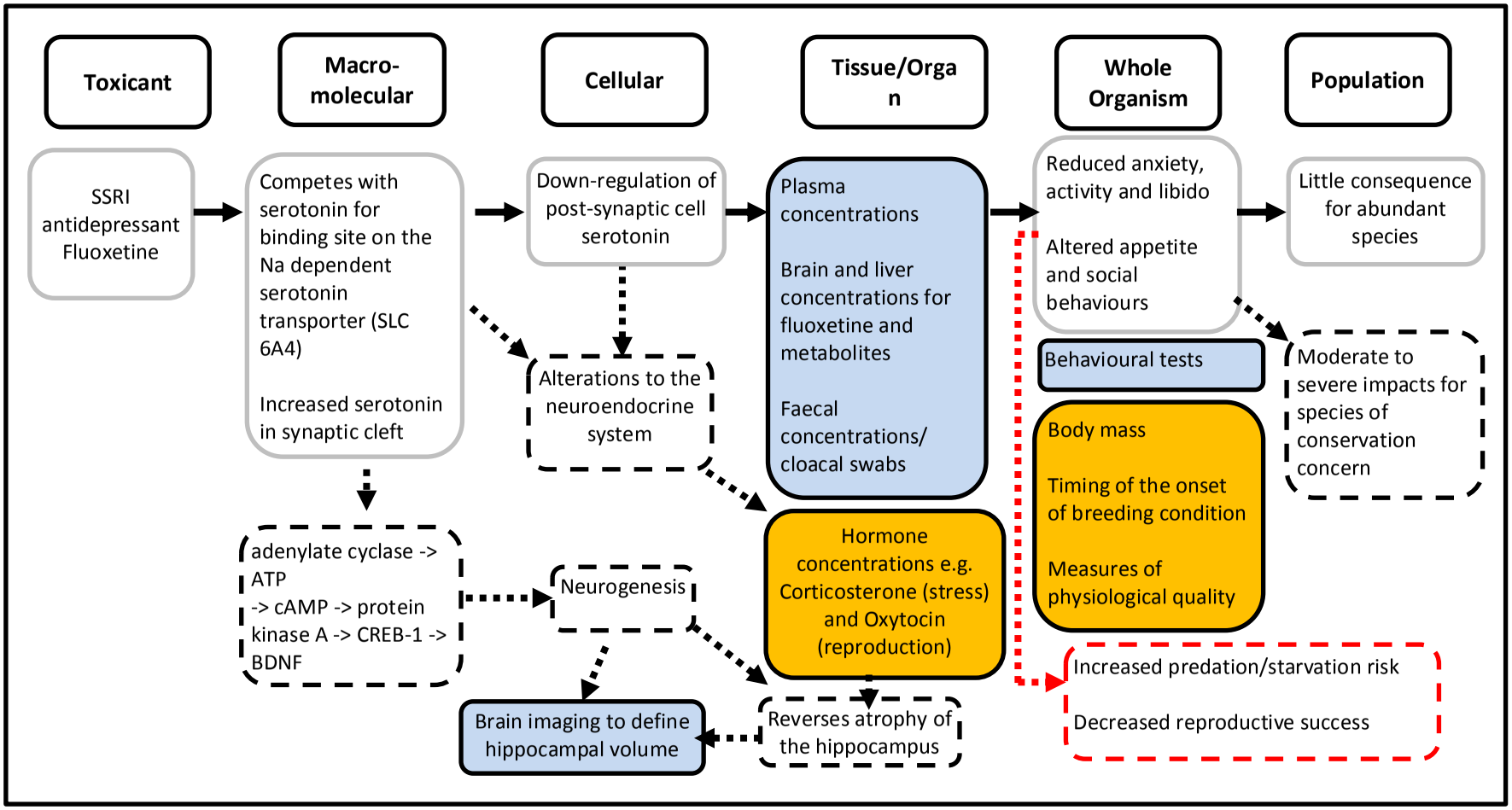



**Figure 1.5: Adverse Outcome Pathway (AOP) for fluoxetine in wild birds. The established, plausible and hypothetical linkages between a molecular interaction and effects at various levels of biological organisation are outlined along with diagnostic tools and biomarkers for these effects.**

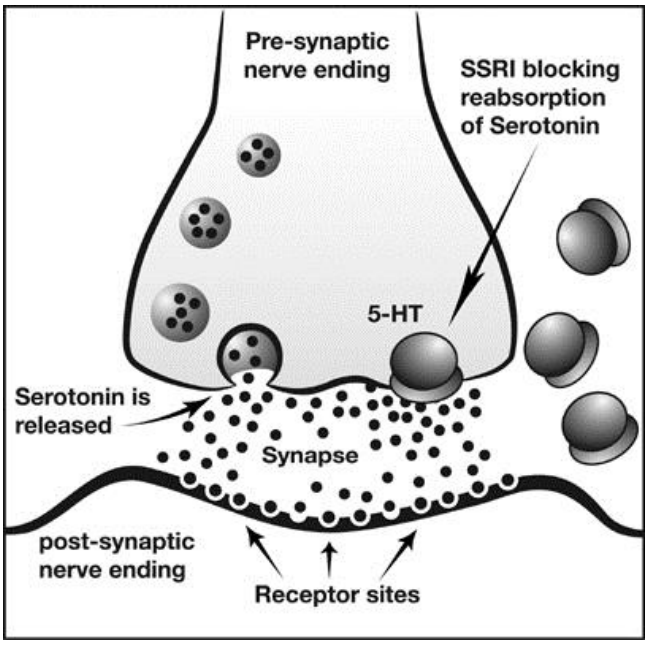



**Figure 1.6: Fluoxetine (large grey circles) acting as a selective serotonin reuptake inhibitor (SSRI) by blockading reabsorption at the pre-synaptic cell causing the serotonin concentration (small black circles) in the synapse to increase. Over time, neurotransmission from the pre-synaptic nerve ending, across the synapse to the post-synaptic cell becomes more efficient in response to treatment with fluoxetine (Lattimore et al. 2005).**

### 1.9.3 Cellular level response

The over activation of the post-synaptic cell receptors leads to decreased release of serotonin from the pre-synaptic cell, with subsequent down regulation of the post synaptic cell serotonin receptors (Serres et al. 2000) (see Figure 1.6). Down regulation is when a cell decreases the quantity of a cellular component, e.g. Ribonucleic acid (RNA) or a protein, in response to an external variable (in this case a pharmaceutical). Axons from the raphe nuclei form a neurotransmitter system that connects to nearly all areas of the brain. Neurons in the hypothalamus trigger the release of a variety of hormones. These neuro-adaptations take weeks rather than days to take effect (Lesch et al. 1991), and thus the therapeutic effects take several weeks to occur and can persist for an extended period after treatment is ended.

Increased serotonin neuro-transmission can lead to long-term neuro-modulating effects that are believed to be achieved through increased neurogenesis (Drugbank 2009). Neurogenesis results from serotonin binding to certain serotonin receptors which then sets in motion a cascade of reactions starting with the activation of the enzyme adenylate cyclase. Adenylate cyclase catalyses the conversion of Adenosine Triphosphate to Cyclic adeno-monophosphate (cAMP) which activates protein kinase A. Protein kinase A activates cAMP response binding protein-1 (CREB-1) which can

enter the cell nucleus and alter the transcription of Brain-derived Neurotrophic Factor (BDNF). BDNF is known to stimulate neurogenesis (Drugbank 2009).

### 1.9.4 Tissue/Organ level response

In humans and model mammals, following oral administration, absorption from the gastro-intestinal tract is slow with peak plasma concentrations not reached until after 4-8 hours (Hiemke and Hartter 2000). The presence of food in the gastrointestinal tract delays absorption but does not reduce the bioavailability of fluoxetine (Altamura et al. 1994). Once in the blood stream, the target organ is the brain. In addition to the down-regulation of post-synaptic serotonin receptors, there is some evidence that fluoxetine can cause neurogenesis (Warner-Schmidt and Durman 2006).

Warner-Schmidt and Durman (2006) have shown that stress hormones (Corticosterone) in rats cause a reduction in the expression of BDNF which leads to atrophy of the hippocampus. A similar atrophy of the hippocampus and other limbic structures of the brain has also been found to occur in chronically stressed humans. Treatment with antidepressants, including fluoxetine, has been shown to increase the expression of BDNF and prevent or even reverse the atrophy of the hippocampus (Drzyzga et al. 2009). Therefore increase in the expression of BDNF and the subsequent neurogenesis is associated with the long term recovery from depression which can outlast the treatment with antidepressants (Brunswick et al. 2002). Fluoxetine has the ability to set in motion a cascade of neuroadaptative measures, which can ultimately result in significant changes to behaviour and physiology. This is believed to result from the connectivity between the serotonin system, upon which fluoxetine acts, the hypothalamus and the neuroendocrine system, which is responsible for regulating body functions (Raap and Van de Kar 1999); see Figure 1.7). For example, serotonin receptors located in the raphe nuclei send out collaterals to the hypothalamus. Neurons in the hypothalamus control the release of a variety of precursor hormones that trigger the glands, e.g. adrenal and pituitary glands to release hormones that can alter behaviour and physiology (Raap and Van de Kar 1999). Therefore, small changes in the levels of serotonin in one part of the brain can have significant consequences for behaviours and physiology unrelated to the therapeutic action of the drug. This connectivity makes the effects of environmental uptake of fluoxetine particularly interesting to ecologists. Prolonged exposure to a low dose will give the possibility for neurological adaptations to take place in a way that short-term acute exposure would not (Lesch et al. 1991). As a pharmaceutical, fluoxetine is designed to act upon a specific drug target rather than have a toxic effect. Therefore, other than preventing/reversing atrophy of the hippocampus, the most relevant data at the tissue/organ level to the AOP are those on the accumulation, distribution, metabolism and elimination (ADME).

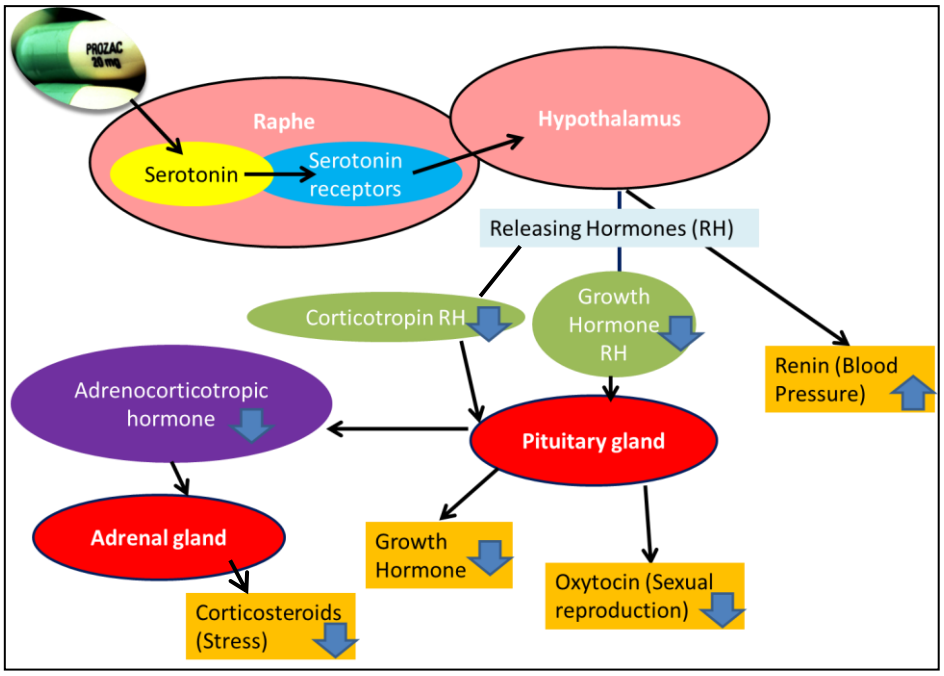



**Figure 1.7: The connection between the serotonin system upon which fluoxetine acts and the neuroendocrine system. In the brain (pink ovals), fluoxetine causes changes to levels of serotonin (yellow oval), which in turn alters the number and functioning of serotonin receptors (blue ovals). The serotonin receptors send collaterals to the hypothalamus. In the hypothalamus, hypothalamic neurons trigger the release of a variety of pre-cursor hormones (green ovals) which triggers the glands (red ovals) to release more pre-cursor hormones (purple oval), which then stimulates the adrenal gland to release hormones (yellow boxes) that cause alterations to behaviour and physiology. The blue arrows represent whether it is believed that fluoxetine acts to increase or decrease the levels of the hormone in the yellow box. This figure has been created based on the information presented in Raap and Van de Kar (1999).**

In the liver, fluoxetine undergoes first pass hepatic metabolism, subsequently oral bioavailability is less than 90% (Catterson and Preskorn 1996), with some sources quoting 60-80% (McEvoy 2003). Fluoxetine is metabolised to its active metabolite by N-demethylation (Hiemke and Hartter 2000). Norfluoxetine has been shown to be at least as potent as the parent compound (Fuller and Perry 1992; Fuller et al. 1992). Cytochrome (CYPs) 450 (CYP 450) are a superfamily of proteins involved in the main enzymatic reactions that metabolise pharmaceuticals (Watanabe et al. 2013). It has been shown *in-vivo* that CYP2D6 is involved in N-demethylation (Dominguez et al. 1996) (the metabolism of fluoxetine is shown in Figure 1.8). However the other enzymes involved in more than 70% of the biotransformation have not yet been characterised. *In-vitro* studies have suggested that CYP2C9 plays more of a pivotal role than CYP2D6 in the N-demethylation of fluoxetine with CYP2C19 and CYP3A4 and 5 having minor roles. Birds have been found not to possess CYP2D6 or CYP3A4 and CYP2C9 and CYP2C19 are located on a different part of the genome to humans (Watanabe et al. 2013). All of which points to different metabolism of fluoxetine in birds compared with humans. A recent study by Hutchinson et al. (2014) has highlighted the importance of comparative metabolism between species when reading-across effects between species.

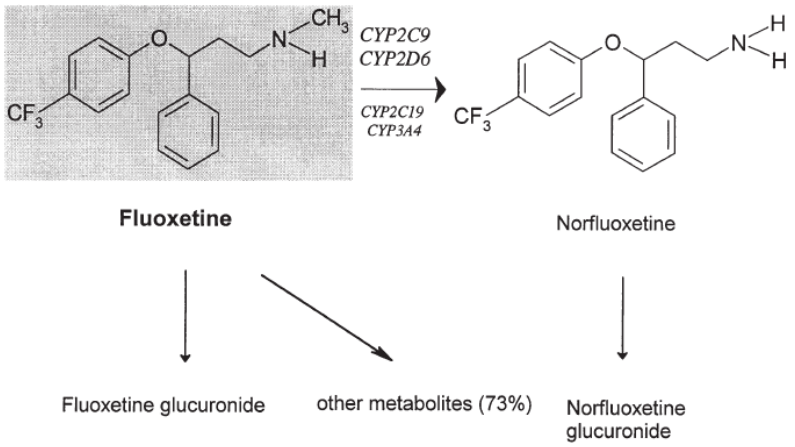



**Figure 1.8: The metabolism of fluoxetine in the human system and the CYP isoenzymes, amine oxidase, and N-acetyltransferase suggested to catalyse the Phase I reactions that result in the production of fluoxetine's metabolites (Hiemke and Hartter 2000).**

Both fluoxetine and norfluoxetine have large volumes of distribution, quoted as 14 – 100 L $Kg^{-1}$ in (Hiemke and Hartter 2000) and 20 – 42 L $kg^{-1}$ in (Lewis et al. 2007) meaning that fluoxetine is more likely to accumulate in tissues than plasma. In post-mortem analysis of human tissues both fluoxetine and norfluoxetine have been found to accumulate in tissues, particularly the lung (Fluoxetine: 60 and norfluoxetine 59 times higher than plasma), liver (38 and 42 times) and brain (15 and 18) (Lewis et al. 2007). The relatively low level distribution of fluoxetine into blood plasma presents challenges in terms of sampling live birds. For example, if concentrations in bird tissues are at ng/g levels as expected, and plasma concentrations are an order of magnitude lower than this, it is likely to be difficult to detect fluoxetine in plasma. However, it remains uncertain whether distribution of fluoxetine for birds will be similar to that in humans, which will be assessed in Chapter 2.

Fluoxetine is metabolised to its active metabolite norfluoxetine in the liver (Hiemke and Hartter 2000). In humans fluoxetine has a plasma half-life ($t_{1/2}$) of 1 – 4 days, in rats 5 hours (Caccia et al. 1990) and in mice 12.9 hours (both rats and mice are plasma half-lives). For its active metabolite, plasma half-lives for norfluoxetine are 7 - 15 days in humans (Hiemke and Hartter 2000) and 15 hours in rats (Caccia et al 1990). In humans excretion rates of unchanged parent compound are quoted at anything between 5 and 24% (Hiemke and Hartter 2000; Jjemba 2006; Lienert et al. 2007) with 20% as norfluoxetine and 20% each of fluoxetine and norfluoxetine glucuronide in humans (Lienert et al. 2007).

About 7% of the human population have reduced activity of the drug metabolising enzyme cytochrome CYP2D6 meaning that S-fluoxetine reaches a higher blood plasma concentration at steady state and S-norfluoxetine is lower (Hiemke and Hartter 2000). R-fluoxetine and R-norfluoxetine concentrations are normal in poor metabolisers. Fluoxetine achieves a steady state in plasma after between 1 and 22 months and does not increase without limit (Hiemke and Hartter 2000). As CYP2D6, is also involved in the metabolism of other pharmaceuticals, there is the possibility for drug interactions (Altamura et al. 1994), which is important to remember as fluoxetine is one of many pharmaceuticals to be detected in the environment e.g. (Grabic et al. 2012; McClellan and Halden 2010). Therefore, although there is accumulation, distribution, metabolism and elimination data for humans (and some model mammals see Table 2.2 in Chapter 2), nothing is known at present about ADME of fluoxetine in wildlife species particularly birds.

### 1.9.5 Organism level response

As an antidepressant fluoxetine is designed to modify behaviour at concentrations well below traditional ecotoxicological endpoints (e.g. mortality and growth inhibition). In humans the behaviours affected are social and anxiety related, with side effects on activity, as well as effects on sexual and feeding behaviours (e.g. reducing compulsive eating) (Eli Lilly 2009). Measuring mode of action related endpoints provides a natural starting point for investigating organism level effects in wild birds.

In rats and mice the Lethal Oral Dose for 50% ($LOD_{50}$) of animals in the trial was established at 452 mg $kg^{-1}$ for rats and 248 mg $kg^{-1}$ for mice (McEvoy 2003). These $LOD_{50}$ values are high given the $LOD_{50}$ of diclofenac to vultures was only 0.1-0.2 mg $kg^{-1}$ (Swan et al. 2006a). In Chapter 2, I predicted (from information on the usage of fluoxetine by the population, fluoxetine's pharmacokinetics and its fate in the environment) and evaluated (in a field monitoring study) as environmentally relevant a daily dose of 0.019 mg $kg^{-1}$ $day^{-1}$ fluoxetine for wild birds in the UK foraging on trickling filter invertebrates. This environmentally relevant dose of fluoxetine for birds in the UK is 2 orders of magnitude lower than has previously been used in studies on birds (Sperry et al. 2003). Table 1.2 summarises some of the endpoints which treatment with fluoxetine has affected in a variety of terrestrial and aquatic organisms. The key point that the data in Table 1.2 highlight are that aquatic studies are more abundant than terrestrial (by a ratio of greater than 4:1). A second point from Table 1.2 is that overall only around one third of the studies used an environmentally relevant concentration and none of these studies were on a terrestrial species. Additionally, half of the studies on terrestrial species administered the dose by injection, meaning that even if the dosing level used had been

environmentally relevant, the uptake route used would not have been and would have led to higher bioavailability, than if ingestion (typical of environmental uptake for a terrestrial species) had been used. Therefore, there is a major knowledge gap regarding the effects of environmentally relevant concentrations on terrestrial vertebrates.

As fluoxetine is designed to modify behaviour, it is possible for apparently subtle, but nonetheless, adverse effects to be caused in non-target species without causing mortality. Fluoxetine is commonly used to treat anxiety related conditions such as depression, obsessive compulsive disorder and bulimia (Eli Lilly 2009) but response is fairly variable with the percentage of patients showing improvements in symptoms of depression ranging from 32 to 72% in different studies (Cook et al. 1999). Concentrations in plasma have been found to be unrelated to whether or not treatment will be effective, and neuroimaging techniques to assess whether a patient possesses certain neurophysiological features have been found to be a better predictor of whether a patient with depression will respond to fluoxetine treatment (Cook et al. 1999). Additionally, side effects such as raised sexual dysfunction (Clayton 2002; Matuszczyk et al. 1998; Shen and Hsu 1995; Uphouse et al. 2006), increased lethargy (Henry and Black 2008; Eli Lilly 2009) and changes to appetite are common, most likely due to the connectivity between the serotonergic system and the neuroendocrine system (Raap and Van de Kar 1999).

Behavioural endpoints are particularly of interest when measuring an antidepressant which is primarily designed to modulate behaviour. Measuring behavioural endpoints in animals has the advantage that they can be measured non-invasively, as highlighted by a number of studies (Clotfelter et al. 2004; Scott and Sloman 2004; Zala and Penn 2004). Behaviour can also be an indicator of higher level physiological or neurological differences between individuals e.g. (Verbeek et al. 1994). Indeed, reflecting such differences in physiology and behaviour, individuals can show consistent responses to stressors (intra-individual consistency) which differ from other individuals of the same species (Bell and Sih 2007). As in humans, wild animals can be considered to possess a suite of personality traits which are consistent responses to a stimulus over time and between contexts (Gosling 2001; Sih et al. 2004). Animals tend to possess a suite of personality traits including boldness/shyness, fast/slow exploration and aggressive/submissive (Bell and Sih 2007; Gosling 2001; Gosling et al. 2003; Sih and Bell 2007; Sih et al. 2004); personality traits can be used to predict how an animal will respond to environmental and social stressors (Herborn et al. 2011). If a pharmaceutical affects the neuroendocrine or serotonergic system, then it is likely to affect behaviour and perhaps personality. A further advantage of using behavioural measures is that it has been shown that personality of birds in captivity reflects personality in the wild (Herborn et al. 2010).

Fluoxetine could alter personality traits of birds, as has been demonstrated in laboratory conditions for aquatic species and shown in humans e.g. (Weinberger and Klaper 2014).

Fluoxetine can also act directly and indirectly to alter physiological endpoints such as body mass, morphological measurements and concentrations of hormones such as Corticosterone (Uphouse et al. 2006) and oxytocin (Cantor et al 1999). These physiological measures can be assayed using non-invasively/sub-lethal approaches and integrated with the behavioural measures (Roberts et al. 2007a; 2007b).

There are disadvantages of using non-invasive/sub-lethal endpoints such as behaviour to assess effects. There is a need for standardisation of behavioural assays (Boxall et al. 2012). Such standardisation would be challenging as tests would need to reflect the mode of action of the pharmaceutical being measured (Boxall et al. 2012) while the large number of different therapeutic classes makes such standardisation impractical. Although the same could be argued for more standard ecotoxicological endpoints, for example using growth as an endpoint: animals fail to grow for many reasons that could be linked to different MOAs. Issues with repeatability are challenging when using behavioural biomarkers, for example, there can be high inter and intra variability in responses in the way that individuals habituate to the test set-up over repeated tests (Saaristo et al. 2010). These issues with non-invasive and sub-lethal endpoints further complicate matters when trying to obtain a repeatable measure (Saaristo et al. 2010). Finally interpreting the relevance of data obtained in captivity to free-living animals (Zala and Penn 2004) can be challenging. However, Herborn et al. (2010) and Minderman et al. (2009; 2010) have shown how behaviour measured in captivity might translate to the natural environment. Moreover, the key point is that the ecological relevance of many standard ecotoxicology tests is yet to be proven.

### 1.9.6 Population level effects

Although individual level effects may be of concern (Figure 1.5), the highest level of biological organisation, and most relevant in terms of ecology for directing policies regarding the management of natural resources, are population level effects (Rattner et al. 2014a). However, in addition to the challenge in translating effects measured in captivity to free-living individuals, it is also challenging to interpret how changes at the organism level translate to the population level (Krebs and Davies 1993; Zala and Penn 2004). To some extent, the significance of population level effects is not only dependent on the severity of the individual level effects, but how robust the population is to changes in population dynamics (i.e. small populations that are widely dispersed are less resilient to

environmental perturbations). For example, in a species of special conservation concern (i.e. endangered or threatened) organism level effects may have consequences for the population as a small decline in their numbers is of greater significance than they are for an abundant or widespread species. Effects at the population level are made more likely if contamination is both high and widespread (Rattner et al. 2014a).

Given the low lethal toxicity of fluoxetine to rats and mice (McEvoy 2003), it is highly unlikely that fluoxetine will have a direct toxic effect in wild birds, e.g. mass mortalities and population declines as resulted from diclofenac in Asian vulture populations since the 1990s e.g. (Cuthbert et al. 2014). However, if fluoxetine has mode of action (MOA) related effects in wild-birds such as reduced responsiveness to stressors, activity levels or a change in foraging behaviours, then birds are likely to become more vulnerable to predation. A reduction in libido is likely to have negative implications in terms of breeding success. These potential effects are made all the more significant given the recent declines in UK garden bird populations. The model species, the starling, has suffered declines of 84% according to the RSPB's Big Garden Bird Watch since the survey started in 1979 (RSPB 2014). This trend of decline is common to many other with 26 species suffering declines of over 50% in the last 31-44 years (Baillie et al. 2014).

### 1.9.7 Conclusions

It is unlikely that fluoxetine alone has caused these drastic population declines in UK wild bird populations; but in a changing environment where there are multiple pressures such as habitat loss, climate change and pollution of the natural environment presents a stressor to wild-bird populations that remains largely unquantified. This thesis presents the first research into the effects of an antidepressant in a terrestrial vertebrate at an environmentally relevant concentration. The first research Chapter presents information on the levels of fluoxetine starlings are expected to be exposed to and the internal concentrations that could result from prolonged exposure at this level.

**Table 1.2: Endpoints affected by treatment with fluoxetine in studies presented in the literature. The species, duration of exposure, effect concentration and an indication as to whether the concentration used was environmentally relevant are also given along with the source of the data. Concentrations of up to 540 ng/L (Brooks et al. 2003) are considered environmentally relevant for aquatic exposure and ng/kg body weight up to low µg/kg body weight is considered likely to be environmentally relevant for an oral dose for terrestrial species based on the monitoring data presented in Chapter 2.**

| Endpoint | Species | Effect Concentration | Exposure period | Env. relevant | Reference |
|---|---|---|---|---|---|
| -Growth | Algae (*Pseudokirchneriella subcaptata*) | $EC_{50}$ = 24 µg $L^{-1}$<br>NOEC = 13.6 µg $L^{-1}$ | Not given | No | (1) |
| -Mortality | Water flea (*Ceriodaphnia dubia*) | $EC_{50}$ = 234 µg $L^{-1}$<br>NOEC = 112 µg $L^{-1}$ | 48 h | No | |
| -Mortality | Water flea (*Daphnia magna*) | $EC_{50}$ = 820 µg $L^{-1}$<br>NOEC = Not available | 48 h | No | |
| -Mortality | Fathead minnow (*Pimephales promelas*) | $EC_{50}$ = 705 µg $L^{-1}$<br>NOEC = Not available | 48 h | No | |
| -Survival/Growth | Crustacean (*Hyalella Azteca*) | $EC_{50}$ > 43 mg $kg^{-1}$<br>NOEC = 5.4 mg $kg^{-1}$ | 10 d | No | |
| -Survival/Growth | Midge (*Chirnomus tentans*) | $EC_{50}$ = 15.2 mg $kg^{-1}$<br>NOEC = 1.3 mg $kg^{-1}$ | 10 d | No | |
| - Phototaxis<br>- Geotaxis | Marine Arthropod (*Echinogammarus marinus*) | 100 ng $L^{-1}$ | 21 d | Yes | (2) |
| - Swimming velocity | *E. marinus* | 1 ng $L^{-1}$ | 1 d | Yes | (3) |
| -Mating behaviour<br>-Predator avoidance<br>-Feeding | Fathead minnow (*Pimephales promelas*) | 1 µg $L^{-1}$<br>1 µg $L^{-1}$<br><br>10 µg $L^{-1}$ | 28 d | No, 2 x env. relevant conc.<br><br>No | (4) |
| -Reduced activity | *Gammarus pulex* | 10-100 ng $L^{-1}$ | 2 h | Yes | (5) |
| -Anorectic effects<br>-Glucose metabolism | Goldfish (*Carassius auratus*) | 54 µg $L^{-1}$<br>540 ng $L^{-1}$ | 28 d | No<br>Yes | (6) |
| -Death<br>-Survival, growth and sex ratio (no effect)<br>-Lethargy<br><br>-Sexual development | Western mosquitofish | 546 µg $L^{-1}$<br>5 µg $L^{-1}$<br><br><br>0.5 µg $L^{-1}$<br>71 µg $L^{-1}$ | 7d<br>91d<br><br><br>91d<br>159d | No<br>No<br><br><br>Yes<br>No | (7) |

| | | | | | |
|---|---|---|---|---|---|
| -Learning and retention<br>-Impaired acquisition capability<br>-Deficit in memory retention | Cuttlefish (*Sepia officinalis*) | 1 ng $L^{-1}$<br><br>1 ng $L^{-1}$<br>10 ng $L^{-1}$ | 15 days perinatal<br>1 month<br>1 month | Yes<br><br>Yes<br>Yes | (8) |
| -Autism like gene expression | Fathead minnow (*Pimephales promelas*) | 10 µg $L^{-1}$ | 18d | No | (9) |
| -Endocrine disrupting effects, not an oxidative stress or neurotoxic inducer | Mussel (*Mytilus galloprincialis*) | 75 ng $L^{-1}$ | 14d | Yes | (10) |
| -Reduced brain serotonin activity<br>-Increased time to capture prey | Hybrid striped bass (*Morone saxatilis×M. chrysops*). | 23.2 µg $L^{-1}$ | 6d | No | (11) |
| -Induced vitellogenin in males | Fathead minnow (*Pimephales promelas*) | 28 ng $L^{-1}$ | 21d | Yes | (12) |
| -No effect on locomotor activity | Roughskin newt (*Taricha granulosa*) | 10 and 100 ng | single intravenous dose | No | (13) |
| -Female paturation of non-visible larvae<br>-Male reproduction<br>-Induced release of spermatozeugmata | Mussel (*Ellipto complanata*) | 300 µg $L^{-1}$<br><br>3000 µg $L^{-1}$<br>3000 µg $L^{-1}$ | 96h<br><br>48h<br>48h | No | (14) |
| -Death ($LC_{50}$) | Japanese Medaka (*Oryzias latipes*) | 5.5 mg $L^{-1}$ at pH 7<br>1.3 mg $L^{-1}$ at pH 8<br>0.2 mg $L^{-1}$ at pH 9 | 96h | No<br>No<br>Yes but pH outside env. range | (15) |
| -Feeding rate ($EC_{10}$) | Fathead minnow (*Pimephales promelas*) | R-fluoxetine 16.1 µg $L^{-1}$<br>S-fluoxetine 3.7 µg $L^{-1}$ | 48h | No<br>No | (16) |
| -Tissue concentrations (ng $g^{-1}$) | Fish *Lepoinis macrochirus,Ictalurus punctatus, Cypirius carpio, Pomoxis nigromaculatus* | Environmental | In the wild | Yes | (17 and 18) |
| | | | | | |

| | | | | | |
|---|---|---|---|---|---|
| -Reproduction | Earthworm (*Lumbruculus variegatus*) | 0.94 mg $kg^{-1}$ | 28d | No | (19) |
| -Reproduction | Mud Snail (*Potamopyrgus antpodaruni*) | 0.81 µg $L^{-1}$ | 56d | No, but same order of magnitude | |
| -No effect on emergence, sex ratio or fecundity | Water flea (*Chironomus riparius*) | up to 400 µg $L^{-1}$ | 28d | Yes | |
| -Reduced aggression<br><br>-Reduced swimming speed/schooling behaviour | Arrabina killifish (*Aphanius dispar*) | 3 µg $L^{-1}$<br><br>0.3 µg $L^{-1}$ | 7 d | No<br><br>Yes | (20) |
| -Reduced depression like behaviour in forced swim and social interaction tests | Mice | 20 mg $kg^{-1}$ | 15d (Intravenous) | No | (21) |
| -Reduced male aggression | Sparrow (*Melospiza morphna*) | 10 mg $kg^{-1}$ | Single intravenous injection | No | (22) |
| -Decreased food intake and interrupts estrous cycle | Fischer Rats (female) | 10 mg $kg^{-1}$ | 12-24d | No | (23) |
| -Reduces female receptive behaviour | Rats | 10 mg $kg^{-1}$ | 7-21d | No | (24) |
| -No effect on aggression or mating behaviour | Syrian and Turkish Hamsters (*Mesocricetus auratus*, *Mesocricetus Brandti*) | 20 mg $kg^{-1}$ | Single intraperitoneal injection | No | (25) |
| Reduced aggression<br><br>No effect on courtship | Japanese fighting fish (*Beta splendens*) | 0.54 µg $L^{-1}$ | 14d | Yes | (26) |
| Decreased ejaculation in males<br><br>Body mass loss | Male Rats | 5 mg $kg^{-1}$<br>5 mg $kg^{-1}$ | 7d | No<br>No | (27) |

1.Brooks et al. (2003); 2. Guler and Ford (2010); 3. Bossus et al. (2014); 4. Weinberger and Klaper (2014); 5 De Lange et al. (2006); 6. Mennigen et al. (2010); 7. Henry and Black (2008); 8. Di Poi et al. (2013); 9. Thomas et al. (2012); 10. Gonzalez-Rey et al. (2013); 11. Gaworecki and Klaine (2008); 12. Schultz et al. (2011); 13. Lowry et al. (2009); 14. Bringolf et al. (2010); 15. Nakamura et al. (2008); 16. Stanley et al. (2007); 17. Brooks et al. (2005); 18. Brooks (2014); 19. Nentwig (2007); 20. Barry (2013); 21. Iniguez et al. (2014) ; 22. Sperry et al. (2005); 23. Uphouse et al. (2006); 24. Matuszczyk et al. (1998); 25. Del Barco-Trillo et al. (2010); 26. Dzieweczynski and Hebert (2012); 27. Cantor et al. (1999).

## 1.10 Aims and Objectives

The aim of this thesis is to assess whether a model species of bird that is known to regularly feed on trickling filter invertebrates is likely to exhibit, and be at risk from, mode of action related effects from environmentally relevant concentrations of a priority pharmaceutical. Using fluoxetine as the priority compound and the European starling (*Sturnus vulgaris*) as the model species, this aim was assessed through the following specific objectives:

- To predict and evaluate an environmentally relevant daily dose of fluoxetine for starlings (Chapter 2 / Appendix A1)
- To quantify Accumulation, Distribution, Metabolism and Elimination (ADME) data for fluoxetine in starlings (Chapter 2)
- To compare fluoxetine ADME for starlings with fluoxetine ADME for humans and model mammals to evaluate the suitability of the read-across hypothesis for fluoxetine in starlings (Chapter 2)
- To assess the likelihood of mode of action related effects occurring in starlings by administering the environmentally relevant dose to birds over an ecologically relevant period of time (Chapters 3 and 4)
- To quantify the bioaccessibility of fluoxetine from invertebrate prey for birds and humans (Chapter 5)
- To compare bioaccessibility of fluoxetine between humans and birds to assess if there are differences in internal uptake (Chapter 5)

## 1.11 Thesis Structure

The aims and objectives described above have been addressed in the following chapters. Chapters 2 - 5 have been written in the format of papers, rather than as a traditional PhD thesis:

*Chapter 2* presents data on the exposure levels of wild birds to fluoxetine and the active metabolite norfluoxetine and an assessment of the internal concentrations that could result from exposure at

this environmentally relevant level. ADME data is presented for fluoxetine in starlings. This is then compared and contrasted with ADME data in model mammals collated from the literature in order to evaluate the application of the read-across hypothesis to the risk scenario that was being assessed.
*Chapter 3* presents results of a long-term chronic effects study in wild-caught starlings. An environmentally relevant concentration of fluoxetine was administered to birds via invertebrates for six months with behavioural and physiological endpoints measured before and after treatment in both treatment and control groups.

*Chapter 4* presents the results from a mate choice experiment using the starlings used in Chapter 3. Specifically I focus on how fluoxetine affected female starlings' association time with males in the mate choice experiment and whether fluoxetine treatment altered traits known to be important in mate choice.

*Chapter 5* examines the bioaccessibility of fluoxetine from earthworms in the avian and human gastrointestinal tract. Using *in-vitro* test systems (Physiologically based extraction tests) to quantify the proportion of fluoxetine ingested in a contaminated earthworm that is mobilised in the digestive tract. Differences in internal exposure to fluoxetine between species are assessed and interpreted in terms of the implications for the read-across hypothesis.

*Chapter 6* contains an overall discussion and conclusions for the work. My recommendations for how to take forward the task of assessing the risks that pharmaceuticals in the environment present to wild birds are presented and discussed.

## 1.12 Experimental structure for all work presented in this thesis

### 1.12.1 Prediction and evaluation of the daily dose of fluoxetine for starlings

Prior to exposing the starlings to fluoxetine experimentally, access could not be gained to wastewater treatment plant trickling filters and so the concentration of fluoxetine in trickling filter invertebrates, and subsequently the daily dose that would be administered to starlings was predicted based on usage data of fluoxetine (see sections 2.2.1 and 2.3.1). However, I was granted permission to sample the invertebrates (biofilm and influent wastewater) in November 2013 (after the starling exposure experiment) so could validate my predicted dosage.

### 1.12.2 Pre and post-treatment measurements of behaviour and physiology

All the starlings used in this thesis were captured in November 2011 and the same animals were used for the all the behavioural and physiological experiments presented in Chapters 3 and 4 before being euthanized for the analyses presented in Chapter 2.

Pre-treatment trials are referred to throughout this thesis as 'Baseline' and trials after 16-weeks of treatment as the 'End' (See Figure 1.9). At Baseline and End; Corticosterone, mass balance, fluoxetine in plasma, activity, exploration and boldness were assessed. Foraging was assessed after dosing had started (as the PIT tag system was not set up until after dosing had started) up until the week when birds were euthanized. Mate choice was only assessed at the End of the experiment because birds would not be in breeding condition at baseline.

Treatment was started one pen at a time, and staggered over four weeks i.e. once each pen had completed the baseline individual and group behaviour trials, treatment began for that pen. A stratified sampling structure was used to allocate birds to their respective treatment groups to ensure that the age structure and sexes of treatment groups were as balanced as possible.

In weeks 20-21 (May 2012), each female took part in a mate choice study where the preference for males was assessed (Chapter 4) (Figure 1.9.).

### 1.12.3 Post mortem dissection and tissue sampling

After 22 weeks, dosing was stopped and birds were euthanized at four time points spaced at 24 hour intervals. At each time point three control and three fluoxetine-treated birds were euthanized. To assess the levels of fluoxetine in faeces, a faecal sample was collected immediately prior to euthanasia. The analysis of tissues and faeces provides the focus for Chapter 2.

### 1.12.4 In-vitro assessment of bioaccessibility

To help with the interpretation of the results presented in this Chapter a separate experiment (which is presented in Chapter 5) was conducted to assess the bioaccessibility of fluoxetine from invertebrate prey for the avian and human system using a two compartment *in-vitro* gastro-intestinal tract model (see Figure 1.9).

### 1.12.5 Ethical Note

All work presented in Chapters 2-5 was carried out under Home Office Licence (PPL 60/4213) and was approved by the ethics committees of FERA and the University of York. Birds were captured under licences from the British Trust for Ornithology and Natural England.

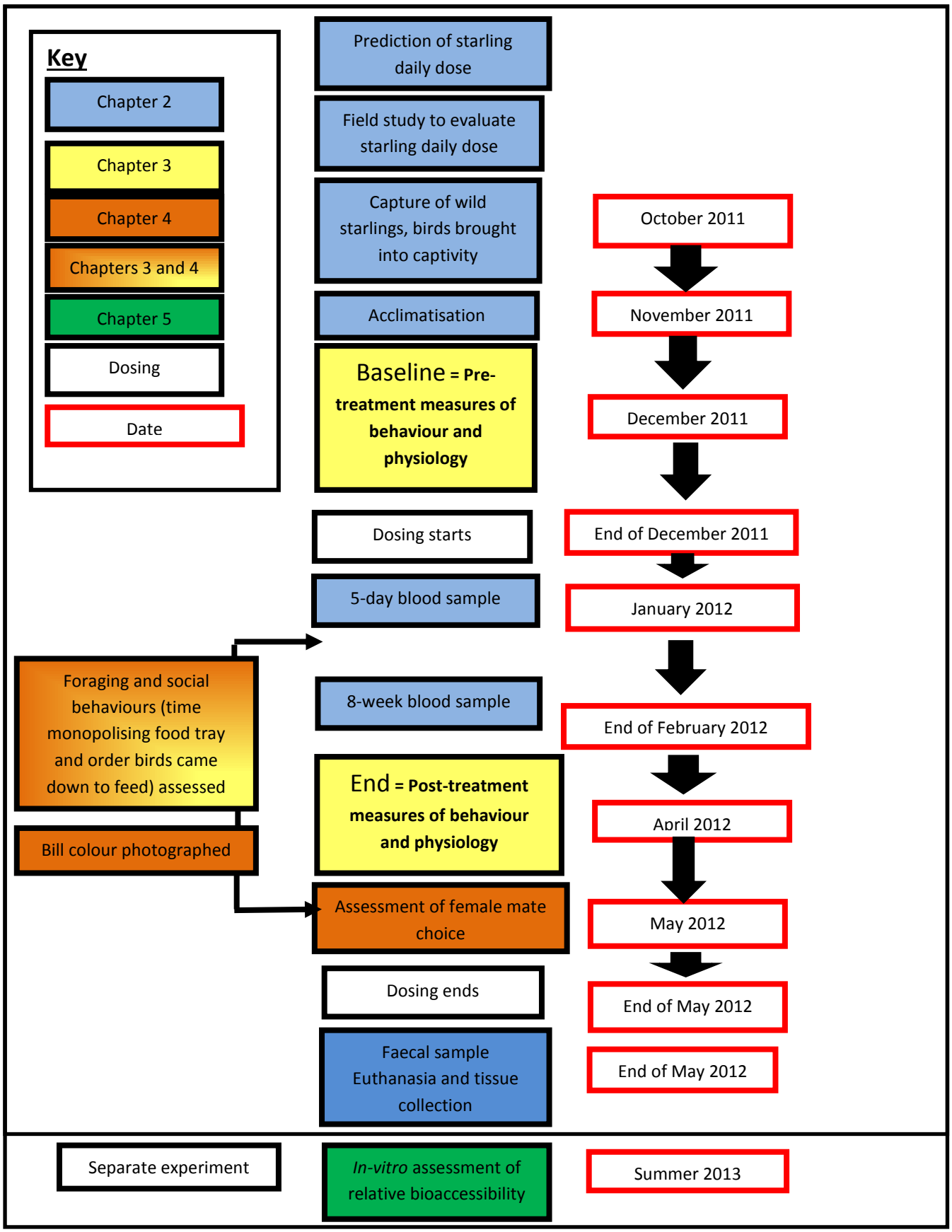



**Figure 1.9: The structure of the experiments presented in Chapter 2 (blue boxes), Chapter 3 (Yellow), Chapter 4 (Orange) and Chapter 5 (Green). The approximate dates of the experiments are given in the boxes with the red outline and information about dosing is given in the white boxes with the black outline. At baseline and end; Day 0 = the day before trials. On this day a blood sample was taken and body mass was measured. Birds were then placed in an individual test cage and left overnight to acclimatise. On Day 1, the first replicate of behaviour trials was done and a faecal sample for Corticosterone analysis was taken. On Day 2, the second replicate of behaviour trials was done and the second faecal sample for Corticosterone analysis was made. At the end of the Day 2 trials, body mass was re-measured before returning birds to their home pens.**

# Chapter 2:

# Accumulation, Distribution, Metabolism and Elimination (ADME) of fluoxetine in a model passerine bird


## Abstract

When humans take pharmaceuticals, a proportion of the dose can be excreted unchanged to wastewater. These excreted pharmaceuticals end up in a wastewater treatment plant (WWTP) where certain pharmaceuticals may bioaccumulate from the sewage into the invertebrates that develop on the trickling filter beds. Ingestion of these contaminated invertebrates by foraging birds presents an exposure pathway for uptake of pharmaceuticals. Fluoxetine is an antidepressant which is commonly prescribed, persistent in the environment and has been shown to affect aquatic species at environmental levels of exposure (ng/L level). In this Chapter, I predicted, and validated using field measurements, an environmentally relevant daily dose of fluoxetine for wild birds feeding on invertebrates at WWTPs to be 928 ng/day. This dose of 928 ng/day is the equivalent of 2.2 to 6.5% of the weight corrected human therapeutic dose. Following 22 weeks of dosing wild-caught starlings in captivity with wax worms injected with this environmentally relevant concentration of fluoxetine, dosing was stopped and birds were euthanized 2, 26, 50 and 74 h after the final dose. Internal concentrations (plasma, brain, kidney, liver and muscle) and excretion levels of fluoxetine and metabolites were measured using methods including ELISA, Liquid chromatography coupled with a triple quadrupole mass spectrometer and Fourier-transform mass spectrometry. The internal concentrations at the different time points were used to assess determine accumulation, distribution, metabolism and elimination (ADME) for starlings in order to evaluate the likely effectiveness of the read-across hypothesis. This hypothesis predicts that evolutionary conservation of a specific pharmaceutical receptor or enzyme in non-target species will result in the potential for pharmaceuticals to cause mode of action related effects in wildlife at the low doses typical of environmental exposure. However, it assumes that internal doses which depend on ADME are the same across species. My ADME results suggest that avian to rodent but not avian to human read-across provides the best support for the use of read-across in avian pharmaceuticals risk assessments.

The relatively poor match between human and avian ADME data is most likely due to differences between species in terms of both metabolic pathway and basal metabolic rate. It is suggested that the low level of accumulation and rapid elimination of fluoxetine in starlings means that in order for the effects observed under experimental conditions to occur in free-living birds, uptake of fluoxetine would have to be high and constant or receptors would have to be more sensitive than those of humans.

## 2.1 Introduction

Worldwide, thousands of different pharmaceuticals are used daily in human and veterinary medicine (Monteiro and Boxall 2010). Many of these pharmaceuticals are only partially metabolised to inactive compounds and remain in the tissues of carcasses or the waste products excreted to the environment (Monteiro and Boxall 2010) (also see Cuthbert et al. 2014). A significant proportion of the human pharmaceuticals that are excreted enter Wastewater Treatment Plants (WWTPs) (Monteiro and Boxall 2010). Here they can be taken up into the invertebrates living on the trickling filters involved in secondary treatment (Markman et al. 2007). Thus, there is a potential for animals such as birds (Fuller and Glue 1980; Gough et al. 2003) and bats (Park and Cristinacce 2006) that forage on WWTPs to be exposed to pharmaceuticals. Moreover, many of the pharmaceuticals that enter WWTPs are incompletely removed by the treatment processes used (Kasprzyk-Hordern et al. 2009; Monteiro and Boxall 2010). Consequently, effluent discharges to surface waters (Ternes 1998) and the application of sewage sludge (McClellan and Halden 2010) to farmland potentially exposes terrestrial and aquatic wildlife beyond those foraging directly on WWTPs (Fick et al. 2010; Rhind 2005; Topp et al. 2008). This is important because pharmaceuticals in the environment are potentially insidious contaminants, designed to alter physiology and behaviour at low concentrations by interacting with receptors many of which are evolutionarily conserved across vertebrate taxa (Gunnarson et al. 2008). To date, studies using environmentally relevant concentrations of pharmaceuticals and non-model animals are rare, particularly for terrestrial species and exposure routes (see Boxall et al. 2012 and Shore et al. 2014).

There is increasing interest in using alternative approaches to *in-vivo* testing to assess the risks pharmaceuticals in the environment pose to non-target species (Boxall et al. 2012). *In-vitro* methods e.g. comparative metabolism (Hutchinson et al. 2014) and *in-silico* approaches (quantitative structure activity relationships and read-across) have been identified as opportunities to replace animal testing in risk assessments. These approaches may also allow extrapolation of observations in one species to other species thus expanding the number of animals covered by Environmental Risk Assessments (Boxall et al. 2012). The read-across hypothesis for extrapolating from pharmacological effects in

mammals to impacts in species in the natural environment was first proposed by Huggett et al. (2003) who noted that pharmaceuticals typically have specific enzyme or receptor based modes of action. In the approach suggested by Huggett et al. (2003), the concentration of a pharmaceutical in the plasma of an environmental organism (usually fish) is estimated and this is then compared to the therapeutic plasma concentration ($_{T}$PC) of the pharmaceutical in humans. If the plasma concentration in the environment is close to or greater than the $_{T}$PC and if the therapeutic target is also present in the environmental organism, then effects related to the therapeutic mode of action of the pharmaceutical might be anticipated.

In addition to the evolutionary conservation of receptors in non-target species, in order for read-across to work, it is necessary to have an understanding of the internal exposure of a pharmaceutical in both the organism of interest and a model species (for which a dose response has been quantified) (Hugget et al. 2003, Rand-Weaver et al. 2013 Gunnarson et al. 2008). An understanding of the similarities and differences between the accumulation (or absorption), distribution, metabolism and excretion (ADME) of a pharmaceutical between the standard organisms used in drug safety testing and organisms in the environment could be very informative in the development and application of the read-across approach. There is an abundance of data on ADME in humans and model mammals as European Medicines Agency guidelines stipulate that mammalian safety tests be carried out (usually in rats and dogs but may include mice, primates or other species if deemed appropriate) prior to clinical drugs trials (EMEA 2005). Indeed, the idea of read-across is not new, pre-clinical drug trials are based on the idea that model mammals such as rats, mice and primates have similar physiology to humans (Owen et al. 2007; 2009). At present ADME data for pharmaceuticals in organisms in the environment is sparse so our understanding of these differences is poor. However, the few studies available on the topic indicate that there is merit in the approach. Recently, Margiotta-Casaluci et al. (2014) tested whether fluoxetine plasma concentrations could be used to predict the likelihood of anxiety related effects in fish. Their study found anxiety-related effects in fish were only observed at fish plasma concentrations that exceeded the human $_{T}$PC. In an earlier study using β-blockers, Owen et al. (2009) found similar support for the use of read-across from mammals to fish with the effective plasma concentration in fish falling within the same range as the effective concentration in mammals. Efficient use of this mammalian data has been proposed to better understand and predict how pharmaceuticals may impact the environment, the potential of which is demonstrated by the work of Owen et al. (2009) and Margiotta-Casaluci et al. (2014). However to date no studies have looked at read-across from humans or model mammals to a species of wild bird.

In this study, I explored the ADME for the selective serotonin re-uptake inhibitor antidepressant, fluoxetine, in wild-caught European starlings (*Sturnus vulgaris*) and compared the observations with findings from ADME studies using some of the standard safety test species and humans. In humans, fluoxetine is used to treat anxiety related conditions such as depression (Eli Lilly 2009). In the brain, fluoxetine competes with serotonin on the post-synaptic receptor cell binding site which sets in motion a cascade of neuro-adaptations (Drugbank 2009); these neuro-adaptations mean the effects of fluoxetine can long outlast the period of treatment (Lesch et al. 1991). In the liver, fluoxetine is metabolised to the active desmethyl metabolite norfluoxetine (also in rats, see Caccia et al. 1990) and multiple other (inactive) metabolites (Drugbank 2014). Elimination is slow one to four days for fluoxetine and up to 15 days for norfluoxetine (Hiemke and Hartter 2000). Figure 2.1 shows the chemical structure of both fluoxetine and norfluoxetine (Hiemke and Hartter 2000). In humans, both fluoxetine and norfluoxetine have relatively large volumes of distribution (Vd) ranging from 14-100 L/kg meaning that both should be widely distributed among tissues (Lewis et al. 2007). The high volume of distribution is due to the lipophilic characteristics contributed to by the presence of three fluorine molecules (Hiemke and Hartter 2000).

Starlings are likely to be exposed to fluoxetine when they feed on contaminated invertebrates at wastewater treatment sites and soils receiving activated sludge (Arnold et al. 2014; Larsson 2014; McClellan and Halden 2010; Redshaw et al. 2008). Chapters 3 and 4 present data that suggest environmentally relevant exposures of starlings to fluoxetine can alter fitness related traits (foraging patterns, response to isolation-stress and social interactions of birds) but nothing is known about how these effects relate to internal concentrations; particularly in the brain which is the key site for pharmacological activity (Hiemke and Hartter 2000). Gaining an understanding of ADME for wild birds will not only provide an assessment of the suitability of mammalian to avian read-across, but will also help with read-across from the captive environment to the wild.

Therefore, the aims of this study were three-fold to 1) Determine what is an environmentally relevant dose of fluoxetine for starlings feeding on trickling filter invertebrates 2) Assess the ADME of fluoxetine in starlings fed the environmentally relevant dose contained in spiked invertebrates over an ecologically relevant period of time and 3) Assess the applicability of the read-across hypothesis to this case study by comparing and contrast the starling ADME with ADME from humans and model mammals.

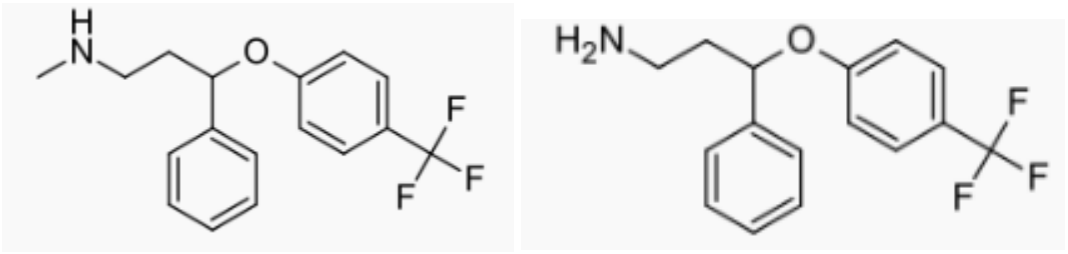



**Figure 2.1: The molecular structure of fluoxetine (left) and norfluoxetine (right), the three fluorine molecules on the right hand side of the structures cause fluoxetine to be relatively hydrophobic (Drugbank 2014).**

## 2.2 Materials and Methods

### 2.2.1 Predicting the daily dose of fluoxetine for wildlife

To calculate a predicted daily dose for starlings, the fluoxetine concentration in influent wastewater was first predicted (section 2.2.1a) and multiplied by a bioconcentration factor (determined in the laboratory by Carter et al. 2011, unpublished data) to give the concentration in invertebrates. The daily dose was then calculated by multiplying the concentration in invertebrates by the mass of invertebrates eaten by starlings in a day.

#### 2.2.1a The predicted concentration in influent wastewater

For a human pharmaceutical, such as fluoxetine, that is excreted to wastewater, the Predicted Environmental Concentration (PEC) is a function of the mass of a pharmaceutical used by a population over time (in this case one year) and the proportion of the drug excreted by patients unchanged in relation to how much the un-metabolised drug is diluted in wastewater.

The PEC for influent wastewater was obtained using Equation 2.1, assuming patient excretion is the main source of pharmaceuticals in the environment (Ort et al 2010). I predicted the concentration of fluoxetine in influent wastewater to be 295 ng/L.

**Equation 2.1**

$$PEC_{influent} = \frac{A * E}{365 * V * P}$$

Where: ***A*** = the mass of the pharmaceutical used by a population $4.639 \times 10^{12}$ (ng $yr^{-1}$) (NHS 2010)

***E*** = the proportion excreted unchanged by patients (24% Lienert et al. 2007)

***365*** = the number of days in a year

***V*** = the volume of wastewater per capita (200 L) per day (Williams 2005)

***P*** = the size of the population (51.7 million in England in 2010 at the time of the prediction www.ons.gov.uk)

### 2.2.1b Predicting the daily dose of fluoxetine for starlings

By multiplying the PEC for influent wastewater by the Bioconcentration factor (BCF) of 133 (Carter et al. 2011 unpublished data), the PEC for invertebrate prey was estimated (ng/kg). The daily dose for starlings was then predicted by multiplying the PEC for invertebrates by the mass of invertebrates consumed by a starling in one day from trickling filters (half of their daily intake of invertebrates which is 23.5 g i.e. 0.0235 kg). For fluoxetine, the predicted concentration in invertebrates was 39.2 ng/g; this equates to a daily dose of 928 ng. The predicted daily dose was later confirmed as environmentally relevant based on analysis of worms from four wastewater treatment plant trickling filters (see section 2.3.1)

### 2.2.2 Pharmaceutical compounds and reagents for analytical and experimental work

Fluoxetine and norfluoxetine for analytical work were purchased from Sigma Aldrich (Dorset, UK). Fluoxetine-d5 internal standard in methanol was obtained from Cambridge Isotopes (Tewkesbury MA). Methanol, acetonitrile, dichloromethane, ethylacetate (High Performance Liquid Chromatography Fluorescence grade, 99.9%), hydrochloric acid (33.3%) and ammonium hydroxide solution (35%) were purchased from Fisher Scientific (Loughborough UK).

The fluoxetine that was spiked into invertebrate prey and experimentally fed to the captive starlings was Prozit® solution (20 mg/5mL solution; PineWood Healthcare, Clonmel, Republic of Ireland).

### 2.2.3 Evaluation of the predicted bird daily dose and its effects of wild birds

#### 2.2.3a Sample collection and preparation

Samples of influent, biofilm (to assess the BCF used in the prediction) and earthworm were collected from the trickling filters at four different WWTPs in the North of England on 26th November 2013 to evaluate whether the predicted values for influent wastewater and invertebrate concentrations were environmentally relevant. At each site, influent samples were collected in solvent rinsed 1 L amber glass bottles. During wastewater treatment, a biofilm on which the earthworms feed builds up around the rocks that made up the filter bed media. To sample the biofilm, a 1 L glass jar was filled with biofilm coated rocks collected from the top 20 cm of the filter bed. Finally, up to 20 g (wet weight) of earthworms (*Eisenia fetida*) were obtained from across the filter beds (from the top 20 cm) and placed into a solvent rinsed glass jar. All samples were placed in cool bags containing ice packs and transported back to the laboratory where any sludge was rinsed away from the earthworms using deionised water. Samples were then frozen at -20°C until extraction and analysis.

#### 2.2.3b Capture of wild starlings, acclimation in captivity and general husbandry

In order to assess the effects that the environmentally relevant dose of fluoxetine could have on wild birds, thirty-two European starlings (*Sturnus* vulgaris) were caught from a reed bed roost site in North Yorkshire over three nights in October 2011. Mist nets were put up about an hour and a half before dusk; all thirty-two birds were captured over three nights. Each night, birds were removed from the net, placed in cloth bags and transported for approximately one hour to a complex of Home Office Licensed outdoor aviaries. On the right leg of each bird, a unique white numbered plastic leg ring (obtained from AC Hughes) and a green leg ring, to which was attached a unique passive integrated transponder tag (PIT tag, 11.5 mm × 2.1 mm, <0.1 g, Trovan Unique) was placed. On the left leg a unique combination of one or two coloured rings was attached to allow easy identification of individuals during behaviour trials. The total mass of all leg rings was less than 1% of body weight.

Birds were placed into one of four outdoor aviaries. All birds were left to acclimatise for four weeks from the day that the final birds were brought into captivity (24th October 2011). Seven birds died during the acclimation period, which revealed atoxoplasmosis on post mortem (PM) dissection at IZVG laboratories (Keighley, UK). Toltrazuril could have been used for treatment of the remainder of the colony as a last resort. However, after treatment with Avipro®, a veterinary probiotic combination of bacteria, enzymes, electrolytes and vitamins, which was added to the water hopper. No further mortalities associated with atoxoplasmosis resulted. When the birds were on Avipro® the water bath was only available for one hour a day, to ensure that they only drank from the water

hopper. In the first two weeks of the experiment one fluoxetine-treated bird died in extreme cold weather (low of -7°C). No disease was associated with this mortality.

The study was designed to have single sex aviaries (two for males and two for females) with the sexes visually isolated. In the final week of the acclimation period, birds were aged (based on body mass, degree of speckling on the throat feather and tarsus length) and sexed by a field ornithologist using the length of the throat feather and colour of the iris ring (Smith et al. 2005). Smith et al. (2005) have found this method of sexing starlings to be 98% accurate compared with DNA testing (N = 100). However, when the birds lower mandibles started to change from winter condition to breeding (pink for females, blue for males) it was found that there had been some mistakes with the sexing. Further mistakes were revealed on post-mortem (PM) dissection. Thus sex ratios in each of the four pens (referred to as Pen 4, 5, 8 and 9) were as follows: Pen 4 (5 females), Pen 5 (1 male and 5 females), Pen 8 (6 males and 1 female) and Pen 9 (5 males and 2 females). In total there were twelve males (eight fluoxetine treated and four controls) and twelve females (five fluoxetine treated and seven controls) that completed the study (the additional mortality was in Pen 4). Pens 8 and 9 and 4 and 5 were on opposite sides of a central observation corridor and so were visually isolated.

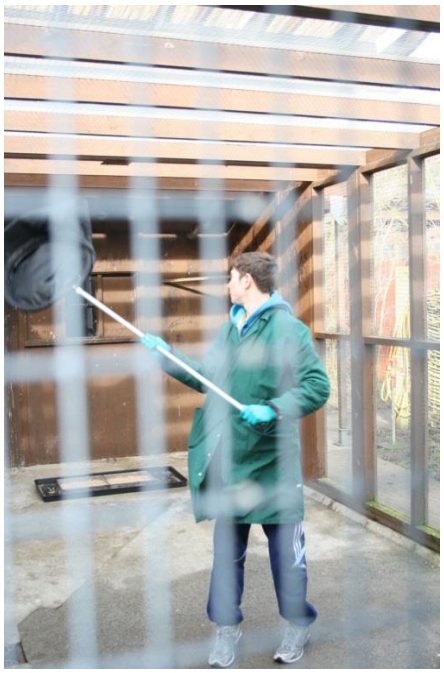
### 2.2.4 Injecting fluoxetine into invertebrates and feeding to starlings

To administer the treatments to the birds, each day live Lesser Wax worms (*Achroia grisella*) were injected with either 1300 ng of fluoxetine solution (Prozit® 20 mg/5mL solution, PineWood Healthcare, Clonmel, Republic of Ireland); 130 µL of Prozit® solution was diluted up to 1 mL with deionised water to give a concentration of 520,000 ng/mL. Invertebrates were then spiked with 2.5 µL of either the 520,000 ng/mL fluoxetine solution, or 2.5 µL of deionised water (controls). Dosing took place five days a week so that birds received their weekly dose over five days. Each day, duplicate treatment and control wax worms were also injected and stored at -20°C. A subset of these additional worms were randomly selected, extracted with methanol and analysed by High Performance Liquid Chromatography (HPLC) as a Quality control (mean concentration 1580 ng/worm, N = 8, Percentage Relative Standard Deviation = 13). In a pilot study injecting dye, I found that although freezing the worms for a short period of time prevented worms from wriggling during their injection, frozen worms leaked more than fresh and so I injected live wax worms.

Oral uptake of the treatment was carried out by catching each bird from its home aviary using a large padded hand net and feeding it just one worm per day (Figure 2.2). Once birds were in the hand net, each was placed into an individual bird bag and stored in a cage measuring 55 cm × 75 cm × 30 cm until all birds in all four pens had been caught (Taking approximately 20 minutes). Each bird was fed one wax worm containing their appropriate treatment by placing the worm at the back of the throat

with a pair of forceps. Birds were given the chance to swallow before massaging the neck if necessary to ensure the worm was swallowed. Birds were then released immediately back into their appropriate home aviary.

a)



b)

c)

**Figure 2.2: a) Daily capture of starlings from home aviaries with a hand net, b) spiking invertebrates with fluoxetine. A 25 µL Hamilton syringe was used to inject an invertebrate held with pointed forceps and c) hand feeding a starling with a spiked invertebrate using blunt ended forceps.**

## 2.2.5 Collection of samples from starlings

### 2.2.5a Collection of plasma from starlings during the 22 week exposure period

Blood plasma samples (≈150 µL) were collected from birds before treatment and at three other times during the experiment, after 5-days of treatment, 8-weeks and 16-weeks of treatment. Using a 25 gauge needle and a 1 mL syringe, 490 µL whole blood (for birds weighing less than 70 g, a pro-rata adjustment was made in line with Home Office guidelines of a maximum sample of 700 µL/ 100 g body weight) was collected, placed into a lithium heparin microtainer (BD, UK) and centrifuged immediately for 3 minutes. After centrifugation, plasma was removed from the surface of the red blood cells and placed in a 1.5 mL microcentrifuge tube (stored on wet ice until sampling was completed).

### 2.2.5b Collection of tissues and faeces from starlings at the end of the 22 week exposure period

After 22 weeks of treatment, birds were randomly allocated to one of four groups (equal numbers of control and fluoxetine-treated birds). To assess ADME in starlings, the four groups were then euthanized at different time points (2, 26, 50 and 74 hours) after the administration of the final dose starting on May 28$^{th}$ 2012 and ending on 31$^{st}$ May 2012. Immediately prior to euthanasia, a sample of faeces was collecting in to a 1.5 mL microcentrifuge tube using a swab and placed on dry ice while post-mortem dissections took place. Approximately three hours later, samples were placed in the oven at 40°C until dry.

Samples of brain, kidney, liver and pectoral muscle were obtained from each bird following euthanasia by PM dissection. Tissue samples were immediately dipped into liquid nitrogen before being placed into labelled glass jars stored on dry ice until all PM dissections for that day had been completed. On each day, once all PM dissections had been completed, samples were placed into storage at -80°C until extraction and analysis. The order in which birds were euthanized was determined using random numbers. To avoid cross-contamination separate dissection kits were used for control and fluoxetine-treated birds and all equipment was cleaned with solvent between individual PM dissections.

## 2.2.6 Analytical methods

### 2.2.6a Influent, Biofilm and Earthworms wastewater extractions

To extract fluoxetine and norfluoxetine from influent wastewater, biofilm and earthworms, the following methods were used. The influent wastewater samples were extracted following the Solid Phase Extraction (SPE) method of Grabic *et al*. (2012). Briefly, samples were given 24 hours to defrost and then acidified to pH 3 with dilute hydrochloric acid. Triplicate 100 mL samples were taken from each sample bottle and 100 µL of 100 ng/mL of Fluoxetine-d5 internal standard was added. An Oasis

HLB cartridge (reversed phase) (6cc, 200 mg) was conditioned with 5 mL methanol, equilibrated with 5 mL deionised water. Each sample was loaded to the cartridge at a rate of 5 mL/min using a vacuum pump, cartridges were washed with 5 mL of 95:5 water:methanol before eluting into a glass tube, containing 100 µL of 9:1 Methanol: ethylacetate (to prevent analytes from sticking to the glass during the concentration phase), with 5 mL methanol and 3 mL ethylactetate. Samples were concentrated under a steady stream of nitrogen on a Turbovap set at 45°C until dry. The residue was reconstituted to 1 mL, first with 0.5 mL of methanol and secondly with 0.5 mL of water (HPLC grade). After the addition of both the methanol and water, samples were vortex mixed for 5 seconds. Samples were then passed through a 0.2 µm polytetrafluoroethylene (PTFE) syringe filter into a 2 mL vial. The vial was sealed with a crimp cap containing a PTFE septum.

Biofilm samples were allowed to come up to room temperature and the biofilm was scraped off the rock using a spatula into a 50 mL centrifuge tube (0.3 g wet weight in triplicate from each site). Biofilm samples were spiked with 100 µL of 100 ng/mL of fluoxetine-d5 internal standard (in methanol) before extracting twice with 5 mL of 7:3 acetonitrile water as in Carter et al. (2014b). Samples were shaken on their sides for 10 minutes at 420 rpm, ultrasonicated for 10 minutes in an ultrasonication water bath and centrifuged for 10 minutes at 4500 × g at 20°C. The supernatant was concentrated in the same way as the influent wastewater.

All 20 g of earthworms were removed from the freezer and defrosted in a 4°C fridge overnight. For each site, a homogenous earthworm sample was created by chopping all earthworms by hand. For each site, triplicate 0.3 g ± 0.03 samples were weighed out. The mass of invertebrates that I extracted was limited by the low number of invertebrates collected at one of the sites. Additionally, I needed to use some of the earthworms collected to use different extraction methods, the extracts of which would be analysed to quantify levels of other pharmaceuticals (data not presented here). One-hundred µL of 100 ng/mL fluoxetine-d5 internal standard (in methanol) was spiked in and samples were extracted by homogenisation with 5 mL of 7:3 Acetonitrile:water followed by centrifugation following the method of Carter et al. (2014b). Earthworms were extracted using 6 mL of 7:3 acetonitrile:water, a method validated by Carter et al. (2014a). No SPE was conducted on the earthworm extracts. Extracts were analysed using the same Liquid Chromatography coupled with a triple quadrupole mass spectrometer (LC-MS/MS) (see section 2.3.6e).

**2.2.6b Blood Plasma**

Starling plasma samples were stored at -20°C and levels quantified within 5 months of collection using a Maxsignal Fluoxetine ELISA (Enzyme-linked immunosorbent assay) kit (Bioo Scientific, Austin, Texas USA; sensitivity 1 ng/mL). The ELISA method was validated by spiking known quantities of

fluoxetine into blue tit (*Cyanistes caeruleus*) plasma (remaining from previous experiments) and following the assay outlined in the manufacturers guide (recovery = 76.6%, sensitivity 1 ng/mL, intra assay Percentage RSD = 28%).

**2.2.6c Starling tissues**

Each tissue sample was cut approximately in half (to ensure sufficient sample remained in case of the need for re-analysis) placed into a 50 mL centrifuge tube and weighed on a tared balance (Sartorius Entris 4202-1s). The mean masses of tissues extracted are given in Appendix A4. Knives, boards and spatulas were all cleaned with solvent between samples. Each sample was spiked with fluoxetine-d5 internal standard in methanol (100 µL of 25 µg/mL) and then extracted by homogenisation with 2 mL of methanol. Samples were homogenised for approximately 10-30 seconds using a Turax homogeniser. Extracts were then diluted with 4 mL of 0.05 M HCl in water which was added in two steps, after each of which the sample was homogenised for a further 10-30 seconds, (mainly to remove any residue from the homogeniser). After homogenisation each sample was briefly vortex mixed, ultrasonicated in an ultrasonication water bath for 10 minutes before centrifuging for 10 minutes (4500 × g, 20°C). The supernatants of tissue sample extracts were then decanted into 8 mL glass vial test tubes. For muscle samples, it was necessary to pass the supernatant extracts samples through a 5 µm PTFE filter prior to the Solid Phase Extraction (SPE).

Extracts were then cleaned up using an SPE method adapted from the method of Chu and Metcalfe (2007). MCX (Cation exchange and reversed phase) cartridges (Oasis 3cc, 60mg, Waters Hertfordshire, UK) were conditioned with 1 mL of methanol and then equilibrated with 1 mL of water (HPLC grade). A 2.4 mL aliquot of the decanted sample supernatant was taken from each 6 mL extract and placed in another 8 mL glass vial. Samples were loaded to the cartridge and then vials were rinsed with 0.6 mL methanol. Cartridges were then washed with 1 mL methanol followed by 1 mL of dichloromethane. Cartridges were eluted with 2.8 mL of 95% methanol 5% ammonium hydroxide (made from a 35% ammonium hydroxide solution) into a glass tube containing 100 µL of 9:1 Methanol:ethylacetate to prevent analytes from sticking to the glass during the concentration phase. The eluate was vortex mixed and blown until dry under a steady stream of nitrogen on a Turbovap set at 45°C. Samples were reconstituted first in 500 µL methanol, vortex mixed briefly before adding 500 µL of HPLC water. Samples were passed through a 0.2 µm PTFE filter into a 2 mL HPLC vial with a crimp cap containing a PTFE septum. Samples were stored at -80°C and analysed within one month of extraction in March 2014.

**2.2.6d Faecal samples**

Faecal samples were dried in an oven in the laboratory at 40°C. Samples were weighed daily until there was no further change in mass was observed. Dry samples were then ground and stored in

small glass vials at -20°C until analysis. Faecal sample extracts were found to be clean enough for analysis without SPE. Each sample was spiked with 100 µL of 25 µg/mL of internal standard (fluoxetine-d5 in methanol) and extracted with 1 mL of methanol in a 1.5 mL micro-centrifuge tube. Samples were shaken on their sides at 420 rpm for 30 minutes, ultrasonicated for 5 minutes before centrifuging for 10 mins in a microcentrifuge at 11,000 × g. The concentration and reconstitution procedures were the same as for tissue samples.

**2.2.6e Analysis using Liquid Chromatography Triple Quadrupole Mass Spectrometry**

The concentrations of fluoxetine and norfluoxetine in the extracted samples (influent, biofilm, earthworm, brain, kidney, liver, muscle and faeces) were determined using the Applied Biosystems/MDS Sciex API 3000 triple quadrupole in positive ion mode for LC-MS/MS analyses. Multiple reaction monitoring (MRM) transitions were Norfluoxetine = 296.4>134.1 (time 50 m/sec) Collison Voltage/Collision Energy (CV/CE) = 9.5; Fluoxetine: 310.2>147.9 (time = 200 msec); CV/CE = 13 and Fluoxetine-d5: 315.2>153.2 (time = 100 msec), CV/CE = 13. For the Liquid Chromatography a Dionex Acclaim® RSLC C18 Polar Advantage II column (2.2 µm 120A 2.1 × 100 mm) was used. A ramp gradient method was used consisting of A: $H_2O$ 0.1% formic acid, B:Acetonitrile 0.1% formic acid was used at a flow rate of 200 µL $min^{-1}$with a total run time of 9 mins. The gradient was as follows 1 min 15% B, 1.5 mins, 40% B, 5.5 mins 45% B, 5.6 mins 95% B, 7 mins 95% B, 7.2 mins 15% B, 9 mins 15% B. Retention times were 5.5 mins for both analytes and internal standard.

For fluoxetine the mean percentage recoveries of the extraction methods (rec ±1 Standard Error (se)), limits of detection (LOD) and limits of quantification (LOQ) (as defined by IUPAC 2014) were: Influent: rec = 95.6% , se = 1.2%, LOD = 0.60 ng/mL, LOQ = 2.00 ng/mL; Biofilm: rec = 126.9%, se = 9.8%, LOD = 0.67 ng/mL, LOQ = 2.20 ng/mL and earthworm was: rec = 111.3% se = 7.2%, LOD = 0.15 ng/mL, LOQ= 0.50 ng/mL. For norfluoxetine the values were: Influent: rec = 101.3%, se = 18.0%, LOD = 0.15 ng/mL, LOQ= 0.50 ng/mL; Biofilm: rec = 71.6%, se = 3.4% LOD = 0.08 ng/mL, LOQ = 0.27 ng/mL and earthworm was: rec = 34.5%, se=8.0% LOD = 0.02 ng/mL, LOQ = 0.07 ng/mL. The low recovery for norfluoxetine in earthworms was accounted for using matrix specific standards.

**2.2.6f Liquid Chromatography-Ion Cyclotron Resonance- Fourier Transform Mass Spectrometry to look for fluoxetine metabolites**

In addition to the active metabolite (norfluoxetine), humans produce six known inactive metabolites of fluoxetine (Drugbank 2014): para-trifluoromethylphenol, hippuric acid, fluoxetine glucuronide, norfluoxetine glucuronide, norfluoxetine alcohol and norfluoxetine acid. The monoisotopic molecular weights and molecular formulas for these six metabolites are given in Table A3 (Appendix A5). For each metabolite, the sodiated (Na+) and the protonated (H+) form was searched for using liquid

chromatography coupled with an Ion Cyclotron Resonance Fourier Transform Mass Spectrometer (ICR-FT-MS). ICR-FT-MS was used because without standards to do an infusion, you cannot optimise methods and quantify the levels of an analyte using LC-MS/MS. There are no standards for these metabolites. ICR-FT-MS can measure mass with high accuracy. All ions are measured simultaneously (i.e. not at different times as with Quadrupole Time of Flight (QTOF)) by passing detection plates (rather than hitting). ICR-FT-MS is a high resolution technique that can be used to determine masses with high accuracy. Therefore, it is a useful technique for assessing the presence of an analyte with unknown chemical properties but known molecular weight and structure. Therefore, ICR-FT-MS is a standard technique for screening samples for a large number of metabolites or transformation products that could potentially be in a sample.

The liquid chromatography conditions were identical to those used on the LC-MS/MS for analysis of the parent compounds and used an Agilent 1200 HPLC and a Dionex Acclaim® RSLC C18 Polar Advantage II column (2.2 µm 120 A 2.1 × 100 mm). For The ICR-FT-MS, the MS analysis was undertaken using a solariX XR 9.4 T (Bruker) FT-ICR mass spectrometer in positive mode electrospray ionisation (ESI). Spray voltage: 4500 V, 4.5 L/min dry gas ($N_2$) at 250°C and 1.6 bar nebuliser gas. The acquisition range m/z was 100-3000, with a transient time of 0.367 s giving an estimated resolution of 66,000 at m/z 400. In total five samples, one for each of brain, kidney, liver and muscle and faeces from one of the three fluoxetine treated birds (each sample randomly selected) euthanized two hours after the final dose were analysed by LC-ICR-FT-MS.

### 2.2.7 Quality Control and Assurance

#### 2.2.7a Validation of extraction of fluoxetine and norfluoxetine from influent biofilm and wastewater

Extraction methods were validated by spiking in fluoxetine, norfluoxetine and fluoxetine d5 internal standards at seven known concentrations between 0.01ng/mL and 1000 ng/mL to create matrix specific standards. Tap water was used for influent wastewater, garden moss (0.3 g) taken from an uncontaminated site for biofilm and *Eisenia fetida* (0.3 g*)* taken from the same captive colony referred to in Chapter 5 and Appendix D. After spiking, the validation standards were extracted and cleaned up using the methods presented in Appendix A2.

#### 2.2.7b Validation of extraction of fluoxetine and norfluoxetine from avian tissues

To validate extraction and (if required) SPE for fluoxetine in avian tissues and faeces, pigeon brain, chicken kidney, pheasant liver, partridge muscle and dry starling faeces (no SPE) was spiked with known quantities of fluoxetine and analysed by High Performance Liquid Chromatography (HPLC) (same conditions as in Appendix A2). The avian tissues were obtained from birds that were

purchased from a local country butchers shop (i.e. all organs still intact). I dissected the birds to remove the required tissues. The tissues were then stored at -20°C until required for laboratory work (within seven days). These species were selected based on the availability of dead birds with all organs still intact.

Once an extraction and SPE method with suitable recoveries (≥70%) for the parent compound had been validated, calibration standards for LCMS were made by spiking known quantities of fluoxetine, norfluoxetine and fluoxetine-d5 internal standard into spare brain (0.41 g wet weight per replicate), kidney (0.07 g), liver (0.22 g), muscle (0.22 g) and faeces taken from the control starlings. Calibration standards were extracted and cleaned up following the same homogenisation, SPE and concentration procedure as the tissue samples. Concentrations ranged from 1ng/mL to 50 ng/mL for tissue samples and 1-100 ng/mL for faeces. $R^2$ values for calibration curves are also presented in supporting information (Table C1). The relative recoveries (± 1 SE) were calculated by relating the peak area given by the matrix specific standards to the peak area given by a solvent standard of the same concentration (Table 2.1). Table 2.1 also contains the limits of detection and limits as defined by (IUPAC 2014) (3 × Root mean square (RMS) of the peak to peak noise). The limits of quantification (LOQ, ng/mL) were set at 10 × RMS (IUPAC 2014) or the signal in the control samples, which ever was less sensitive. The LOQ ng/g wet weight for tissues and dry weight for faeces was calculated using the mean mass of that matrix that was extracted (Appendix A4).

**Table 2.1: Validation of analytical methods: matrix effect, percentage recovery, limits of detection (ng/ml) and limits of quantification given as both ng/mL and ng/g (which is converted to a wet weight tissue concentration except for faces which is dry weight) The LOD was 3 × RMS (Root mean square of the peak to peak noise) and LOQ was 10 × RMS (as defined by (IUPAC 2014)) unless the peak to peak noise in the control samples (slightly greater mass of tissue extracted than the calibration standards) exceeded 10 × RMS, in which case the LOQ was set above the signal in the controls (values marked by *).**

| | **Fluoxetine** | | | | **Norfluoxetine** | | | |
|---|---|---|---|---|---|---|---|---|
| | Relative recovery (%) | LOD (ng/mL) | LOQ (ng/mL) | LOQ (ng/g) | Relative Recovery (%) | LOD | LOQ (ng/mL) | LOQ |
| Brain | 71.6.5 ± 4.1 | 0.02 | 0.07 | 0.15 | 81.6 ± 6.8 | 0.05 | 0.04 | 0.09 |
| Kidney | 72.9 ± 0.15 | 0.0034 | 0.03 | 0.53* | 65.9 ± 0.24 | 0.2 | 0.04 | 3.43 |
| Liver | 103.0 ± 3.0 | 0.027 | 0.09 | 0.41 | 132.0 ± 3.8 | 0.05 | 0.11 | 0.52 |
| Muscle | 80.6 ± 4.6 | 0.01 | 0.30 | 2.97* | 72.0 ± 5.7 | 0.05 | 0.47 | 5.14* |
| Faeces | 135.7± 3.2 | 0.3 | 1.00 | 5.92 | 59.8 ± 22.0 | 0.05 | 0.18 | 1.06 |

### 2.2.8 Data analysis: Calculation of pharmacokinetic variables

- The plasma concentration for humans and birds was calculated assuming first order kinetics (Equation 2.2)
- Accumulation in tissues was calculated relative to body mass using Equation 2.3).
- Distribution was calculated by dividing the tissue concentration by the plasma concentration.
- The ratio of fluoxetine to norfluoxetine was calculated by dividing the fluoxetine concentration by the norfluoxetine concentration.
- The rate constant of the elimination half-life was calculated using Equation 2.4
- The proportion of the dose excreted unchanged in 2 hours was calculated using Equation 2.5.

**Equation 2.2**

$E_{peak}$ = (Dose remaining) $e^{-kt}$ + Dose (just after dose)

In equation 2.2 $E_{peak}$ is the peak plasma concentration, which is determined by adding the daily dose to the fraction of previous doses remaining.

The fraction of previous doses remaining is given using first order kinetics where k is the rate constant (given by rearranging Equation 2.4) and t is time.

**Equation 2.3**

Percentage accumulation = (Concentration in tissue (ng/g)) / (dose (ng)/ body mass (g))

**Equation 2.4**

$t_{1/2} = \ln(2)/k$

Where k is the rate constant and $t_{1/2}$ is the elimination half-life

**Equation 2.5**

proportion of dose excreted unchanged = Concentration in faeces (ng/g) × mean mass of faeces produced in 2 hours (0.169g)/ dose contained in the worm (1300 ng).

## 2.3 Results and Discussion

### 2.3.1 Evaluation of the predicted dose

Figure 2.3 shows the overall mean concentrations of fluoxetine and norfluoxetine in influent wastewater (ng $mL^{-1}$), biofilm and *Eisenia fetida* (earthworms) (both ng $g^{-1}$ wet weight) which were

taken from four different wastewater treatment plants. Although both parent compound and active metabolite were detected in all the samples of influent, biofilm and earthworm (norfluoxetine in all but one sample for earthworms) it is clear from Figure 2.3 that the fluoxetine concentrations were much higher than norfluoxetine. Note that wastewater and biofilm concentrations in Figure 2.3 have been expressed as ng $mL^{-1}$ and ng $g^{-1}$ to illustrate bioaccumulation; these concentrations are usually expressed as ng $L^{-1}$ (Calisto and Esteves 2009) or ng $kg^{-1}$ (McClellan and Halden 2010). For fluoxetine the mean concentration in influent was 1,310 ng/L, which is more than four times higher than the predicted concentration of 295 ng/L (section 2.2.1). However, when the concentrations in influent wastewater and biofilm are compared with the environmental monitoring studies in other countries, there is a good agreement with my data (Calisto and Esteves 2009; McClellan and Halden 2010).

The mean concentration of fluoxetine in earthworms was 26.2 ng $g^{-1}$ (range of values 2.5-53.8 ng $g^{-1}$ and range of means across the four sites 6.9 -35.5 ng $g^{-1}$) which corresponds to a daily dose of 620 ng $day^{-1}$ (with the range of the means being = 59- 1264 ng $day^{-1}$). Mean concentrations at each WWTP (± 1 Standard Error) were: WWTP 1= 6.9 ng $g^{-1}$ ±4.3, WWTP 2= 35.5 ng $g^{-1}$ ±3.4, WWTP 3= 26.9 ng $g^{-1}$ ±9.6 and WWTP 4=35.4 ng $g^{-1}$ ±9.5. The range of concentrations in the individual replicates across was 2.7-53.8 ng/g. Concentrations of norfluoxetine in earthworms were negligible by comparison. The standard errors in Figure 2.3 were greater for fluoxetine in earthworms than influent and biofilm. I found fluoxetine concentrations to vary considerably within and between sites.

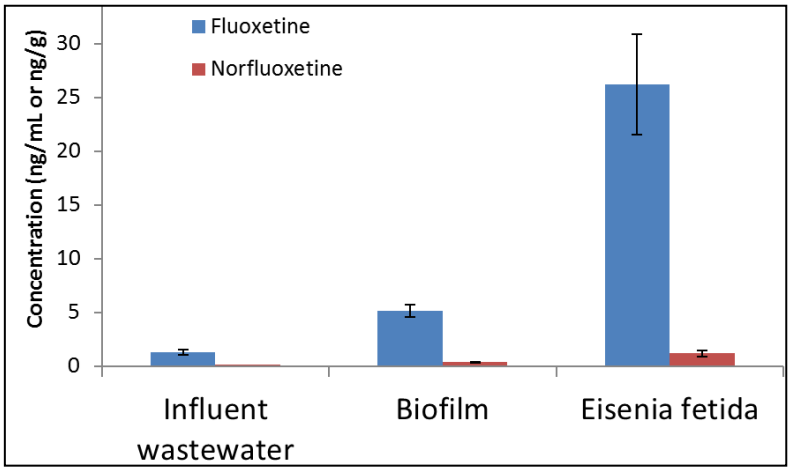



**Figure 2.3 Mean (±1 Standard Error) fluoxetine (blue, left bars) and norfluoxetine (red, right bars) concentrations in influent wastewater (ng $mL^{-1}$) (N = 6 i.e. triplicates from 1 WWTP and 1 pooled set of triplicate for the other three WWTPs), biofilm (ng $g^{-1}$, N = 12 i.e. triplicates from four sites) and *Eisenia fetida* (ng $g^{-1}$, N = 12 i.e. triplicates from 4 WWTPs) samples collected from four wastewater treatment plants (WWTP).**

Figure 2.3 clearly shows that bioaccumulation of both parent compound and active metabolite occurs from wastewater, to biofilm and into earthworms. For fluoxetine, the mean bioconcentration factor (BCF) (N = 12) from influent wastewater into biofilm was 5.18 (range across the four WWTPs 2.83-9.36); from biofilm into earthworms the Bioaccumulation factor (BAF) was 5.78 (range 0.61 to 13.49) and the BCF from influent wastewater into earthworms (if biofilm is ignored) was 26.27 (range 3.07-56.88). Norfluoxetine concentrations were typically 1 to 2 orders of magnitude lower than fluoxetine, nevertheless, BCFs were similar to the parent compound: Influent into biofilm = 9.67 (range 4.24 to 22.96), biofilm into earthworms = 4.00 (range 0 to 13.3) and influent into earthworms = 30.14 (range 0 to 74.5).

My predicted daily dose of 928 ng/day was based on birds consuming 23.5 g/day of invertebrates (on a wet weight basis) that have accumulated fluoxetine from the biofilm/sludge using a BCF of 133. Although the experimental BCF used for predictions was around four times higher than that measured in the environment (see Appendix A1), the predicted influent wastewater concentration was around four times lower than the measured concentration and so the predicted concentration in invertebrates 39.2 ng/g fell within the measured range (2.5-53.4 ng/g). Therefore so long as feeding rates are similar to the levels that have been suggested in the literature (e.g. Tinbergen 1981, Markman 2008 and Feare 1986), then the predicted dose of 928 ng/day fell within the range of concentrations detected in invertebrates.

### 2.3.2 Plasma: Was the starling plasma concentration what was expected given the dose and the levels found in humans and model mammals?

Analysis of plasma from before treatment (baseline), after five-days of treatment and eight-weeks of treatment found fluoxetine concentrations to be at or around the LOD (1 ng/mL), with similar values for both fluoxetine-treated and control birds, most likely due to cross reactivity (data not presented here). For the purpose of calculating tissue to plasma ratios, the plasma concentration was assumed to be half the limits of detection (i.e. 0.5 ng/mL), but I must highlight the point that these values are indicative only. However, the key point is that detection of fluoxetine in the plasma should have been possible based on data from humans and model mammals.

As I was unable to detect fluoxetine in plasma, it is difficult to compare the mammalian plasma/serum/blood values e.g. (Caccia et al. 1990; Holladay et al. 1998; Mukherjee et al. 1998; Pawluski et al. 2014) to the starling data collected here. The reasons why fluoxetine could not be detected in starling plasma were 1) the ELISA appears to not work well for birds (intra assay variation

28%) and 2) concentrations were much lower than would be expected based on the human therapeutic plasma concentration. Brunswick et al. (2002) quote the stable human therapeutic plasma concentration to be around 89 ng/mL. This stable concentration is reached after around four weeks of treatment. The elimination half-life is one to four days in humans (Hiemke and Hartter 2000). Using the first-order kinetics equations presented by Lazarus et al. (2014) (see Equation 2.2), I calculated the human therapeutic plasma concentration $_{T}$PC to be 70 ng/mL for an adult weighing 70 kg (if the elimination half-life is four days and the daily dose is 20 mg). I calculated the Human $_{T}$PC to be 109 ng/mL if the dose is 60 mg/day (four-day elimination half-life). Therefore there is a good match between the human therapeutic plasma concentrations calculated using Equation 2.2 and those quoted in the literature (Brunswick et al. 2002). When plasma concentrations were calculated for starlings using the same first-order kinetics equations, a daily dose of 1300 ng and the elimination half-lives ranging from 5 hours (Caccia et al. 1990) in rats and 12.9 hours in mice (Holladay et al. 1998) then the expected plasma concentrations for starlings ranges from 18.5 to 24.1 ng/mL (lowest = rat $t_{1/2}$ and a blood sample taken after the two-day rest, highest = mouse $t_{1/2}$ and blood sample taken after five consecutive days of dosing). Therefore the starling plasma concentrations at around the LOD were clearly much lower than what is expected based on humans and model mammals.

It was decided to use the ELISA for analysis of plasma as the sensitivity (1 ng/mL) was theoretically good enough to see down to the expected level (18.5-24.1 ng/mL). However, cross reactivity meant that the ELISA did not work well on plasma collected at baseline, five-days and after eight-weeks. With hindsight, time and money would have been better used developing Solid Phase Extraction and LCMS/MS methods. The 16 week (end) plasma has now been through the same solid phase extraction procedure as the tissue samples (see section 2.2.6c) and I am awaiting the results from LCMS/MS analysis using highly sensitive instrumentation (Waters Xevo TQS tandem quadrupole mass spectrometer coupled to an Acquity UPLC I-Class system (Waters, Manchester, UK) in 2015. Using this instrumentation, I should be able to see down to the sub pg/mL level required for detection in plasma. These results will be integrated into the publication that will result from this Chapter at a later date.

### 2.3.3 Tissue accumulation: did fluoxetine accumulate in starling tissues to the levels expected based on the dose and what is found in humans and model mammals?

Both parent compound and active metabolite were detected in all four tissues types collected from birds euthanized within two hours of administering the final dose (Figure 2.4, Table 2.2).

As tissues can only be obtained following euthanasia, many mammalian studies only look at accumulation of fluoxetine in plasma. The high volume of distribution of fluoxetine means detection

in tissues is more likely than in plasma (Hiemke and Hartter 2000). An Eli Lilly study in 1974 (Parli and Hicks 1974) that looked at ADME in rats and dogs is cited widely in the literature e.g. (Caccia et al. 1990; Holladay et al. 1998) but appears to be unavailable via the Web of Knowledge and Google searches. The other studies that have looked at accumulation of fluoxetine in tissues in mammals only looked at accumulation in the brain e.g. (Caccia et al. 1990; Holladay et al. 1998; Mukherjee et al. 1998). In humans Karson et al. (1993) looked at accumulation in the brain while Lewis et al. (2007) provide the most extensive tissue data from a study looking at residues in airline pilots involved in fatal crashes. These data for humans and model mammals were collated and compared with the data I collected for starlings (Table 2.2).

In comparison to human data it is clear that accumulation of both fluoxetine and norfluoxetine in starling tissues was typically one to two orders of magnitude lower (Karson et al. 1993; Lewis et al. 2007). In rat brains Caccia et al. (1990) found levels of fluoxetine accumulation to be around four times higher than I did for starlings, although this level of accumulation is contradicted by Mukherjee et al. (1998), a study which found levels of fluoxetine accumulation in rat brains were similar to, if not lower, than I found. The work of Mukherjee et al. (1998) and Caccia et al. (1990) highlights the variability in fluoxetine accumulation even within the same species. It is possible that the reason for different accumulation levels in the brain of rats (Caccia et al. 1990; Mukherjee et al. 1998) is a result of certain studies targeting different areas of the brain for extraction. In my study, the brain was simply sliced in half lengthways so that ether the left or right side was extracted. However Mukherjee et al. (1998) looked at uptake into different areas of the rat brain, finding that lipid rich (and serotonin receptor poor) regions of the brain such as the cerebellum have high accumulation compared to the areas with lower lipid content and greater density of receptors such as the hypothalamus.

Another factor that was not quantified in the studies presented in the literature, but may be an important reason for the differences in accumulation are differences between species in internal uptake. Internal uptake of an oral dose depends on both bioaccessibility from the food item and bioavailability of the pharmaceutical; a dose injected into the blood stream is 100% bioavailable. Therefore one challenge with reading-across between accumulated tissue concentrations is that it is difficult to account for these differences in internal uptake. This study used oral ingestion to mimic uptake by birds in the natural environment but many of the mammalian studies use intraperitoneal injections which will have greater levels of internal uptake e.g. (Caccia et al. 1990; Holladay et al. 1998; Mukherjee et al. 1998).

**Table 2.2 Accumulation of fluoxetine (flx) and norfluoxetine (Nflx) in plasma and tissues for starlings, humans and model mammals The level of accumulation is related to dose and body mass using the following equation to enable comparison between species (Conc. in tissue/(dose/body mass)): The approximate percentage accumulation per gram of body mass is given in brackets and was calculated using the following body masses: starling 70 g, human 70 kg, rat 300 g, mouse, 15-30 g, the body mass of dogs not given in the source of the data. Data collected by this study are given in bold.**

| Species | Flx or Nflx | Dose | Plasma | Brain | Kidney | Liver | Muscle | Source |
|---|---|---|---|---|---|---|---|---|
| **Starling** | **Flx** | **1300 ng (Oral)** | **<LOD (LOD = 1 ng/mL** | **3.1 ng/g (16%)** | **7.3 ng/g (39%)** | **14.4 ng/g (78%)** | **3.0 ng/g (16%)** | **My study** |
| | **Nflx** | | **Not measured** | **0.2 ng/g (0.9%)** | **1.3 ng/g (7%)** | **25.6 ng/g (137%)** | **10.5 ng/g (57%)** | |
| Human | Flx | 20-60 mg/day (Oral)<br><br>20 mg/day (Oral)<br><br>20-40 mg/day (Oral) | 430 ng/mL (blood)<br><br>89-100 ng/mL<br><br>90-250 ng/mL | 4,200 ng/g (490%) | 3,000 ng/g (350%) | 9,000 ng/g (1050%) | 800 ng/g (93%) | 1<br><br>2<br><br>3 |
| | Nflx | 20-60 mg/day (Oral)<br><br>20 mg/day (Oral) | 410 ng/mL (blood)<br><br>114-130 ng/mL | 6,500 ng/g (758%) | 3,500 ng/g (408%) | 13,500 ng/g (1575%) | 700 ng/g (82%) | 1<br><br>2 |
| Rat | Flx | 5 mg/kg/day (Oral)<br><br>10 mg/kg/day (Oral | 60 ng/mL (serum)<br><br>80 ng/mL (serum) | | | | | 4, 5 |
| | Nflx | 5 mg/kg/day (Oral)<br><br>10 mg/kg/day (Oral | 210 ng/mL (serum)<br><br>250 ng/mL (serum) | | | | | |
| Mouse | Flx | 20 mg/kg intraperitoneal injection<br><br>12.5 µg/hour subcutaneous infusion | 1,370 ng/mL (7%) 30 mins after dose<br><br>280 ng/mL | 31,300 ng/g 30 mins after dose (157%)<br><br>6,300 ng/g | | | | 6 |
| | Nflx | | 210 ng/mL (2%) 30 mins after dose<br><br>470 ng/mL | 3,970 ng/g 30 mins after dose (20%)<br>8,900 ng/g | | | | |
| Dog | Flx | 8 mg/day (oral) | Not detected after 24 hr | | | | | 7 |

1.Lewis et al. (2007); Brunswick et al. (2002); 3. Karson et al. (1993); 4. Pawluski et al. (2014); 5. Caccia et al. (1990); 6. Holladay et al. (1998); 7. EMEA (2008)

### 2.3.4 Relative distribution of fluoxetine between tissues of key organs and blood for starlings compared with model mammals and humans

For starlings the highest fluoxetine concentrations in the tissues collected were found in liver, followed by the kidney; levels in the brain and muscle were very similar (Figure 2.4a). For norfluoxetine, the order from highest to lowest concentration was liver > muscle > kidney > brain. As the brain is the main location of the serotonin receptors upon which fluoxetine acts (Lesch et al. 1991), distribution into the brain is of particular interest. Table 2.3 shows that the distribution coefficients for brain to plasma (or serum) range from 3.3 to 40 for model mammals. This compares to an indicative value of 6.1 for starlings (indicative value calculated using half the LOD for plasma to calculate the brain to plasma distribution ratio) and so the brain: plasma distribution ratio is likely to be at the lower end of the range of distribution coefficients for model mammals.

As the brain is the primary site for fluoxetine's pharmacological activity particular attention was paid to distribution into the brain. Distribution is normally expressed as a ratio of tissue concentration relative to blood/plasma or serum concentration e.g. (Lewis et al. 2007). A volume of distribution, which is the theoretical volume that would be required for the drug concentration to be equal to plasma (i.e. the larger the volume of distribution, the more it distributes into tissues and the lower the concentration in plasma), may also be calculated by pharmacokinetic studies. Fluoxetine is said to be reasonably lipophilic and so has a large volume of distribution in humans of up to 100 L/kg (Hiemke and Hartter 2000). Therefore, given the low fluoxetine and norfluoxetine tissue concentrations obtained from the starlings it is not surprising that I had difficulties detecting fluoxetine in the plasma (see section 2.3.2).

The different routes of administration could go some way to explaining the differences between Holladay et al.'s (1998) brain distribution values and mine. For example, Holladay et al. who found a brain: serum ratio of over 20 used an intraperitoneal injection, which will have higher bioavailability than the oral dose I administered to starlings. Another possible reason for the lower accumulation in starling brains relative to mice is the assumption of plasma concentrations being at half the LOD (0.5 ng/mL) may well over estimate plasma concentrations. For this second reason, I decided not to attempt to calculate the volume of distribution for starlings. The lower than expected accumulation in starling tissue (Table 2.2) suggests that plasma concentrations would have to be 0.1 ng/mL or lower in order for the volume of distribution to be similar to mammals and humans.

**Table 2.3: The relative distribution ratios of fluoxetine (Flx) and norfluoxetine (Nflx) into key tissues relative to plasma or serum. Data are given for starlings (data collected by this study in bold), humans and model mammals. *As fluoxetine could not be detected in starling plasma, the level in plasma was assumed to be half the LOD to give an estimate which can be compared across species; these data are indicative only of what the tissue to plasma ratio might be. As plasma concentrations were not measured for norfluoxetine in starling plasma, distribution ratios were not calculated for norfluoxetine.**

| | | Distribution coefficient (tissue ng/g divided plasma ng/mL | | | | |
|---|---|---|---|---|---|---|
| Species | Flx or Nflx | Brain | Kidney | Liver | Muscle | Source |
| **Starling** | **Flx** | **6.1*** | **14.7*** | **28.8*** | **5.9*** | **This study** |
| | **Nflx** | ND | **ND** | **ND** | **ND** | |
| Human | Flx | 15<br><br>20 (combined Flx and Nflx) | 9 | 38 | 2.2 | 1<br><br>2 |
| | Nflx | 18 | 9 | 42 | 2 | 1<br><br>3 |
| Rat | Flx | 20 to 40<br>3.3-10 | - | - | - | 4<br>5 |
| | Nflx | 20 to 40 | - | - | - | 4 |
| Mouse | Flx | 22.9-26.5 (serum | - | - | - | 6 |
| | Nflx | 14.1-20.6 (serum) | - | - | - | |

1. Lewis et al. (2007); 2. Brunswick et al. (2002); 3. Karson et al. 1993; 4. Caccia et al. (1998); 5. Mukherjee et al. (1998); 6. Holladay et al. (1998)

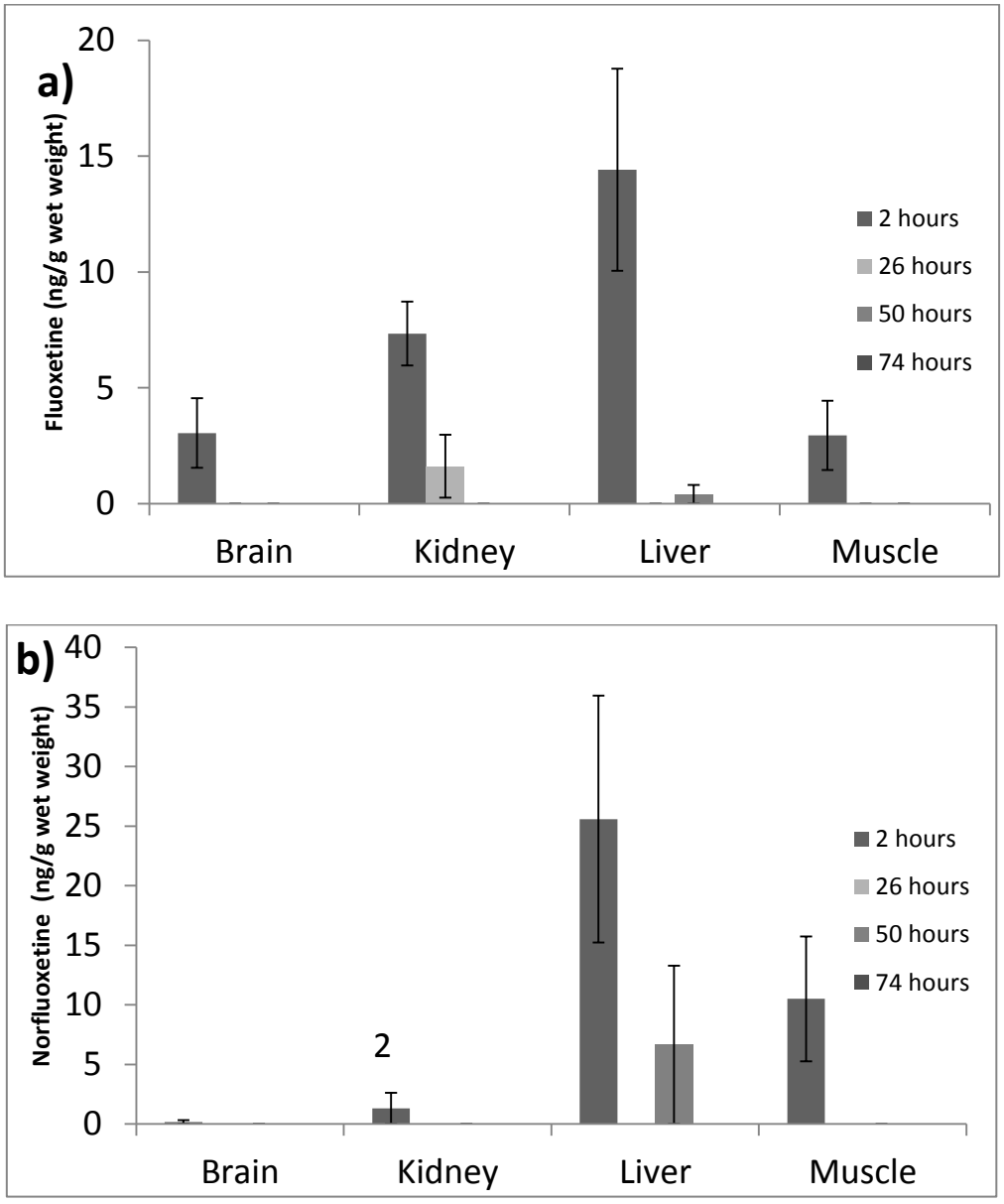



**Figure 2.4: Mean (n = 3) ±1 Standard Error fluoxetine (a) and norfluoxetine (b) concentration (ng/g wet weight) in starling brain, kidney, liver and muscle from birds euthanized 2, 26, 50 and 74 hours after the final dose. The daily dose was 1300 ng/day administered five days a week for 22 weeks (i.e. average daily dose 928 ng/day). Numbers above bars indicate the number of samples that were not included in the mean as they fell below LOQ but concentrations were detected above LOD. For norfluoxetine in kidney after 2 hours, the standard error was calculated by including the values below the LOQ.**

### 2.3.5 Metabolism: were the relative concentrations of fluoxetine and norfluoxetine in starling tissues what would be expected based on data from humans and model mammals?

The ratio of fluoxetine to norfluoxetine in the brain was 19.63, for kidney it was 2.27, for liver it was 0.58 and for muscle it was 0.22 (i.e. norfluoxetine was higher than fluoxetine in liver and muscle). In humans, Lewis et al. (2007) found the fluoxetine to norfluoxetine ratios to be 0.65, 0.85, 0.67 and 1.14 for brain, kidney, liver and muscle respectively. Therefore, with the exception of the liver, the

different ratios of fluoxetine and norfluoxetine in tissues indicate different distribution and/or metabolism between species.

In humans the main enzymes responsible for fluoxetine –*N*-demethylation to norfluoxetine are CYP 3A4, 2C9 and 2D6 (Margolis et al. 2000) although 3A5 and 2C19 are also involved (Drugbank 2009)(see Table 2.4). CYP3A4 and 2D6 are also involved in the production of hippuric acid and *para*-trifluoromethylphenol. Conjugation to fluoxetine and norfluoxetine glucuronide involves UDP-glucuronosyltransferase (UDP-GT) (Drugbank 2009). No data on which enzymes are specifically involved in the production of norfluoxetine acid and norfluoxetine alcohol could be found (Drugbank 2009).

Information on the enzymes possessed by starlings are not available at present and so data from a study on birds (Chicken *Gallus gallus*, Zebra finch *Taeniopygia guttata* and Turkey *Meleagris gallopavo*) was used (Watanabe et al. 2013). Birds do not possess many of the enzymes responsible for metabolism of fluoxetine in humans, particularly CYP 2D6, 3A4 and 3A5. Some of those they do possess (CYP 2C9 and 2C19 are located on different parts of the genome to humans (Watanabe et al. 2013). Rats and mice also do not possess many of the enzymes responsible for fluoxetine metabolism (Martignoni et al. 2006), see Table 2.4); in fact, rodents appear to possess even less than birds which is surprising given the fact that rodents are used in pre-clinical trials due to their similar physiologies to humans (Rand-weaver et al. 2013). The fact that birds appear to have more similarities with humans than rodents is possibly an artefact of the data available in the literature, although it is worth noting that both birds and rodents possess only one of the main enzymes responsible for fluoxetine metabolism (shaded grey in Table 2.4). Additionally, both rodents and birds have some level of UDP-GT activity which is responsible for biotransformation to glucuronide conjugates (Drugbank 2009; McLeary 2001). Therefore I would have expected to see glucuronide metabolites in the starling tissues and faeces.

**Table 2.4: Do birds and model mammals possess the enzymes responsible for metabolism of fluoxetine in humans? The main enzymes are given in bold. The avian data was collected by other studies in model species as no starling data were available. CYP = cytochrome P 450, UDP-GT = UDP Glucurosyltransferase. The columns of the main enzymes involved in metabolism are shaded in grey.**

| Humans | CYP 2C9 | CYP 2D6 | CYP 3A4 | CYP 2C19 | CYP 3A5 | UDP GT | Source |
|---|---|---|---|---|---|---|---|
| **Birds (Turkey, chicken and Zebra finch)** | **Yes** | **No** | **No** | **Yes** | **No** | **Yes** | **1, 2** |
| Rats | No | Some expression | No | No | No | Some | 2, 3 |
| Mice | No | Some expression | No | No | No | Some | |

1. Watanabe et al. 2013; 2. McLeary (2001); 3 Martignoni et al. (2012)

### 2.3.5a: Could any of the six inactive metabolites of fluoxetine be detected in starling tissue and faeces?

None of the six metabolites searched for were detected in any of the tissue (brain, liver, kidney and muscle) samples analysed by ICR-FT-MS. While, the sensitivity of ICR-FT-MS is not as good as LC-MS/MS and so the absence of detection may not mean the metabolites are not there; the fact that fluoxetine (but not norfluoxetine) could be detected by ICR-FT-MS suggests that if these metabolites are present, concentrations will be much lower than observed for the parent compound. It is interesting that I was unable to detect any of these six metabolites in starling tissues as it appears they are extensively produced in humans (Drugbank 2009 also see section 2.3.6).

In humans, metabolites other than norfluoxetine are detected at levels that are equivalent to a significant proportion of the administered dose. However, as I was unable to detect any of these metabolites in starling tissue or faeces, it suggests that their production is not significant. The fact that Watanabe et al. (2013) suggest that starlings do not possess three of the five enzymes responsible for metabolism of fluoxetine (and two of the main enzymes responsible for fluoxetine metabolism are included in the three enzymes that starlings do not possess) suggests metabolism of fluoxetine is likely to be very different between humans and birds.

## 2.3.6 How did the elimination of fluoxetine and norfluoxetine from tissues compare between starlings and mammals?

The tissue and faecal concentrations at the four time points were used to calculate elimination half-lives. When nothing could be detected the level was assumed to be half the limit of detection.

Elimination of the parent compound and active metabolite was found to be rapid and in samples taken after 26 hours, fluoxetine was only detected in muscle and kidney and no norfluoxetine was found (Figure 2.4). After 50 hours, fluoxetine and norfluoxetine were both detected in the liver even though neither were detected the day before, indicating depuration and accumulation is highly variable between individuals. Neither fluoxetine nor norfluoxetine could be detected in any of the tissue samples seventy-four hours after the final dose. Elimination half-lives for fluoxetine and norfluoxetine from starling tissue were calculated using Equation 2.4. For fluoxetine the elimination half-lives were as follows: brain = 3.1 h, kidney = 8.3 h, liver = 8.4 h, muscle = 10.8 h) while for norfluoxetine the elimination half-lives were: brain = 5.4 h, kidney = 6.7 h, liver = 8.7 h and muscle = 3.3 h.

In model mammals and humans, no data on elimination half-lives in tissues could be found and so I was only able to compare with plasma elimination half-lives. In rats the elimination half-lives of fluoxetine from plasma are 5 and 15 h respectively (Caccia et al. 1990) while in mouse plasma the elimination half-life of fluoxetine is 12.9 hours (Holladay et al. 1998). These elimination half-lives in rodents are similar to what I found for starling tissue. Mice have a similar Basal Metabolic Rate (BMR) to starlings (Koteja 1991) (Rat BMR not presented by Koteja 1991). Humans have a BMR an order of magnitude lower than starlings and mice and a plasma elimination half- life around an order of magnitude higher for fluoxetine (24-96 h) and one to two orders of magnitude higher for norfluoxetine (168-360 h). Therefore the elimination rate appears to be inversely related to BMR. Different metabolic pathways between birds and mammals is supported by the fact that the proportion excreted unchanged by starlings was higher than most human studies quote e.g. (Hiemke and Hartter 2000; Jjemba 2006) and also higher than has been found for dogs (EMEA 2008).

### 2.3.7 How did the proportion of the dose excreted unchanged by starlings compare with humans and model mammals?

The mean concentration of fluoxetine excreted unchanged within two hours of the final dose was 1487 ng/g. For norfluoxetine only 2.25 ng/g was measured (i.e. 660 times higher for fluoxetine than norfluoxetine) (Figure 2.5). The large error bars for fluoxetine indicates that the proportion of fluoxetine excreted as the parent compound was highly variable between individuals, which is also typical for humans (see Table 2.5 and Hiemke and Hartter 2000; Jjemba 2006; Lienert et al. 2007). Given that the mean dry faecal mass produced by starlings was 0.169 g, a concentration of 1487 ng/g equates to around 19% of the dose (the dose was 1300 ng five days per week). As elimination half-lives are so short, then virtually no fluoxetine from the previous day's dose remains in the system. For the purpose of working out the proportion excreted unchanged, the mass detected in faeces was expressed relative to 1300 ng and not the mean daily dose of 928 ng ) excreted as the unchanged

parent compound and less than 0.01% as norfluoxetine (Table 2.5). As with the tissue samples, none of the six metabolites searched for in faecal samples analysed by ICR-FTMS were detected which is interesting given that their presence in faeces is equivalent to a significant proportion of the dose.

**Table 2.5: The proportion of the dose excreted as parent compound (Flx) and metabolites (NFLX= Norfluoxetine, Flx gluc. = Fluoxetine glucuronide, Nflx gluc. = norfluoxetine glucuronide, HA= Hippuric acid, NFLX alc. = norfluoxetine alcohol, Nflx acid = norfluoxetine acid, P-tml = Para –trimethylphenol) by starlings, humans and model mammals. Nd = not detected, no data for fields with a – in them.**

| Species | Flx | Nflx | Flx gluc. | Nflx gluc. | HA | Nflx alc. | Nflx acid | P-tml | Source |
|---|---|---|---|---|---|---|---|---|---|
| **Starlings** | **19%** | **0.1%** | **Nd** | **Nd** | **Nd** | **Nd** | **Nd** | **Nd** | **My study** |
| Humans | 5-26% | 6.8-25% | 7.4-25% | 8.2-25% | >20% | Up to 46% combined | | 1-5% | 1, 2, 3 |
| Dogs | 10.9 % | - | - | - | - | - | - | - | 4 |

1. Jjemba et al. (2006); 2. Lienert et al. (2007); 3. Drugbank (2014); 4. EMEA (2008)

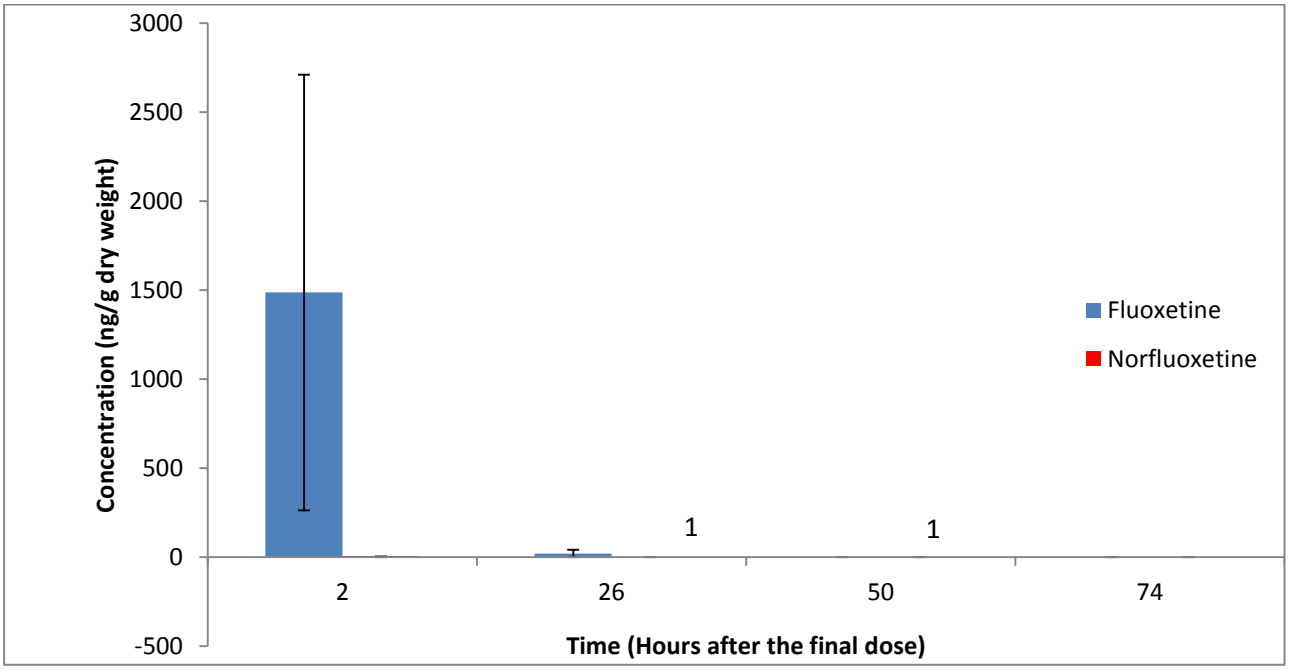



**Figure 2.5: Mean (n=3) ±1 Standard Error faecal concentration of (a) fluoxetine and (b) norfluoxetine ng/g dry weight for birds euthanized 2, 26, 50 and 74 hours after the final dose. The mean dry mass of faecal samples collected in the 2 hours between dosing and euthanizing the birds was 0.169 g. Numbers in place of bars represent the number of samples that exceeded LOD but not LOQ.**

### 2.3.8 Implications of results for the read-across hypothesis

Fluoxetine was found to accumulate in plasma and tissues to a lower extent than was predicted based on the dose and what is known about accumulation in humans and model mammals - particularly the relative distribution into the brain, the organ containing the receptors fluoxetine targets, was much lower than expected. The relative concentrations of fluoxetine and norfluoxetine were very different from that which is found in humans and none of the inactive metabolites could be detected suggesting that their presence is unlikely to be significant in starlings. Elimination rates were found to be much more rapid for starlings than for humans, but were similar to those found for rodents. The proportion of the dose excreted unchanged was at the upper end of what is found in humans and higher than what has been found for dogs. As a significant proportion of the dose (19.3%±15.3%) was recovered in the faeces of starlings after only 2 hours (compared to 5-26% and 10.9% in humans and dogs after 24 hours (Jjemba 2006; Lienert et al. 2007; EMEA 2008)), and metabolite profiles were different in starling tissues and faeces compared to humans and mammals. Different metabolic processes appear to be the most likely explanation for the observed differences in ADME between species.

Birds do not possess many of the CYP 450s that humans do (and neither do rodents). It is plausible that different metabolic pathways combined with the higher BMR are responsible for the lower accumulation observed in starlings. The higher the BMR, the quicker the metabolic processes will occur and the faster the drug will be eliminated. The starling metabolic rate (43 kJ/hr/kg body weight) is an order of magnitude higher than it is for a human (≈3.6 kJ/hr/kg body weight) and is even 30% higher than it is for a mouse (33 kJ/hr/kg body weight) (Koteja 1991). The influence of BMR is supported by the differences in elimination half-lives I found between starlings and humans.

Another potential explanation for the lower levels of accumulation in birds than in humans is differences in bioavailaibility between species as fluoxetine is a basic pharmaceutical (Pka 10.06 Brooks et al. 2003), fluoxetine would be primarily in the ionised form (and polar) in the acidic environment of the gastrointestinal tract. The ionised form of the drug is more polar than the non-ionised form; this may lead to insufficient time at the absorption site (Oh 2002). As the avian gastrointestinal tract (Martinez-Haro et al. 2009) is more acidic than the human gastrointestinal tract (Ruby et al. 1999), then bioavailability could be lower for birds than humans. In Chapter 5, I assess bioaccessibility (but not bioavailability) to provide an indication as to whether differences between species in the amount of fluoxetine available for uptake could have contributed to the lower level accumulation of fluoxetine observed in starlings than humans.

### 2.3.9 Wider implications and recommendations for further work

This study demonstrates the potential for read-across from mammalian to avian species for the assessment of the risks posed by pharmaceuticals in the environment. In starlings, the low level accumulation of fluoxetine in the brain in combination with the lengthy exposure period mean that it is plausible that neurological adaptations could occur and lead to birds exhibiting mode of action effects (see Figures 1.5-1.7 in Chapter 1). However, the rapid elimination means that in order for any effect I observe in the laboratory to be translated to the wild, birds would have to be feeding each day on fluoxetine contaminated invertebrates to maintain fluoxetine in their system. Pharmacological effects at this low level of internal concentration are plausible if the receptors of birds are more sensitive to fluoxetine than humans, something which has been found previously in birds for other pharmaceuticals (Hutchinson et al. 2014). Determining the extent to which individual birds routinely forage on wastewater treatment plants is likely to be a challenging one, but one that I believe could be overcome through a combination of technology (e.g. an extensive passive integrated transponder tag system) and skilled and experienced labour (see Chapter 6). Given the rapid elimination of both fluoxetine and norfluoxetine and their high volumes of distribution which makes detection in plasma unlikely (Hiemke and Hartter 2000) then faecal sampling or rinsing cloaccal swabs may provide a useful, non-invasive way of assessing the extent to which the potential risk identified here is likely to occur in wild birds. Assessing the risks pharmaceuticals pose to terrestrial wildlife species is an important knowledge gap (Shore et al. 2014). To conduct these environmental risk assessments, data on levels of exposure and the resulting internal concentrations and pharmacokinetics are required (see Chapter 6). Once data for a range of wildlife and model species have been collected, it would then be possible to read-across with greater certainty and extend the coverage of environmental risk assessments for pharmaceuticals for a wider range of terrestrial wildlife species.

# Chapter 3:

# Behavioural and physiological responses of birds to environmentally relevant concentrations of an antidepressant


## Abstract

Many wildlife species forage on sewage-contaminated food, e.g. at Wastewater Treatment Plants and on fields fertilised with sewage sludge. The resultant exposure to human pharmaceuticals remains poorly studied for terrestrial species. Based on predicted exposure levels in the wild, I administered the commonly prescribed antidepressant fluoxetine or control treatment via prey to wild-caught starlings (*Sturnus vulgaris*) for 22 weeks over winter. To investigate responses to fluoxetine, birds were moved from their group aviaries into individual cages for two days. Boldness, exploration and activity levels showed no treatment effects but controls and fluoxetine-treated birds habituated differently to isolation in terms of the concentration of Corticosterone (CORT) metabolites in faeces. The controls that excreted higher concentrations of CORT metabolites on day 1 lost more body mass by day 2 of isolation than those which excreted lower levels of CORT metabolites. CORT metabolites and mass loss were unrelated in fluoxetine-treated birds. When I investigated the movements of birds in their group aviaries, I found the controls made a higher frequency of visits to food trays than fluoxetine-treated birds around the important foraging periods of sunrise and sunset, as is optimal for wintering birds. Although individual variability makes interpreting the sub-lethal endpoints measured challenging, my data suggest that fluoxetine at environmentally relevant concentrations can significantly alter behaviour and physiology.


## 3.1 Introduction

In Chapter 1, the selective serotonin re-uptake inhibitor (SSRI) antidepressant fluoxetine (Prozac) was identified as the priority compound. Fluoxetine, along with other antidepressants, has been detected in effluent and surface water at concentrations up to the µg $L^{-1}$ level and in fish tissue at the ng $g^{-1}$ level (Brooks et al. 2005; Calisto and Esteves 2009). Fluoxetine prevents the re-uptake of serotonin by the pre-synaptic cells in the brain, thus increasing the neurotransmission of serotonin (Lesch et al.

1991). Fluoxetine is commonly used to treat anxiety-related conditions such as depression, obsessive compulsive disorder and bulimia (Eli Lilly 2009). Moreover, there is growing evidence that SSRI antidepressants not only reduce depression and anxiety, but can also change personality traits, for example resulting in people becoming less neurotic and more extroverted (Du et al. 2002; Tang et al. 2009). In animals, personality traits are defined as behavioural differences that are stable within individuals measured repeatedly across a range of situations or contexts (Gosling 2001), and include aggression, activity, exploration and boldness (Bell and Sih 2007; Sih et al. 2004). An individual's combination of personality traits essentially determines how it will cope with environmental and social stressors. Thus, exposure to fluoxetine could alter behavioural responses to stimuli and potentially also personality traits in non-target wildlife (Gross-Sorokin et al. 2006).

Fluoxetine also induces a range of side effects in humans, for example sexual dysfunction (Clayton 2002), lethargy (Eli Lilly 2009) and weight and appetite changes are all common (Fichtner and Braun 1994). This is most likely due to the connection between the serotonergic system and the neuroendocrine system (Raap and Van de Kar 1999, see Figure 1.7 in Chapter 1). If fluoxetine causes some of the changes to behaviour and physiology in free-living animals that are commonly observed in humans, then there could be negative implications for survival chances. In the wild, food resources vary and are inevitably probabilistic, so in a wide range of taxa (from bees, fish, birds to mammals), animals are generally risk averse unless they are at a high risk of starvation (Caraco et al. 1980; Kacelnik and Bateson 1996). Altered activity levels, food intake, mass balance, and stress-responsiveness are predicted to shift how an individual manages the trade-off between starvation and predation risk. In birds, there is a very finely balanced trade-off between maintaining the fat reserves required to prevent starvation, particularly in cold weather, and retaining the ability to fly away from predators (Wingfield and Romero 2001; Kitaysky et al. 2006; Kitaysky et al. 2001; Kitaysky et al. 2007; Muller et al. 2007; Romero et al. 2000). The physiological mechanisms underpinning this trade-off involve corticosterone (CORT) which is the main glucocorticoid in birds. Levels of CORT are known to affect body mass and behaviour in birds (Roberts et al. 2007a; Roberts et al. 2007b). CORT is elevated in response to environmental perturbations to adjust physiology and behaviour, e.g. foraging, appropriately for the prevalent conditions (Wingfield and Romero 2001). The release of CORT promotes gluconeogenesis, which can mobilise fat reserves for energetically demanding activities such as escaping predators (Wingfield and Romero 2001) and responding to thermal conditions. CORT responses to environmental stressors are, therefore, predicted to be affected by exposure to fluoxetine.

Unlike previous studies on fluoxetine in terrestrial species, (Sperry et al. 2003) I assessed the effects of exposure to environmentally relevant levels of fluoxetine, administered in prey items over an ecologically relevant period of time, on wildlife using starlings as the test species. Starlings are a good model as they forage on WWTPs throughout the year (Fuller and Glue 1978) with up to 50% of their diet coming from trickling filters in the breeding season (Markman et al. 2008). To maintain body mass in captivity, starlings must consume at least 60% of their diet dry mass as invertebrates, this equates to about 35 g of larvae per day (Feare and McGinnity 1986); higher energetic demands in the wild will inflate this value further (Tinbergen 1981). Thus I assumed that wild starlings would consume 45-50 g of invertebrates per day (wet weight), with 50% coming from trickling filters (see Chapter 2 section 2.2.1 and 2.3.1 for details of the predicted dose). The fluoxetine concentration in invertebrates was predicted and evaluated as environmentally relevant in Chapter 2. A second reason for selecting starlings is that their behaviours are relatively well characterised (Minderman et al. 2009).

Overall, I aimed to investigate whether exposure to fluoxetine modified behavioural and physiological end points. Specifically I addressed whether experimental exposure to fluoxetine altered: i) Diurnal variations in visits to food trays; ii) Risk taking behaviours and personality traits (exploration in a novel environment and boldness); iii) Activity levels; iv) Physiological stress responses to isolation; v) The relationship between CORT metabolite levels in isolation and change in body mass.

## 3.2 Materials and Methods

The same twenty-four wild starlings as referred to in Chapter 2 were used in this experiment. The structure of the experiment is outlined in Figure 1.9 (Chapter 1); briefly behaviour and physiology were measured on consecutive days, referred to as Day 1 and Day 2 (Day 0 is the day before Day1 on which body mass was measured) at Baseline (pre-treatment) and End (after 16 weeks of treatment). Birds were dosed with 1300 ng/worm fluoxetine five days a week i.e. 928 ng/day by capturing from their home aviaries with a hand net (see sections 2.2.1, 2.2.3 and 2.3.1 for details of the dosing). While every care was taken to minimise the stress of capture and handling (the help of an experienced animal technician was used to capture and feed birds), capture and handling are likely to represent stressors to which birds are unlikely to fully habituate (Herborn et al. 2014). Total capture time was typically less than 20 minutes and it usually took around ten seconds to remove a bird from its bird bag, feed it a worm and release it to its home aviary. Both treatment groups experienced the same capture process. Many individuals voluntarily took their treated invertebrate from the forceps (see Chapter 2, Figure 2.2).

### 3.2.2 Diurnal variation in foraging

In order to assess diurnal variation in foraging behaviour of individuals in their home aviary, each bird was fitted with its own unique Passive Integrated Transponder Tag (see Chapter 2, section 2.2.3b) and a system of electronic tag readers (Figure 3.1 shows a tag reader on one of the perches). Two antennas (8 cm x 5 cm; Trovan, www.trovan.com) were positioned flat in the two food trays (40 cm x 20 cm x 6 cm). The monitoring system was set up to read at one second intervals, recording the unique PIT tag code along with a date and time thus enabling me to calculate the total number of feeding visits (a visit was classed as an absence of > 4 s, based on pilot data) per bird per hour. After 48 hours of acclimatisation to the recording equipment, visits to feeders were recorded for 48 hours. The readers were rotated around the aviaries so that foraging behaviour was recorded twice per aviary for a period of two days between 15th February and 26th April 2012.

**Figure 3.1: A Passive Integrated Transponder tag reader covered by a waterproof bag, positioned under an uncovered perch in one of the home aviaries. In this case, the antennas were positioned on the perch to record movements of birds when they came into range. A similar set up was also used to monitor visits to the food tray.**

### 3.2.3 Behavioural assays

At the baseline and end trials, behavioural and physiological responses of individuals to standardised stressors were assayed in isolation over a two day period. The test cages (Kent Cages, Kent, UK) (127 cm wide by 39 cm deep and 36 cm high) were positioned in an outdoor aviary so that birds were

exposed to natural weather and light conditions but visually occluded from other birds. The set-up of the outdoor test aviary is shown in Figure 3.2.

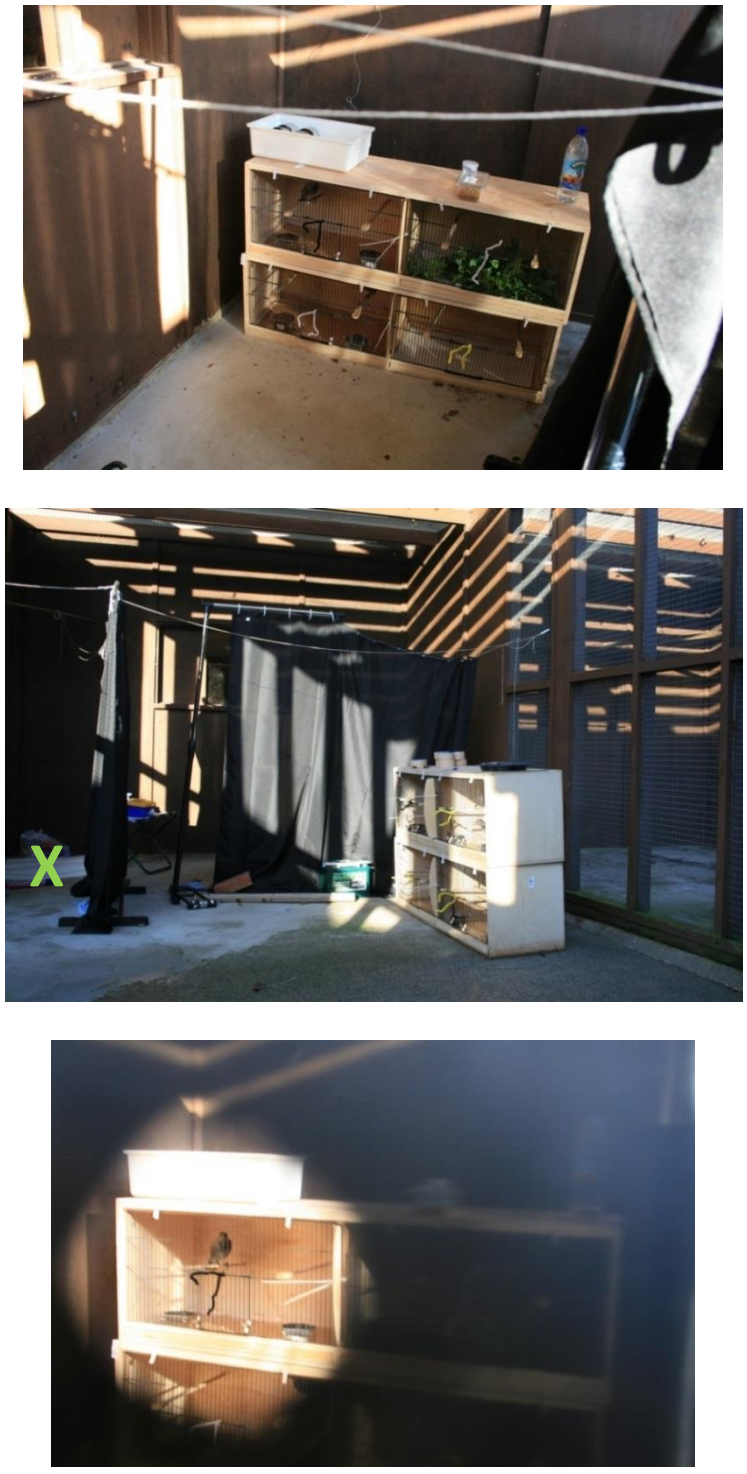



**Figure 3.2: The set-up of the outdoor test aviary for the assessment of activity, boldness and exploration. The aviary contained two banks of two cages divided by a screen. To observe the birds, I sat behind the left hand screen (marked with a green X). From this position, both banks of cages could be seen through a 2 cm × 2 cm hole.**

Exploratory tendency was assayed over two trials, one each on consecutive days (see Figure 1.9 in Chapter 1), at baseline and again at the end (adapted from Herborn et al. (2010)). Each bird was moved from its home aviary to one half of a randomly selected test cage at least one hour before dusk. By containing the bird within one half of the cage a familiar half and a novel half (behind the wooden divider) were created. One half was lined with white paper and contained two perches wreathed with vines of plastic 'sycamore' leaves while the other half had brown paper and plastic 'ivy' vines to create two 'habitats'. In other respects both halves of the cages were identical. The familiar half of the cage and the familiar 'habitat' type were randomly selected prior to the trial. Birds were provided with food *ad libitum* (usual diet of chick starter crumb, wild bird seed and insectivore mix, as well as a few meal worms) and water. The following morning ('day 1'), food and water were removed (typically between 08:00 and 09:00) an hour before the start of the trial to standardise hunger. All spilt food was removed from the cage bottom, faeces collected and the lining paper replaced (see section 3.2.2 'Corticosterone Metabolites').

To start the trial, the wooden divider between the two cage halves was removed and the observer retreated behind a screen with an observation hole (2 cm x 2 cm). When the bird was perched, a movement was defined as a hop or a flight. When on the ground any movement of the feet, or a flight, was defined as a movement, with the end point of a movement used to define its location (i.e. novel or familiar and ground or perched). When the end of a movement was on the central ridge of the cage (on average 4.8% of total movements at baseline and 7.6% at the end), then the direction in which the bird was facing defined the end point of the movement (novel ground or familiar ground). As starlings are ground feeders (Minderman et al. 2009), exploratory tendency was defined as the number of total movements on the ground in the novel half during the 10 minute trial as a proportion of the total moves (Minderman et al. 2009). General activity level was scored as the total number of movements in the 10 minute exploration trial. The exploration trial was repeated the following day ('day 2') at both baseline and end. For the end trials, after each exploration trial, each bird was captured and immediately given its experimental treatment before it was returned to its test cage. The bird then had access to the whole cage and was given one hour to feed and drink undisturbed before the boldness trial. Figure 3.3 shows the cage set up.

Forty-five to sixty minutes before the start of the boldness trials, the food (along with any spilt food) and water were removed to induce hunger. To start the trial, the food bowl containing three to four meal worms (*Tenebrio molitor*) as well as the usual food was returned to outermost side of the cage. The latency of the first approach to the food bowl (defined as less than one body length) was recorded. Birds that approached before the watch could be started were given a latency of 0.1s with

trials lasting for up to 1800s. Trials were terminated as soon as the bird had approached for a second time or after 1800s, whichever came first. The latency to approach rather than to feed was recorded as not all birds fed during the experiment (Herborn et al. 2010); however, in all trials latency to approach and latency to feed were correlated ($p < 0.05$ in all cases). After the day 1 trials the birds were switched between cages, the divider was replaced and the arrangements of habitats were changed in preparation for the exploration trial on day 2.

**Figure 3.3: A similar set up of the test cage to that used in the exploration trials (although food and water was not available during the exploration trials). The bird was isolated in one half of the cage overnight. The left and the right halves of the cage were different. Here the bottom cage has a left half with brown paper and sycamore shaped vine leaves while the right half has white paper and ivy shaped vine leaves. To start the trial, the wooden divide (resting at the left end of the cage here), was removed from the central wooden ridge where the bird is perched in this photo. The latency to enter the novel half was recorded as well as the total number of movements in the ten-minute trial.**

### 3.2.2 Corticosterone metabolites

I analysed faecal CORT metabolites rather than plasma CORT to avoid additional stress from handling and blood sampling which could have affected the second day's behaviour trials. Also, I wanted to measure how the birds habituated to individual isolation rather than testing their responses to repeated handling stress. At baseline and end trials, each bird was put in an individual test cage on the afternoon of day 0. The following morning (day 1) fresh faeces were removed from the paper lining the cage and placed in a 1.5 mL sealed tube. The lining paper was again replaced prior to dusk

on day 1 and the faeces collected the following morning. Faeces were placed into a freezer at -20°C, while behavioural measures were completed, before being weighed to ±0.001g later the same day and dried at 40°C until there was no change in mass. Dried faecal samples were stored at -20°C until analysis in September 2012. While there is a risk of freeze thaw artificially inflating levels of faecal Corticosterone metabolites, Washburn and Millspaugh (2002) found the changes due to freeze thaw to be relatively small and not too dissimilar to changes that take place over time anyway in white-tailed deer faeces (*Odocoileus virginianus*). Herring and Gawlick (2009) have found that faecal Corticosterone metabolites are stable in the freezer for 165 days for two species of bird (whistling ducks *Dendrocygna bicolor* and white ibis *Eudocimus albus*). Therefore, without the equipment being available for sample lyophilisation, it is unlikely that this procedure would have significantly inflated faecal glucocorticoids.

In the laboratory, analysis took place on consecutive days using the CORT OCTEIA ELISA kit (ID Labs, Boldon, UK). This ELISA has been validated for a range of species (Sensitivity = 11 ng $g^{-1}$, Mean recovery using faeces = 93%, Mean linearity = 100%, Intra assay mean = 4.95% CV, Inter assay mean = 7.9% CV).

### 3.2.3 Change in Body Mass

Body mass was recorded on in the late afternoon on day 0 and again at a similar time on day 2 just before birds were returned to their home aviaries.

### 3.2.4 Statistical Methods

All data were analysed using R version 3.0.2 (R Core Team 2013). To assess diurnal variation in feeder visits, I used a zero-inflated repeated measures model with a Poisson error structure in R-package glmmADMB. It was important to control for zeroes in the model as they were generated in different ways, e.g. a bird was not feeding or a bird was not in range of the antenna when it fed. For each day's data, I took the first and last four hours of recordings and took the number of visits per bird per hour as the response variable. I corrected time relative to sunrise and sunset respectively. Initially I looked for the linear and the quadratic relationship, however, only the quadratic relationship (indicative of the expected bimodal peaks in feeding effort around sunrise and sunset) was significant and so I dropped the linear relationship from the models. Treatment and day effects were tested using Generalised Estimation Equations (GEE) which were fitted with R package geepack (Højsgaard et al. 2006) to account for data from the same individual being repeated (i.e. day 1 and day 2). Repeatability between days was quantified using the effect of day in the GEE. The boldness and natural log-transformed activity data were normally distributed and exploratory tendency (number

of ground movements in novel half out of total number of movements) showed a binomial distribution. In each case, the difference between the treatment and control groups was tested using the Wald statistic produced by the GEE, by comparing it to a $\chi^2$ distribution with 1 degree of freedom. When analysing the changes in body mass in response to CORT, we used a Linear Model. In all the analyses below, factors were tested against the 5% significance level.

## 3.3 Results

### 3.3.1 Diurnal variation in foraging behaviour

Within their home aviaries, control birds visited the food trays more than the fluoxetine-treated birds (Z = -2.20, N = 768, p = 0.028). Time after sunrise and before sunset was also significant in the model (Z = -2.45, N = 768, p = 0.014). Variation in feeding rate was described by quadratic relationships with time which differed between treatment groups (Z = 2.53, N = 768, p = 0.011): Controls increased their frequency of visits to food trays in the three hours after sunrise (Fig. 3.4a) compared with fluoxetine-treated birds. There was a less pronounced peak in food tray visits just before sunset in controls but not fluoxetine-treated birds (Fig 3.4b).

### 3.3.2 Behavioural assays

For both baseline (Appendix B, Table B1) and end trials (Table 3.1), exploratory tendency, activity and boldness were repeatable between days 1 and 2. Exploratory tendency (Table 3.1 and Fig. 3.5a for end and Appendix B Table B1 in for baseline), activity levels (Tables 3.1 and A1 and Fig. 3.5b) and boldness (Tables 3.1 and B1 and Fig. 3.5c) did not vary with treatment, day (1 or 2) or in the way that the birds of the different treatment groups habituated from day 1 to day 2, as shown by the non-significant interactions for treatment and day in Table 3.1 (end) and Table B1 (baseline, Appendix B).

### 3.3.3 Corticosterone metabolites

The mean faecal CORT metabolite concentration was obtained from two replicates. For each sample, the variability between replicates was significantly repeatable (Single factor ANOVA) given the level of variation between individual birds (baseline day 1: $F_{23,24} = 33.95$, r = 0.94, $p < 0.001$; baseline day 2: $F_{23,24} = 34.51$, r = 0.94, $p < 0.001$; end day 1: $F_{23,24} = 33.06$, r = 0.94, $P < 0.001$ and end day 2: $F_{23,24} = 7.78$, r = 0.76, $p < 0.001$).

In the baseline trials, the levels of faecal CORT metabolites did not differ significantly between treatments or in the way it changed from day 1 to day 2 for the two treatment groups (Appendix B Table B1). In the end trials CORT metabolites were significantly lower for all birds on day 2 compared

with day 1 (Fig. 3.6a and Table 3.1). The variances in mean CORT metabolites for fluoxetine-treated birds were the same on days 1 and 2 ($F_{1,22}$ = 0.979, p = 0.33), but they were significantly higher on day 1 than on day 2 for controls ($F_{1,22}$ = 22.3, p < 0.001).

**Table 3.1: Effects of treatment and day on behaviour (Exploration, Activity and Boldness), Corticosterone metabolites and body-mass measured after 16 weeks of treatment (end). Wald statistics from repeated measures GEE compared to a $\chi^2$ distribution with 1 degree of freedom. The $\chi^2$ value along with P> $\chi$ are reported for each of the explanatory variables.**

| Endpoint | Treatment | | Day | | Interaction | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | P | $\chi^2$ | P | $\chi^2$ | P |
| **Exploration** | 0.60 | 0.44 | 1.93 | 0.17 | 1.83 | 0.18 |
| **Activity** | 2.48 | 0.12 | 0.30 | 0.58 | 0 | 1.00 |
| **Boldness** | 0.26 | 0.61 | 0.020 | 0.89 | 0.033 | 0.86 |
| **Corticosterone metabolites** | 0.016 | 0.90 | 4.47 | 0.035 | 1.38 | 0.24 |
| **Body-mass** | 3.02 | 0.082 | 7.11 | <0.01 | 0.026 | 0.87 |

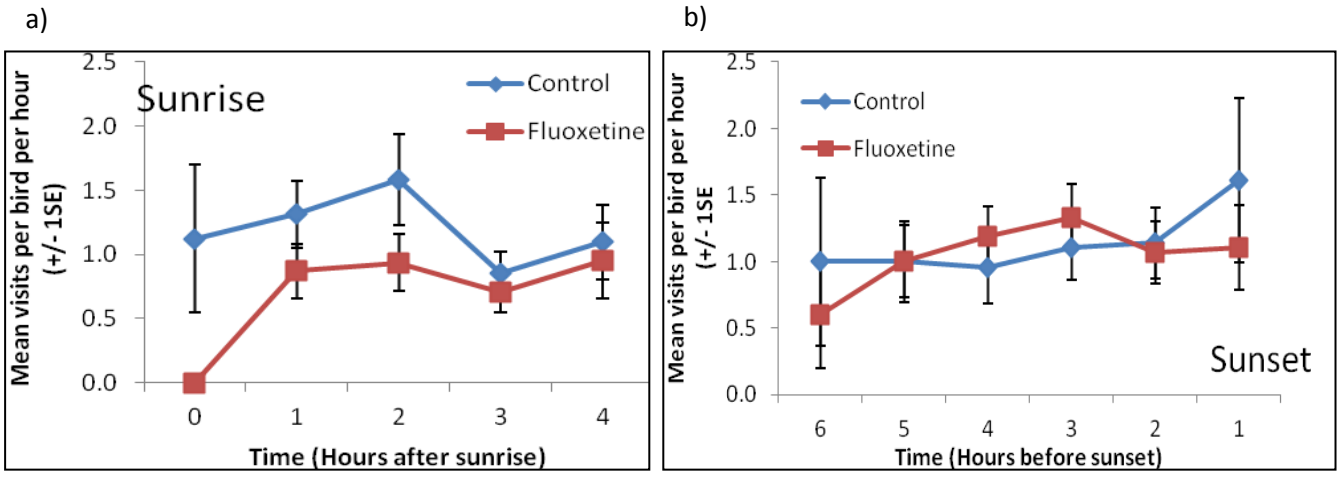



**Figure 3.4: Mean (±1 Standard Error) feeder visits per hour per bird for the first and last 4-h of data against time for control (blue diamonds) and fluoxetine-treated birds (red squares). Time is expressed relative to (a) sunrise and (b) sunset and so the 4-h period for which observations were taken depended upon the time when birds first fed in the morning and last fed at night.**

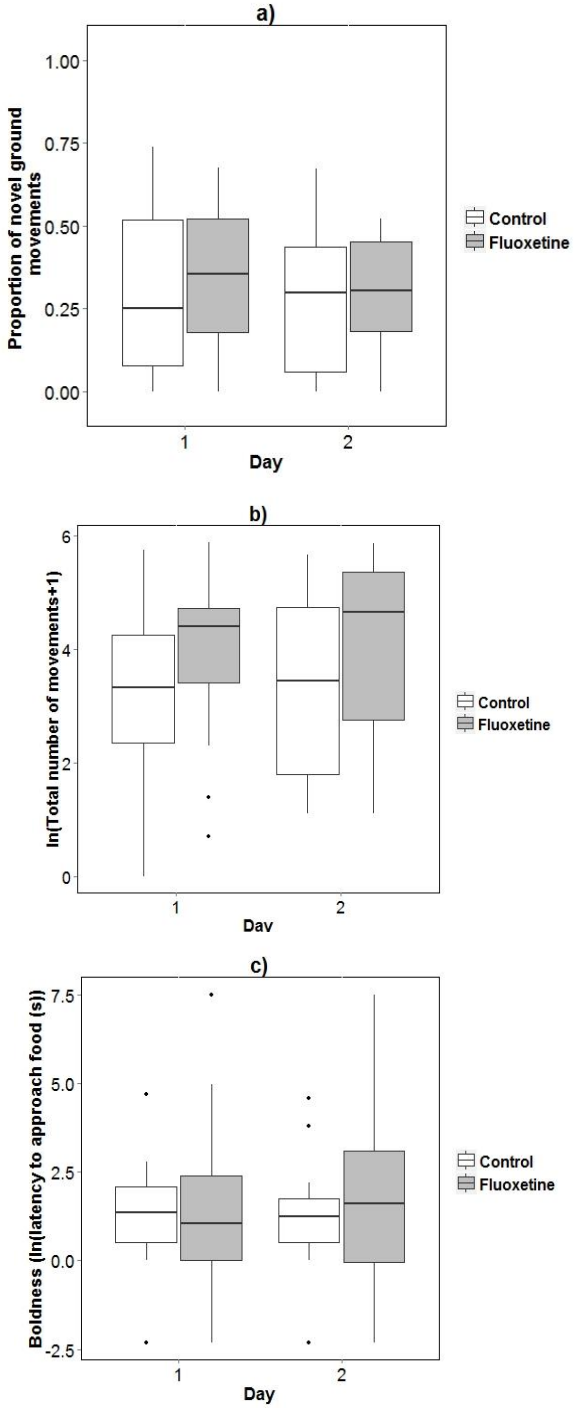



**Figure 3.5: Median behaviour at the end trials (Day 1 and Day 2) for control (white) and fluoxetine-treated birds (Grey): a) Individual exploration (proportion of total movements that were novel ground movements), b) Individual Activity (ln (total number of movements+1)) and c) Boldness (ln (latency to approach food (s)). Boxes represent the upper and lower quartiles and points beyond the ends of the whiskers represent outliers as defined by Tukey (1977).**

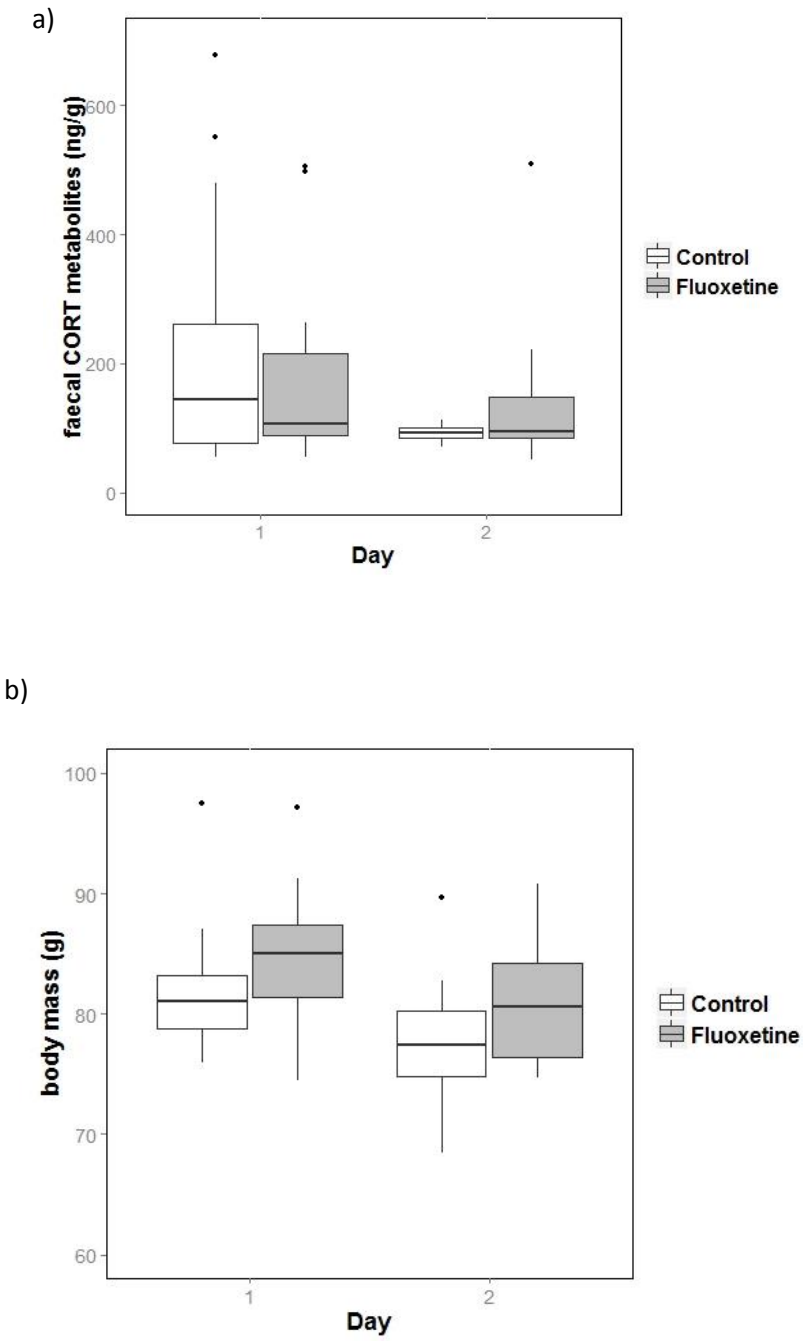



**Figure 3.6: (a) Median faecal Corticosterone metabolite concentration (ng/g of dry faeces) on days 1 and 2 for the end trials and b) Median body mass (g) on day 0 (the afternoon before the day 1 trials) and day 2 for the end trials for control birds (white) and fluoxetine-treated birds (grey). Boxes represent the upper and lower quartiles and points beyond the ends of the whiskers represent outliers as defined by Tukey (1977).**

### 3.3.4 CORT metabolites and Body Mass change

In isolation, the birds in both treatment groups lost weight during the two days that they were held in individual test cages for the behaviour trials at baseline and end. There was no treatment effect on weight loss at baseline or end (Table 3.1, Table B1 in Appendix B and Figure 3.6b). At baseline, there was no relationship between faecal CORT metabolites on the morning of day 1 ($F = 1.35$, $p = 0.26$), Treatment ($F = 0.17$, $p = 0.69$) or their interaction ($F = 1.37$, $p = 0.26$) and body mass change over the two days in isolation. At the end there was a significant interaction between CORT metabolites on day 1 and treatment ($F = 5.67$, $p = 0.027$): Control birds that exhibited high CORT metabolites on the morning of day 1 lost more mass over the two days in individual cages, than those that showed a lower physiological stress response at the start. In comparison, in the fluoxetine-treated birds there was no relationship between CORT metabolites and mass change.

## 3.4 Discussion

In Chapter 2 I found that the fluoxetine treatment experimentally fed to starlings was within the correct order of magnitude to be considered environmentally relevant and that fluoxetine can accumulate in the starling brain (which is the key tissue for fluoxetine's pharmacological activity). Therefore, it is intriguing that there was no effect of fluoxetine on boldness, exploratory tendency or activity levels in individual isolation. Whether this was due to the environmentally relevant, but low, dose administered, the lower than expected levels of accumulation in the brain; the subtle, non-standard, endpoints investigated or the small sample size, from a statistical but not an ecotoxicology perspective, is difficult to interpret. Even though our environmentally relevant exposure for birds corresponds to only 2.2–6.5% of the human therapeutic dose (when corrected for body mass differences), I found evidence that fluoxetine could potentially affect mass balance in starlings through both behavioural and physiological mechanisms. It is plausible that the prolonged dose could have led to subtle changes in neurophysiology over time. These neuro-adaptations appear to have caused effects similar to side effects of fluoxetine treatment in humans rather than therapeutic effects on anxiety. I suggest that this is because I was not measuring effects in 'depressed' subjects.

In terms of behavioural effects I found the diurnal patterns in food tray visits of fluoxetine-treated individuals did not show the normal peaks at times of highest nutritional need, that is immediately before and after the overnight fast (Macleod et al. 2005). Moreover fluoxetine-treated birds overall made less visits to food trays than controls, in the relatively low stress surroundings of their home aviaries within their familiar flock.

From a physiological perspective, I found no relationship between the levels of CORT metabolites in faeces and body mass loss during a stressful situation for fluoxetine-treated birds, but there was the expected relationship with high CORT metabolites causing greater body mass loss in controls. However, the relationships between faecal CORT metabolites and circulating CORT (Goymann 2005) are notoriously difficult to interpret. Further experiments on the effects of fluoxetine on mass balance and circulating concentrations of basal and acute CORT would allow us to establish the proximate mechanisms and ultimate impacts of fluoxetine-induced changes in foraging and stress responsiveness

To place these findings in context, maintenance of body mass plays a vital role in birds and they can change their body mass considerably in a short space of time (Krebs and Davies 1993). Over the course of 24 hours, birds must regulate their body mass precisely to ensure that they lay down enough fat reserves to provide sufficient insulation and energy to survive periods without food, while not gaining so much mass so that it inhibits their ability to avoid predators (Krebs and Davies 1993). Under predation pressure, optimal foraging models suggest that birds' daily foraging patterns should exhibit bimodal peaks around the hours of sunrise, to stave off starvation risk and to build up energy reserves, and sunset, to build up fat reserves (McNamara et al. 1994). Controls showed the expected bimodal peaks in feeding around sunrise and sunset but for fluoxetine-treated birds the peaks occurred later in the morning and early in the afternoon. Additionally, the control birds had a higher overall feeding effort than fluoxetine-treated birds. Fluoxetine has been shown to alter appetite in humans (Visser et al. 1993), and it is believed that the serotonergic nerve terminals play a role in regulating feeding behaviour (Broberger and Hokfelt 2001); with serotonin decreasing food intake and increasing energy expenditure (Guimaraes et al. 2002). It is possible that I have found evidence that fluoxetine, as in humans, caused individuals to be less sensitive to physiological signals stimulating foraging behaviour as displayed by their lower feeding effort and delayed foraging peaks.

When individuals were moved into test cages for two days all birds showed relatively high levels of CORT metabolite in the faecal samples collected from their first night in isolation, as expected. Stress hormone metabolites in samples collected from the birds' second night in isolation were significantly lower than in the first sample. The evidence for the effects of fluoxetine on glucocorticoids in other species is inconsistent. For example, DeBellis *et al.* (1993) found evidence that fluoxetine decreased levels of corticotropin releasing hormone (CRH) in humans, a precursor hormone to the release of cortisol. In arctic char (*Salvelinus* alpinus), Hoglund et al. (2002) found acute activation of serotonin receptors reduced levels of glucocorticoid precursor hormones, while chronic activation of the serotonin receptors elevated production of the glucocorticoid precursor hormones in a non-stressful

situation. Thus, the effects of fluoxetine on CORT are likely to be complex and probably context dependent. Analysing CORT in blood samples might help differentiate between effects of fluoxetine on basal and stress-induced CORT concentrations. Metabolites contained in faeces can be used as a non-invasive measure of CORT (Millspaugh and Washburn 2004), which provides an integrated measure of CORT levels over a period of several hours, which has a number of benefits over blood sampling, but also have several limitations such as the effect of sex, diet and digestive efficiency (Goymann 2005).

Interestingly, control birds that had higher CORT levels on day 1 lost more body mass over two days than those with lower levels of CORT metabolites, as expected from other studies  if CORT metabolites. For example work by Dickens et al. (2009) on chukars (*Alectoris chukar*) showed that on the first day of captivity both baseline and stress-induced CORT concentrations were raised and birds lost weight. However, for fluoxetine-treated starlings there was no relationship between CORT metabolites and mass change. CORT is intrinsically linked to energetic status through its role in the glucose regulation (Sapolsky et al. 2000) and has been linked to environmental conditions (Kitaysky et al. 2007; Muller et al. 2007), diet (Kitaysky et al. 2006; Kitaysky et al. 2001) and body condition in birds (Love et al. 2005; Pike and Petrie 2005). Therefore it is plausible that disruption to CORT production could also have influenced the way individuals responded to environmental stimuli associated with diurnal variation in starvation probability (Sapolsky et al. 2000).  In order to establish whether the feedback pathways linking CORT and body mass have potentially been disrupted I would need to repeat these experiments and collect plasma samples before and after isolation (Angelier et al. 2007b). This was not done in this experiment as blood sampling in the middle of two days of behaviour trials would have been a significant stressor.

Exploratory tendency and boldness, but not activity, were repeatable across trials in both treatment groups, indicating that they were personality traits in the starlings that reflect stable individual differences in how individuals responded to environmental stressors. However, I found no evidence of a treatment effect on these behaviours. Previous studies have found contradictory evidence for the effects of fluoxetine on activity but have focussed on aquatic species, see Brodin et al. (2014). A reduction in activity was observed in mosquito fish (*Gambusia affinis)* by Henry and Black (2008) but at much higher concentrations than De Lange *et al.* (2006), who found no effect at environmental concentrations (Brooks et al. 2003; Mennigen et al. 2010). These studies support my findings that suggest that pharmaceuticals designed to treat anxiety-related conditions may not have the expected effects in wild animals based on therapeutic or side effects in humans.

Although effects on boldness, exploration and activity may not have been observed due to the low dose, the aim of this study was to use an environmentally relevant exposure concentration. The null results could have been produced for a variety of reasons other than the low dose. These reasons include the small sample size, individual variation, simply looking at the wrong endpoints or the stress of the daily capture. One of the more challenging aspects of working with terrestrial species is administering the dose via a vehicle that closely mimics uptake in the environment, without inducing capture and handling stress that outweighs the effects of the experimental treatment. It is known that birds are unlikely to habituate to repeated capture and handling stress and that the resultant repeated short-lived peaks in CORT can have lasting effects on physiology (Herborn et al. 2014). Therefore, it was important that the control group experienced the same stress as the fluoxetine-treated group. There is a realistic possibility that I was only measuring the effect of fluoxetine in stressed birds. However, the presence of significant variation in faecal CORT levels (regardless of the treatment group) suggests that there was still variation in stress level. However, factors such as differences in digestive tract efficiency make differences in faecal CORT metabolites difficult to interpret

## 3.5 Conclusions

Previously (Chapter 2), I have shown that starlings can accumulate fluoxetine in the brain, the organ responsible for its pharmacological activity. Here I have demonstrated the potential for an antidepressant to alter behaviour and physiology in birds at environmentally relevant concentrations. This is one of few studies to have investigated the effects of environmentally relevant concentrations of pharmaceuticals in either wild or terrestrial vertebrates. To place my findings in a wider context, many terrestrial species forage on food sources contaminated with human sewage and consequently will uptake a range of pharmaceuticals (Gough et al. 2003). However, the proportion of diet that is made-up from prey items obtained from trickling filters is poorly defined and difficult to obtain. Having found effects on fitness related traits after administering starlings with an environmentally relevant dose of fluoxetine (dose based on values obtained from the literature and the University of York/FERA laboratories and validated as environmentally relevant in Chapter 2), my findings are noteworthy. I found that fluoxetine may alter diurnal variation in food tray visits and disrupt the relationship between CORT metabolites and mass balance worthy of further investigation. Importantly fluoxetine is not the only pharmaceutical, or indeed the only antidepressant, to be detected in the environment (Brooks et al. 2005; Calisto and Esteves 2009); through additive or synergistic interactions, mixtures of pharmaceuticals could potentially be more potent than single compounds increasing the likelihood of adverse effects. More research is now required in both the

field and the laboratory to determine the extent to which pharmaceuticals bioaccumulate in prey items, their uptake by wildlife via food and their potential to impact upon fitness-related traits.

# Chapter 4:

# The effects of fluoxetine on female mate choice in starlings (*Sturnus vulgaris*)


## Abstract

Chemical contaminants in the environment have been shown to alter a variety of behaviours and sexually selected traits such as courtship, sexual development and parental care. Recently there has been increased attention on the potential for pharmaceuticals in the environment to cause effects in non-target species. Antidepressants are one group of pharmaceuticals that are commonly prescribed and frequently detected in environmental samples. They are of particular interest to behavioural ecologists as they are designed to modulate behaviour in human patients. Although fluoxetine is designed to alter anxiety related behaviours, it also has a range of side effects in humans including decreased libido. Patient excretion of the unchanged parent compound to wastewater, followed by bioconcentration and bioaccumulation into the invertebrates that develop on trickling filters, represents an exposure pathway for uptake of fluoxetine into wild birds. By feeding wild-caught captive starlings an environmentally relevant dose (928 ng $day^{-1}$) of fluoxetine via food for 22 weeks, the potential for fluoxetine to alter mate choice and individual quality was assessed. Mate choices of both fluoxetine-treated and control females were assessed by presenting them with a fluoxetine-treated or a control male, although there was also the option to spend time in front of neither male (no-choice). Compared with control females, fluoxetine-treated females spent 49% more time in the 'no-choice' zone. The rate at which the fluoxetine-treated females' bill's changed colour from over-winter to breeding condition was found to lag behind that of the control-females in the early breeding season. At the time of the first two measurements of bill colour (End of March and beginning of April 2012), the fluoxetine–treated females' bills had 19.8% less pink on their bills than the control-females. However, at the time of the mate choice trials there were no treatment differences in breeding condition bill colour. With the exception of bill colour, none of the measures of female social rank or physiology were found to differ between the treatment groups. Male social rank, body mass, bill colour and relative gonad size did not differ between the treatment groups or

significantly affect male attractiveness. These results present another (in addition to the findings of Chapter 3) example of the potential for fluoxetine to cause changes to avian behaviour at environmentally relevant concentrations. The observed effects on female association time with males could be a result of interactions between the serotonergic system, upon which fluoxetine acts, and the neuroendocrine system which regulates many aspects of behaviour and the development of breeding condition. Given the recent declines in the populations of many UK wild birds, it is important to discover the extent to which pharmaceuticals such as fluoxetine influence wild populations.

## 4.1 Introduction

Environmental contaminants have been reported to alter behaviour and sexually selected traits, including courtship, sexual development and parental care in aquatic species (Saaristo et al. 2009; Saaristo et al. 2010; Soeffker and Tyler 2012) but to date there has been relatively little focus on terrestrial species. Recently, the importance of including behavioural endpoints in ecotoxicology has been highlighted by a number of studies, as behaviours are sensitive to environmental changes linked to fitness and can be measured non-invasively (Clotfelter et al. 2004; Scott and Sloman 2004; Zala and Penn 2004). A group of contaminants of emerging concern, but to date understudied are pharmaceuticals (Arnold et al. 2013). Many pharmaceuticals are incompletely metabolised by the target organism and subsequently are excreted into the natural environment (Monteiro and Boxall 2010). Once in the environment, pharmaceuticals are known to partition to a range of matrices including water, biosolids and the tissues of living organisms which potentially exposes a range of non-target species (Ayscough et al. 2000; Brooks et al. 2005; Markman et al. 2007; McClellan and Halden 2010; Monteiro and Boxall 2009).

The selective serotonin re-uptake inhibitor (SSRI) antidepressant fluoxetine was selected as a compound of potential concern for wild birds feeding at WWTPs (see Chapter 1). In humans, fluoxetine (the active ingredient in Prozac) is used to regulate mood in patients suffering from anxiety related conditions (Eli Lilly 2009). Fluoxetine causes biochemical changes in the central nervous system (Lesch et al. 1991); by binding to the pre-synaptic serotonin receptors to inhibit re-uptake from the synapse, fluoxetine potentiates the effect of serotonin on the post-synaptic receptors (Newman et al. 2004), thus improving the neurotransmission of serotonin (Lesch et al. 1991; Newman et al. 2004). Serotonin has an important role in the regulation of several physiological and behavioural processes such as fear and anxiety, appetite, wakefulness, aggression, libido and locomotion (Mennigen et al. 2011). Subsequently side effects such as lethargy, changes in body mass, social and reproductive behaviours are common (Eli Lilly 2009). The incidence of sexual dysfunction in humans taking fluoxetine (Park et al. 2009) is highly variable between studies, with values

reported anywhere between 8 and 75% (Clayton 2002; Madeo et al. 2008; Matuszczyk et al. 1998; Montgomery et al. 2002; Shen and Hsu 1995; Uphouse et al. 2006). Given predicted evolutionary conservation of vertebrate endocrine systems (Gunnarson et al. 2008), it is predicted that similar side effects may be seen in wildlife to humans. However this remains to be tested.

Recently pharmaceuticals have been shown to bioaccumulate in the invertebrates that develop on the trickling filters of wastewater treatment plants (WWTPs) (Markman et al. 2007) with the potential to adversely affect birds including starlings (*Sturnus vulgaris*), a species known to forage on trickling filters throughout the year (Markman et al. 2008; 2011). In Chapter 2, I determined an environmentally relevant daily dose of fluoxetine to be 2.2-6.5% of the human daily dose (corrected for differences in body mass).

Previously (in Chapter 3) I have found the environmentally relevant concentration of fluoxetine wild birds are likely to be exposed to was sufficient to alter (in what is expected to be a negative way) fitness related traits (foraging behaviours and mass balance), after dosing individuals in captivity over winter. Other studies have previously shown that fluoxetine can affect social, mating and aggressive behaviours in terrestrial species e.g. Mongolian gerbils (*Meriones unguiculatus*) (Cheeta et al. 2001), Prairie voles (*Microtus ochrogster*) (Villalba et al. 1997) and Song sparrows (*Meolspezia meoldia morphna*) (Sperry et al. 2003), but at concentrations several orders of magnitude higher than are present in the environment (see Chapter 1 Table 1.2 and Chapter 2 section 2.3.1). Recently, effects on mating behaviour, specifically nest building and defending in males, have been observed in aquatic species (*Pimephales promelas*) (Weinberger and Klaper 2014). Similarly, the anti-anxiety drug, oxazepam, designed to have similar therapeutic effects to fluoxetine has been shown to cause reduced sociality in European Perch (*Perca fluviatilis*) at environmentally relevant concentrations (Brodin et al. 2013). A range of antidepressants and anxiolytic drugs, all with similar therapeutic effects have been detected in the environment (Calisto and Esteves 2009). While effects of pharmaceuticals on the behaviour of aquatic species have been demonstrated at environmentally relevant concentrations, terrestrial wildlife species remain largely unstudied.

In model species, the role of serotonin in the modulation of social behaviours is well-established (Ferris and DelVille 1994). Changes in the serotonergic system control aggression via the arginine vasotocin/vasopressin (AVT/AVP) system, which in turn mediates dominance/submissive and reproductive behaviours (Barry 2013; Ferris and Grisso 1996). High levels of serotonin are also believed to inhibit vasopressin and lead to individuals being less aggressive and more submissive (Barry 2013). At low levels of serotonin, vasopressin increases. High levels of vasopressin are associated with social dominance and aggression (Delville et al. 1998; Ferris and Grisso 1996). In a

gregarious species such as the starling (Boogert et al. 2006; Stahl et al. 2001) subordinate individuals are expected to incur greater fitness costs than their more social and dominant con-specifics, as they are forced to occupy more perilous positions within the flock and allocate a greater proportion of time to surveillance (Apfelbeck and Raess 2008; Powell 1974). Additionally, subordinate individuals may also suffer fitness costs as a consequence of a reduction in their ability to attract a mate (Feare 1984), whether this is due to lower body mass, reduced sociality (Fernandez-Juricic et al. 2005), inferior courtship displays (Markman et al. 2008) or poorer breeding condition (Bennett et al. 1997) remains uncertain.

Although the reasons why fluoxetine causes sexual dysfunction related side effects remain less certain than effects on sociality, it has been suggested that alterations in the production of oxytocin as a result of changes to the serotonin system may contribute alongside changes in vasopressin (Cantor et al. 1999; Raap et al. 1999; Serres et al. 2000; Uphouse et al. 2006). Gonad size in birds undergoes a marked seasonal cycle of growth in the breeding season and regression during the remainder of the year. In birds, larger gonads are generally indicative of a higher quality male (Calhim and Birkhead 2009; Graves 2004). In terms of breeding condition, starlings of both sexes have similar glossy black feathers with iridescent green and purple feathers in the breeding season (Feare 1984). Bill colour is sexually dimorphic; in the spring all but the base of the lower mandible turn from black to yellow, while the base turn pale blue in males and pale pink in females (Feare 1984). Therefore the extent to which the bill has changed colour is indicative of when a bird has entered breeding condition, with the individuals that lag behind potentially left with an inferior choice of mate.

Using the same twenty-four wild caught starlings referred to in Chapters 2 and 3, this study examined the effect of fluoxetine on female association with males was investigated in standard two-way mate choice trials. Additionally, behavioural and physiological measures that could potentially explain mate preferences, such as body mass, gonad size, rank in the dominance hierarchy (social rank) and breeding condition, were also assessed.

This study aimed to test whether exposing starlings to environmentally relevant concentrations of fluoxetine affected: 1) male attractiveness, 2) female preference for males 3) male and female social rank, body mass, relative gonad size and the development of breeding condition and 4) whether male social rank, body mass, relative gonad size and the development of breeding condition affected female mate choice.

## 4.2 Materials and Methods

Full details of the structure of the experiment, capture, husbandry and dosing can be found in Chapter 1, section 1.12 (specifically Figure 1.9) and details of capture, husbandry and exposure are

presented in Chapter 2, section 2.2.3. Briefly, the same 24 birds were used in this experiment as in Chapter's 2 and 3. Fluoxetine-treated birds (N = 12) were captured from their home pens in a hand net and hand fed with invertebrates spiked with 1300ng/worm of fluoxetine for five days a week (i.e. 928 ng/day) for 22 weeks. Controls were fed invertebrates spiked with deionised water. After 15-20 weeks of treatment, social rank was assessed using monopolisation of the feeding resource at key foraging times (see Bean et al. 2014 or Chapter 3) and the foraging hierarchy (order in which birds came down), remotely using a system of passive integrated transponder (PIT) tags (see Chapter 2 section 2.2.3b). Female mate choice was assessed between weeks 19 and 21 (Chapter 1, Figure 1.9).

### 4.2.1 Female mate choice

For the mate choice study, all females were assessed individually between 10$^{th}$ and 23$^{rd}$ May 2012 (days 137-148 of treatment) between the hours of 10:00 and 15:00. Each individual female (either fluoxetine-treated or control) was presented with two males (one fluoxetine-treated and one control) randomly selected and captured from their home pens using a hand net. Each female was only tested once but stimulus males were re-used in different pairings (only once per female). A standard two-way mate choice was set up as in (Arnold et al. 2002), in a separate, visually occluded outdoor aviary. The female could see both males but the males could not see each other (Figure 4.1). A trial for each female consisted of two phases, each lasting 30 minutes during which the time she spent in the half that was facing the control male, the half facing the fluoxetine treated male or the central no-preference zone facing neither male was recorded.

The female was placed into a central cage, a large double breeder (Kent Cages, UK) consisting of two halves each measuring 63.5 cm × 38.7 cm × 35.6 cm, separated by a wooden ridge 2 cm high. Each cage half was lined with white paper and the female was given full access of this cage. Facing to the female's cage was a second cage into which two stimulus males were to be placed, each isolated in one half of the cage by the wooden divide which slides into the grove of the central wooden ridge. The observer sat behind the stimulus cage behind a screen from where the female's position in her cage could be observed. Natural lighting conditions were used as test cages were in outside aviaries.

To set up each trial, the males were placed in separate halves of the stimulus cage (sides were randomly selected prior to the trial) and covered over with a sheet. The female was then placed into her test cage and all birds were given 20 minutes to acclimatise. No food or water was available during the trial.

To begin the trial, the sheet was removed from the stimulus male's cage and the stopwatch started when the female was in the centre of her cage. A trial for each female consisted of two phases, each lasting 30 minutes during which I recorded how long she spent in the half that was facing the control

male, the half facing the fluoxetine treated male or the central no-preference zone facing neither male.

After the first 30 minute phase, food and water was placed in all halves of the cages in use (i.e. two for the female, to prevent the creation of a side bias in the second phase of the trial, and one each for the males). The female's cage was then covered and the males were swapped between the cage halves to control for side bias. The cover was then removed from the female's cage and placed over the males' cage. Birds were given 20 minutes to feed, drink and acclimatise. The second phase of the trial was then conducted exactly as the first.

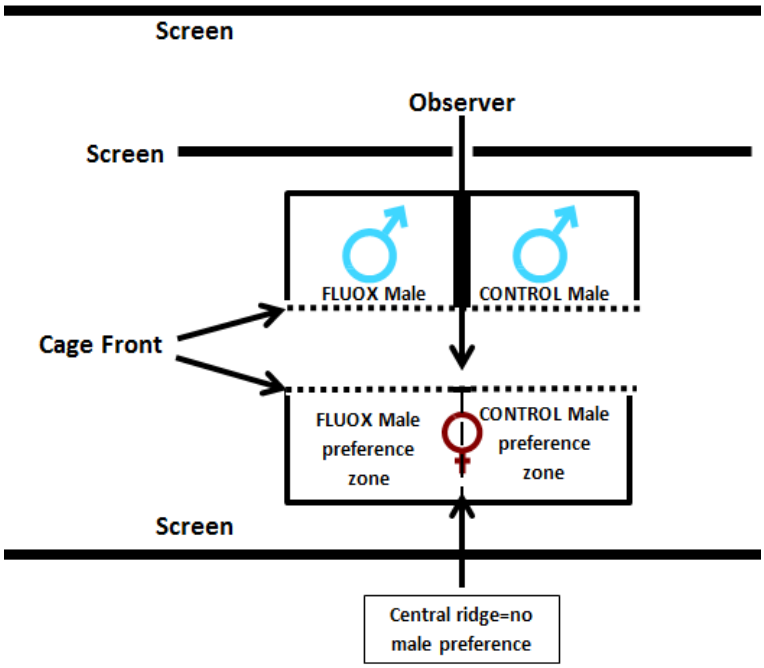



**Figure 4.1: The set-up of the test aviary for the mate choice trials. One female per trial (fluoxetine-treated or control) was placed in the test cage facing to a cage containing a fluoxetine treated male in one half and a control male in the other half. The female had access to both halves of her test cage as well as the option to remain in front of neither male (centre). Two trials were conducted for each female, each lasting half an hour so that the males could be swapped sides to control for left or right side bias in the females. The arrow from the males' cage shows the view of the observer. The test aviary was in an outside aviary to and so screens were placed on the sides of the aviary to obscure the view of birds in other aviaries.**

### 4.2.2 Measures of social rank

In order to assess variation in social rank, foraging data were collected from each of the four aviaries. Each aviary contained two food trays and three perches, using the food tray as the resource for which birds competed, a remote Passive Integrated Transponder (PIT) tag system (full details in Bean et al. 2014, Chapter 2 section 2.2.3b and Chapter 3 section 3.2.2) was used to quantify social rank remotely without the effect of human disturbance. Two antennae (8 cm x 5 cm; Trovan, www.trovan.com) were positioned flat in the two food trays (40 cm x 20 cm x 6 cm). The monitoring

system was set up to read at one second intervals, recording the unique PIT tag code along with a date and time (full details in Bean et al. 2014, Chapter 2 section 2.2.3b and Chapter 3 section 3.2.2)

The total time birds were detected at feeders in the first four hours and last four hours before sunset by the PIT tag readers was used to establish which birds were monopolising the food trays as a measure of social dominance; the assumption being dominant birds spend longer at feeders than subordinates (Boogert et al. 2006). Repeated measures on consecutive days were made, with each bird detected for an average of 175 seconds (range 0 to 1270 seconds) in multiple foraging bouts. This measure is referred to as 'monopoliser rank' hereafter.

Secondly, the order in which the birds came down to feed at the start of the day (four sets of measurements taken on two sets of two consecutive days between March 1$^{st}$ and May 21$^{st}$ 2012) was used to rank birds. This social measure was used because there is growing evidence that individuals differ in their social roles with leaders having a greater influence over group behaviour than those that tend to follow (Jolles et al. 2014). This measure is referred to as 'leader-follower rank' hereafter.

For each measure of social behaviour, consecutive days of data were used for each of the four social groups (home pens, see Chapter 2 section 2.2.3b) and the repeatability of individuals' rank (for both monopoliser rank and leader-follower rank) was assessed using a single factor ANOVA (see below). A mean was then created for each variable to give two values for each bird, one for monopoliser rank and one for leader-follower rank which was then adjusted for group size as there were different numbers of birds in the four social groups. Rank orders were expressed on a scale of 0 (for the bird that monopolised the food resource most or was first down i.e. the leader in the foraging hierarchy) to 1 (monopolised the food for the shortest time or for foraging hierarchy, the last bird down) using Equation 4.1.

**Equation 4.1**

$$BR = R-1 / n-1$$

Where:

**BR** = Rank on a binomial scale

**R** = the rank order from1 to n

**n** = the number of birds in the social group

Repeatability for both the monopolisation of the food resource and the foraging hierarchy was assessed for males and females separately (calculated using a single factor ANOVA). Both measures

of social rank in males (monopolisation: $F_{11\,12} = 209.4$, $r = 0.99$, $p < 0.01$; foraging hierarchy: $F_{11,36} = 2.30$, $r = 0.25$, $p = 0.03$) and females (monopolization: $F_{11,12} = 9.61$, $r = 0.81$, $p < 0.01$; foraging hierarchy: $F_{11,36} = 3.12$, $r = 0.35$, $p < 0.01$) were found to be significantly repeatable within individuals and so a mean rank was given to each bird for both monopolization and foraging hierarchy.

### 4.2.3 Bill colour

To quantify breeding condition, bill colour change from winter condition (black) to breeding condition (yellow upper mandible with pink base for females and light blue for males (Bennett et al. 1997; Feare 1984) was measured, for a two month period prior to the mate choice trials, to assess whether fluoxetine delayed the onset of breeding condition. The head and beak of each bird was photographed each week onto a white background between 26th March and 21st May (day 88 -144 of receiving treatment), using a digital camera (Canon EOS 400D Digital), giving a total of nine photographs per bird. The proportion of the beak that had changed colour from black was quantified using ImageJ (downloaded from http://rsb.info.nih.gov/ij/) to give a series of time lapse beak colour change proportions for each individual. Proportions (from 0 to 1) were arcsine square root transformed to standardise the variance.

The bill colour data were used in three ways:

1) I assessed whether there were treatment effects on bill colour at the time of the mate choice trials. For this assessment the last three of the nine photos were used. These three photos were taken immediately before, during and immediately after the mate choice trials were combined into a mean. The proportion of the bill that had changed colour in these three photos was found to be marginally repeatable within individuals for males (single factor ANOVA: $F_{11,24} = 1.93$, $r = 0.24$, $p = 0.086$) and significantly repeatable for females (single factor ANOVA: $F_{11,24} = 4.03$, $r = 0.50$, $p = 0.002$). So the creation of a mean proportion to test for treatment effects was justified. The effect of treatment was assessed separately for males and females as secondary sexual characteristics differ between the sexes (Feare 1984).

2) The effect of male bill colour on female mate choice was assessed by expressing the proportion of the fluoxetine males' bill that had changed colour relative to the control male it was paired with in the trial. For this analysis, the measurement of bill change taken closest to the mate choice trial in which the male took part was used.

3) I assessed whether treatment with fluoxetine affected the rate of bill colour change over the period for which the nine photos were taken. For males and females separately, the rate at which the

bill colour changed from black to pink or blue was assessed using all nine photos taken between March 26th and May 21st.

### 4.2.4 Body mass and Relative gonad size

Body mass was recorded immediately prior to birds being euthanized in the week following the mate choice trials (Chapter 2). To assess the effect of relative male gonad size on association with males the gonads were removed during post mortem dissection and their weight was expressed relative to body mass. Sex specific treatment effects on relative gonad size were also assessed.

### 4.2.5 Data analysis and Statistical Methods

All analyses were conducted using R (version 3.0.2) and SPSS (Version 19). In the mate choice trials, the overall preference for males (looking at all data together and irrespective of female treatment) was assessed using a two-tailed matched pairs t-test.

Secondly, in order to assess the effect of female treatment on association time with males, the time each female spent in each of the three possible locations within the cage (control male, no-male, fluoxetine-treated male) was natural log transformed. The effect of female treatment on the ratio of time females spent in front of fluoxetine-treated males and no-male was assessed in an ANOVA. The analysis was repeated for the ratio of control male time to no-male time. In order to assess whether the single dependent variable approach was missing the effect of female treatment group, both the dependent variables (ln(fluoxetine-treated male association time/ no male) and ln(control male association time/no-male)) were looked at together using a multivariate approach (MANOVA).

Next, to assess whether the relative quality of the fluoxetine-treated and control male pair presented to the female was a confounding factor with male treatment for the female's preferences, the MANOVA referred to in the previous paragraph was repeated five times with the addition of one of five measures of relative male quality as a covariate. Thus the MANOVA becomes a MANCOVA due to the addition of the covariate and the interaction of this covariate and female treatment (independent variable). The five covariates used were measures of relative male quality (fluoxetine-treated relative to control) for each of: gonad size relative to body mass, bill colour, body mass, monopoliser rank and leader-follower rank). Body and gonad masses were natural log transformed to meet assumptions of normality.

In order to assess whether the fluoxetine-treated females selected the lower quality male (compared with the preference of control females), regardless of the males treatment, linear correlations were used. To calculate these correlations, new dependent and independent variables were calculated. The dependent variable became the natural log transformed ratio of association times between the

preferred (the male she spent more time with) and rejected males. For the independent variable in each analysis, the five covariates used in the MANCOVA (above) were recalculated so that instead of being a relative measure of fluoxetine to control male, they now became relative measures of preferred to rejected male quality. Five separate linear correlations were done with the measure of relative male quality being the independent variable and the measure of relative association time being the dependent variable (see Figures 4.3a-e). In each of the five analyses, I calculated the correlation coefficient and two tailed significance for control females (N=7), fluoxetine –treated females (N=5) and overall (N=12).

Finally, in order to assess the effect of treatment on relative gonad size, body mass, bill colour (at the time of the mate choice trials), monopoliser rank and leader-follower rank separate independent sample t-tests were performed for males and females. To assess the effect of treatment on the rate at which the bill colour changed from winter condition to breeding condition, a repeated measures linear effects model was used in R package nlme.

## 4.3 Results

In the mate choice study, females did not differ in their association times with control and fluoxetine-treated males (Two-tailed matched-pairs t-test: df = 11, t = -0.11, P = 0.91; mean time with fluoxetine male = 1195 s, mean time with control male = 1209 s).

There was no effect of female treatment on the time spent with fluoxetine treated males compared with no male ANOVA: $F_{1,10} = 3.02$, p = 0.11 or for the equivalent analysis in control males $F_{1,10} = 0.53$, P = 0.48. However, Figure 4.2 shows that the fluoxetine-treated females appeared to spend less time with the fluoxetine-treated males than they did with control males, preferring to allocate this time to 'no males'. Control females spent similar time with both males and much less time with no male. The multivariate approach found that there was a significant effect of female treatment on association time (MANOVA: approximate $F_{1,10} = 6.37$, p = 0.022), i.e. the fluoxetine female spent less time with both males because she allocated more time to sitting in the centre of the cage disinterested in either male than the control female did. This effect does not come out when the data are analysed in isolation but does when the two response variables are analysed together.

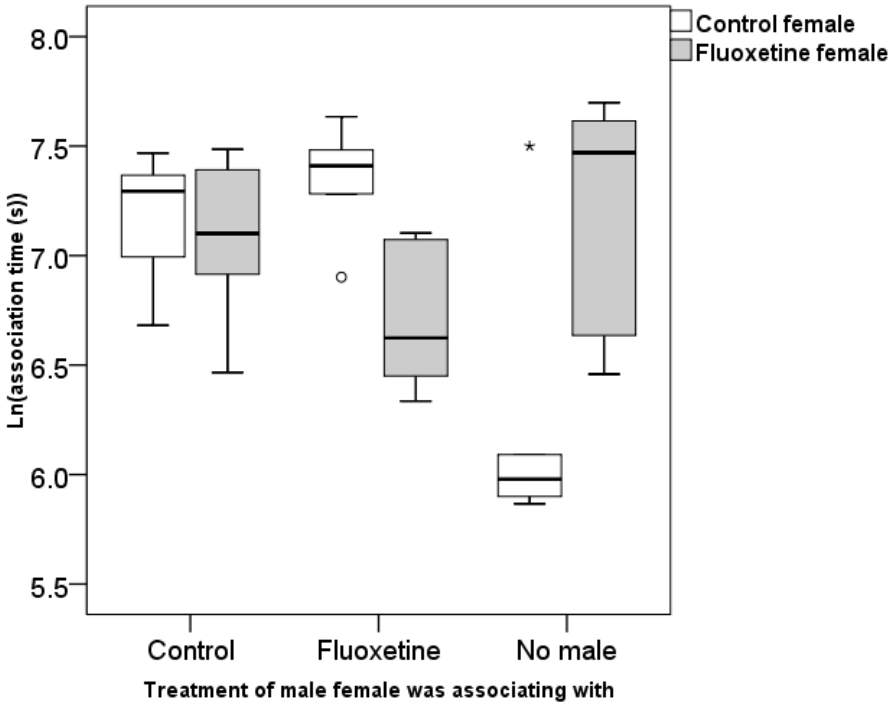



**Figure 4.2: Median of the natural log transformed female association times with control, fluoxetine and no male for control female (white bars) and fluoxetine female (grey bars). Boxes represent the 25th and 75th percentile respectively with points beyond the whiskers being the outliers as defined by Tukey (1977). This figure contains the data that were used to create the logarithmic ratio variables used in the ANOVA and MANOVA analyses.**

### 4.3.1 Did male quality interact with female treatment?

The data presented in Table 4.1a-e show that the addition of the relative male quality variables to the MANCOVA did not cause the treatment effect to disappear, but none of them were significant and neither was their interaction with female treatment.

**Table 4.1a-e: The association times of females with males (dependent variables) explained by female treatment (independent variable), one of five measures of relative male quality (covariate) and their interaction in a MANCOVA, the Approximate F and P value (on 2 and 7 degrees of freedom) are given in five separate tables (a-e). In each table (a-e), the analysis used a multivariate approach where the same two dependent variables (ln(Association time with fluoxetine male/ time no male) and the equivalent for control males association time were looked at together. The covariates in the five separate analyses are expressed as the fluoxetine male's quality relative to that of the control male that it was paired with. A subheading is given above each table to explain what the covariate was in each analysis. P-values marked with an * are significant at the 5% level.**

**Figure 4.1a: The measure of male quality used as the covariate in this analysis is the ratio of the fluoxetine-treated male's gonads mass to his body mass minus the ratio of the control male's gonads mass to his body mass**

| Independent variables and covariates | | | | | |
|---|---|---|---|---|---|
| Female treatment | | Relative male quality (gonads relative to body mass) | | Interaction of female treatment and relative male quality (gonads relative to body mass) | |
| approx. F | P | approx. F | P | approx. F | P |
| 5.70 | 0.03* | 0.23 | 0.80 | 0.80 | 0.48 |

**Figure 4.1b: The measure of male quality used as the covariate in this analysis is the arcsine square root transformed proportion of the fluoxetine-treated male's bill that had turned from black to light blue minus the equivalent measure of bill colour change for the control male.**

| Independent variables and covariates | | | | | |
|---|---|---|---|---|---|
| Female treatment | | Relative male quality (bill colour change) | | Interaction of female treatment and relative male quality (bill colour change) | |
| approx. F | P | approx. F | P | approx. F | P |
| 5.81 | 0.03* | 0.45 | 0.65 | 1.93 | 0.22 |

**Figure 4.1c: The measure of male quality used as the covariate in this analysis is the natural log of the fluoxetine-treated male's body mass (g) minus the equivalent measure of body mass for the control male.**

| Independent variables and covariates | | | | | |
|---|---|---|---|---|---|
| Female treatment | | Relative male quality (body mass) | | Interaction of female treatment and relative male quality (body mass) | |
| approx. F | P | approx. F | P | approx. F | P |
| 6.21 | 0.03* | 0.09 | 0.92 | 0.78 | 0.51 |

**Figure 4.1d: The measure of male quality used as the covariate in this analysis is the monopoliser rank of the fluoxetine-treated male minus the equivalent measure of monopoliser rank for the control male. Monopoliser rank was determined from total amount of time spent at the food tray (recorded by the Passive Integrated Transponder Tag system) in the home pens for the first four hours after sunrise and the last four hours before sunset. The measure of monopoliser rank goes from 0 (most monopolising bird) to 1 (least monopolising bird) and was scaled like this to account for the different numbers of birds in the home pens (see section 4.2.2).**

| Independent variables and covariates | | | | | |
|---|---|---|---|---|---|
| Female treatment | | Relative male quality (monopoliser rank) | | Interaction of female treatment and relative male quality (monopoliser rank) | |
| approx. F | P | approx. F | P | approx. F | P |
| 6.63 | 0.02* | 0.46 | 0.65 | 1.15 | 0.37 |

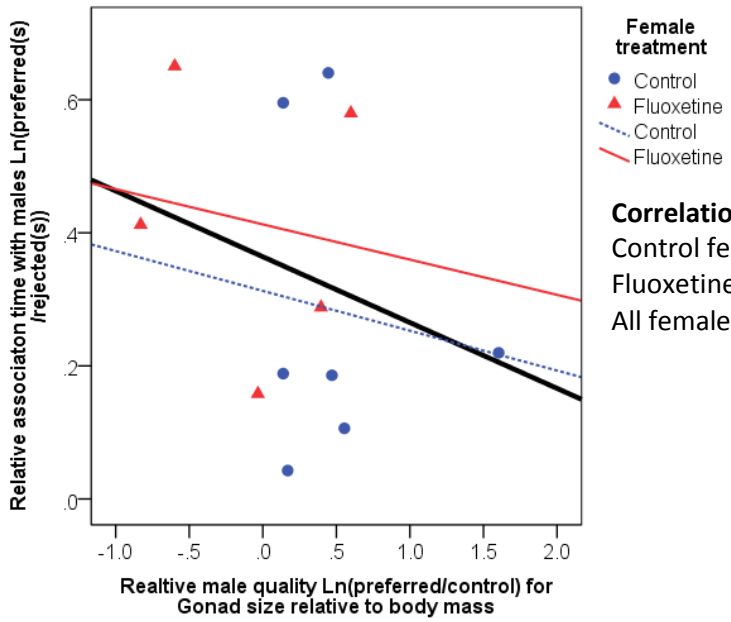
**Figure 4.1e: The measure of male quality used as the covariate in this analysis is the leader-follower rank of the fluoxetine-treated male minus the equivalent measure of leader-follower rank for the control male. Leader-follower rank was determined from the order in which the birds came down to the food tray at the start of the day (recorded by the Passive Integrated Transponder Tag system). The measure of leader-follower rank goes from 0 (leader) to 1 (follower) and was scaled like this to account for the different numbers of birds in the home pens (see section 4.2.2).**

| Independent variables and covariates | | | | | |
|---|---|---|---|---|---|
| Female treatment | | Relative male quality (leader follower rank) | | Interaction of female treatment and relative male quality (leader-follower rank) | |
| approx. F | P | approx. F | P | approx. F | P |
| 8.34 | 0.01* | 0.20 | 0.83 | 1.68 | 0.25 |

### 4.3.2 Effect of male quality on female association time: Preferred male association time relative to the rejected male association time

Figures 4.3a-e show that there was no significant correlation between any of the five measures of relative male quality and relative association time for the control females and the fluoxetine treated females. Thus, fluoxetine did not significantly affect the association time of females with males of 'higher quality';

**a)**



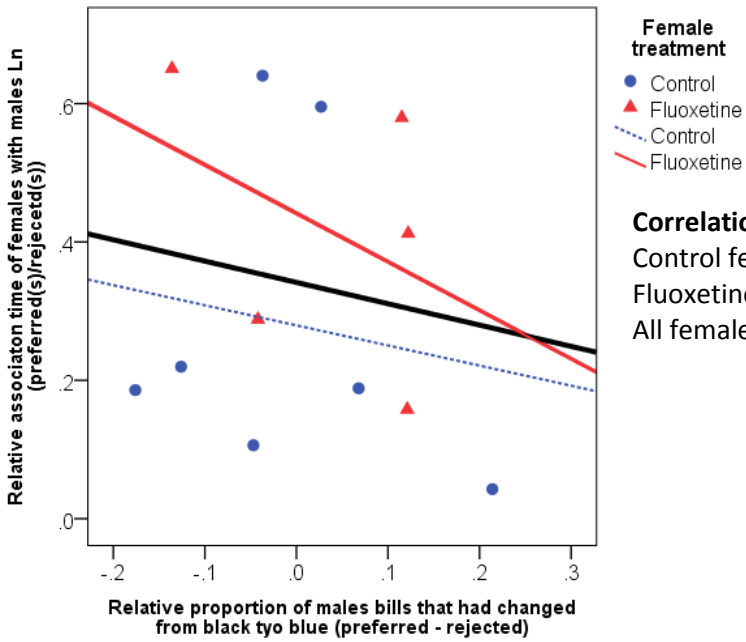

**Correlations**

Control female: r= -0.13, P= 0.78

Fluoxetine females: r= -0.16, P = 0.80

All females: r = -0.27, P = 0.40

**b)**



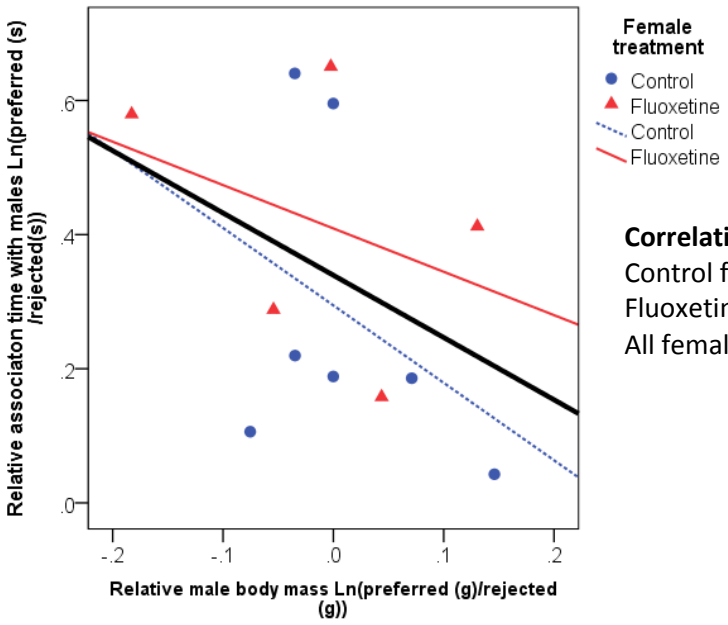

**Correlations**

Control female: r= -0.16, P= 0.73

Fluoxetine females: r= -0.41, P = 0.49

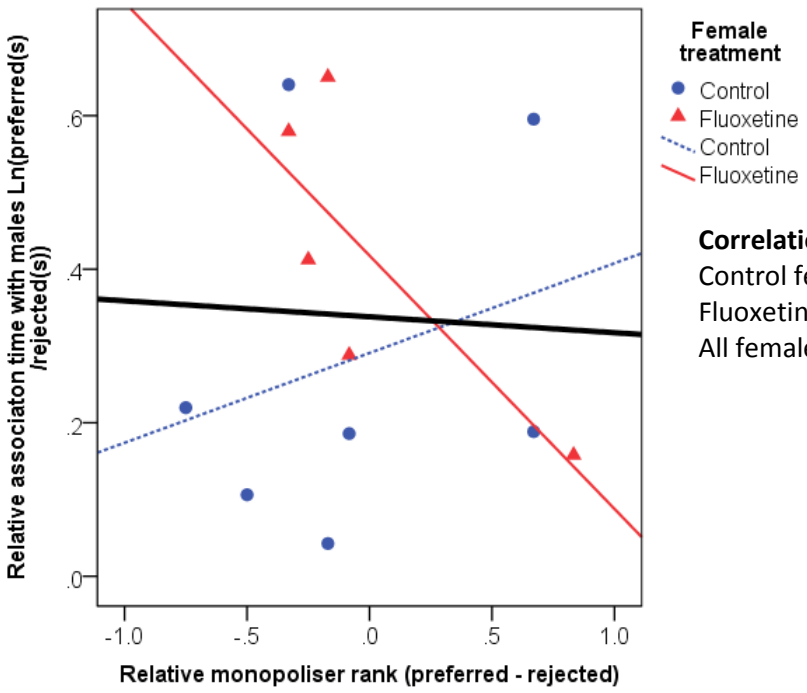
All females: r = -0.17, P = 0.60

c)




**Correlations**
Control female: r= -0.25, P= 0.59
Fluoxetine females: r= -0.37, P = 0.54
All females: r = -0.37, P = 0.23

d)




**Correlations**
Control female: r= 0.27, P= 0.56
Fluoxetine females: r= -0.77, P = 0.13
All females: r = -0.05, P = 0.89

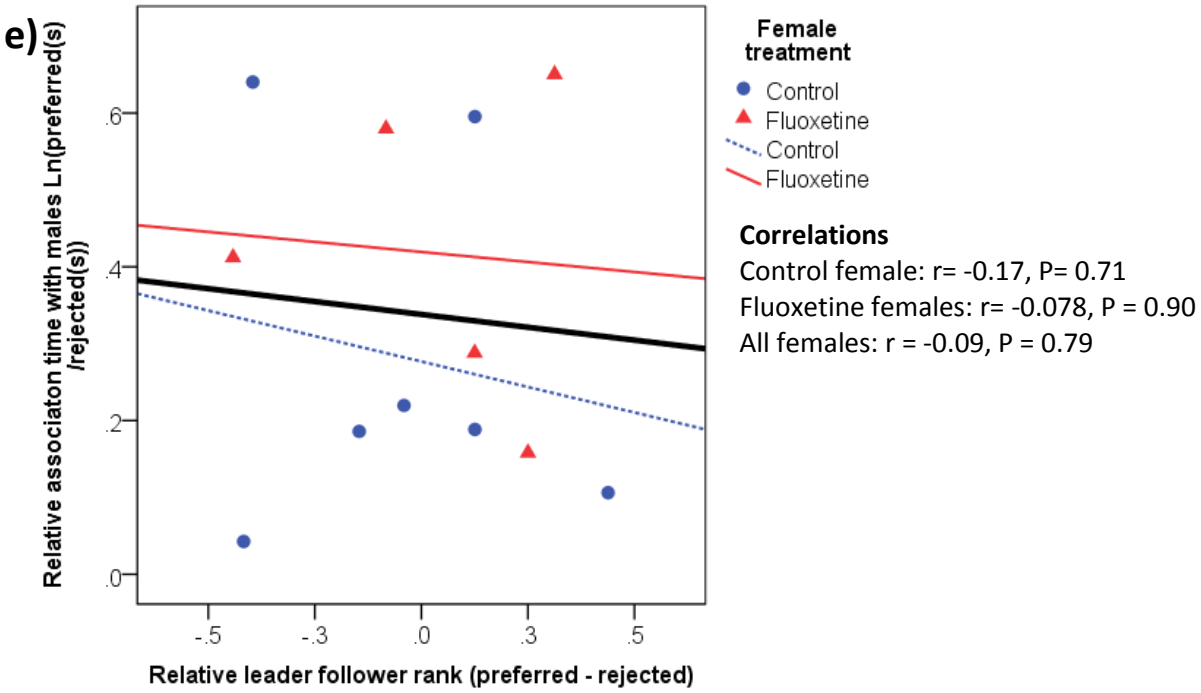



**Figure 4.3a-e: The lack of significant correlation betwee relative association time of females with the preferred and the rejected male (dependent variable in all figures) and the independent variables which are measures of relative male quality (preferred relative to rejected). The independent variables are all looked at in separate analyses. They are a) Gonad size relative to body mass Ln(preferred/rejected), b)arcsine square root transformed proportion of the males' bills that had changed from black to light blue (preferred – rejected), c) body mass(preferred/rejected), d) Monopoliser rank, the extent to which a bird monopolises the food tray (0 to 1, 0 being the most dominant) for preferred - rejected and e) Leader follower rank which is the same as Monopoliser rank but instead of time spent feeding it is the order the birds came down in (preferred – rejected). The control females are represented by blue circles and a dashed blue linear trend line (N = 7), the fluoxetine-treated females are represented by red tringles and a solid red line (N = 5), the overall linear trend line for all females regardless of treatment is given by the bold black line 'All' (N = 12). Pearson's correlation coefficients (r) and 2-tailed significance (P) are given alongside for each of Figures 4.3a-e for control female, fluoxetine-treated females and all females regardless of treatment 'All'.**

### 4.3.3 Effects of treatment on male and female quality

For both males and females, there was no significant difference between the fluoxetine and control groups in terms of gonad size, bill colour at the time of the mate choice trials, body mass, monopoliser rank or leader-follower rank (Table 4.2).

Although no birds' bills started to turn yellow, the lower mandibles did turn pink in females and blue in males. Fluoxetine-treated females developed pink beak colouration more slowly than controls (repeated measures linear mixed effects model: F = 9.00, df = 94, p = 0.0035, Figure 4.4) in the rate that their bills turned pink, but there was no significant difference in bill colour change for males (F = 1.20, df = 94, p = 0.27) (see photos in Figure 4.5. Although the rate of bill colour change for the fluoxetine-treated females lagged behind that of the control females in the early part of the breeding season, they had caught up by the time of the mate choice trials in mid-May (Figure 4.5).

**Table 4.2: The effects of treatment on 1) Relative gonad size (gonad size / body mass), 2) arcsine square root transformed bill colour at time of mate choice, 3) ln(Body mass g), 4) Monopoliser rank (0 monopoliser to 1 monopolised) and 5) Leader-follower rank (0 to 1) assessed using independent t-tests. The test statistic (t-value) and the significance of the test (p-value) are reported along with the means and standard errors for the groups. All tests assumed equal variance following a Levene's test (see Appendix C1) and were on 10 degrees of freedom.**

| | | t-test | | Control | | Fluoxetine | |
|---|---|---|---|---|---|---|---|
| | | t | p | Mean | Standard Error | Mean | Standard Error |
| Males | Relative gonad size | -0.85 | 0.42 | 0.00057 | 0.00011 | 0.00043 | 0.00002 |
| | Proportion of bill colour changed | -1.65 | 0.13 | 0.82 | 0.04 | 0.88 | 0.02 |
| | Body mass | -0.49 | 0.96 | 79.76 g | 2.51 g | 79.58 g | 1.90 g |
| | Monopoliser Rank | 0.42 | 0.69 | 0.48 | 0.11 | 0.57 | 0.16 |
| | Leader-follower rank | 0.62 | 0.55 | 0.42 | 0.10 | 0.50 | 0.09 |
| Females | Relative gonad size | 0.81 | 0.44 | 0.00023 | 0.00006 | 0.00028 | 0.00005 |
| | Proportion of bill colour changed | -0.17 | 0.87 | 0.93 | 0.03 | 0.92 | 0.02 |
| | Body mass | 1.46 | 0.18 | 74.71 g | 3.02g | 81.40g | 3.69g |
| | Monopoliser Rank | 0.25 | 0.81 | 0.45 | 0.11 | 0.50 | 0.15 |
| | Leader-follower rank | 0.42 | 0.68 | 0.51 | 0.10 | 0.57 | 0.08 |

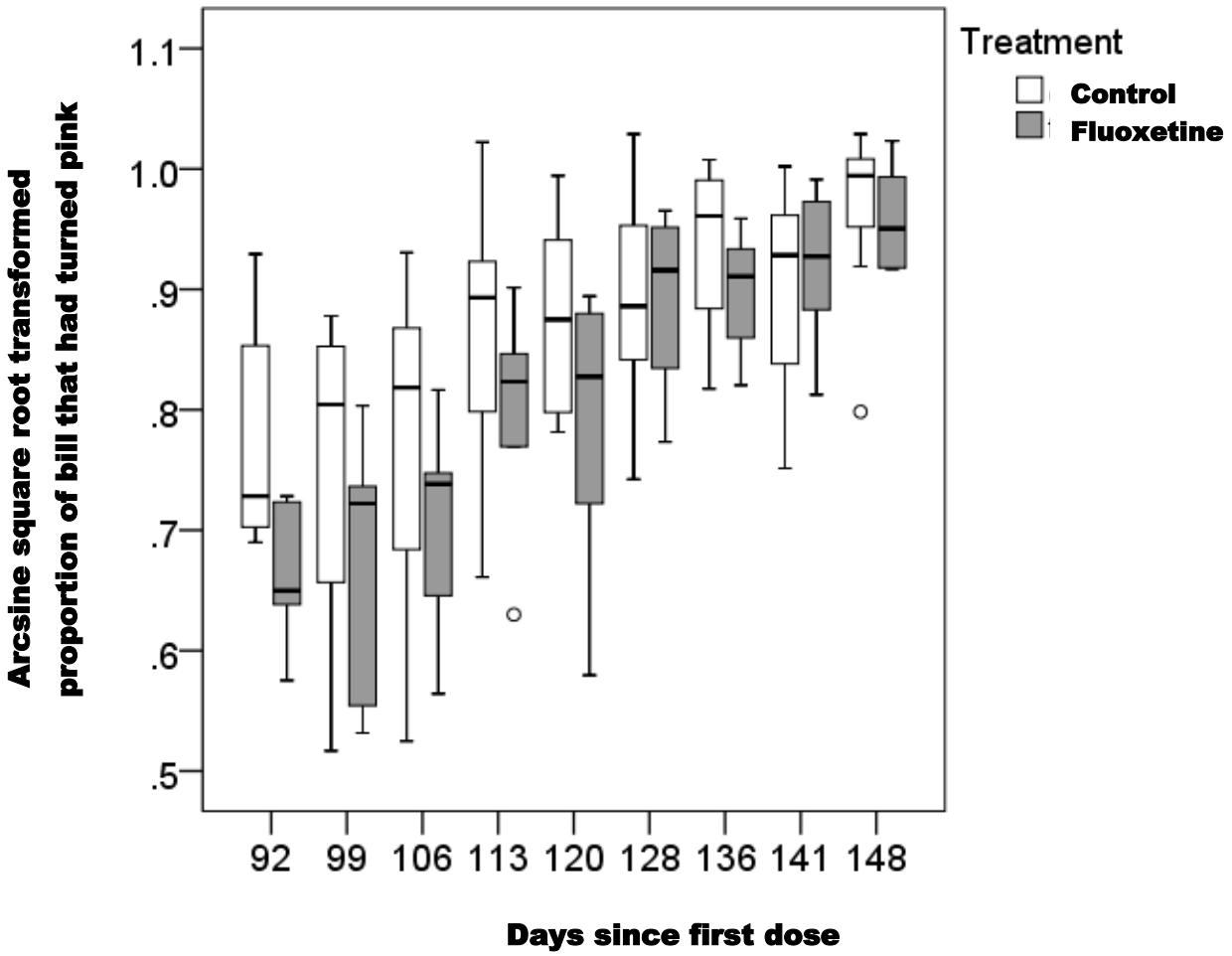



**Figure 4.4: Median proportion of females' bills that had changed from black to pink over time (Day 92 of treatment was 26th March when the first photo was taken) for control (white boxes) and fluoxetine-treated (grey boxes) birds. Proportions were arcsine square root transformed to meet assumptions of normality. Boxes represent the 25th and 75th percentile respectively and points beyond the whiskers represent outliers as defined by Tukey (1977).**

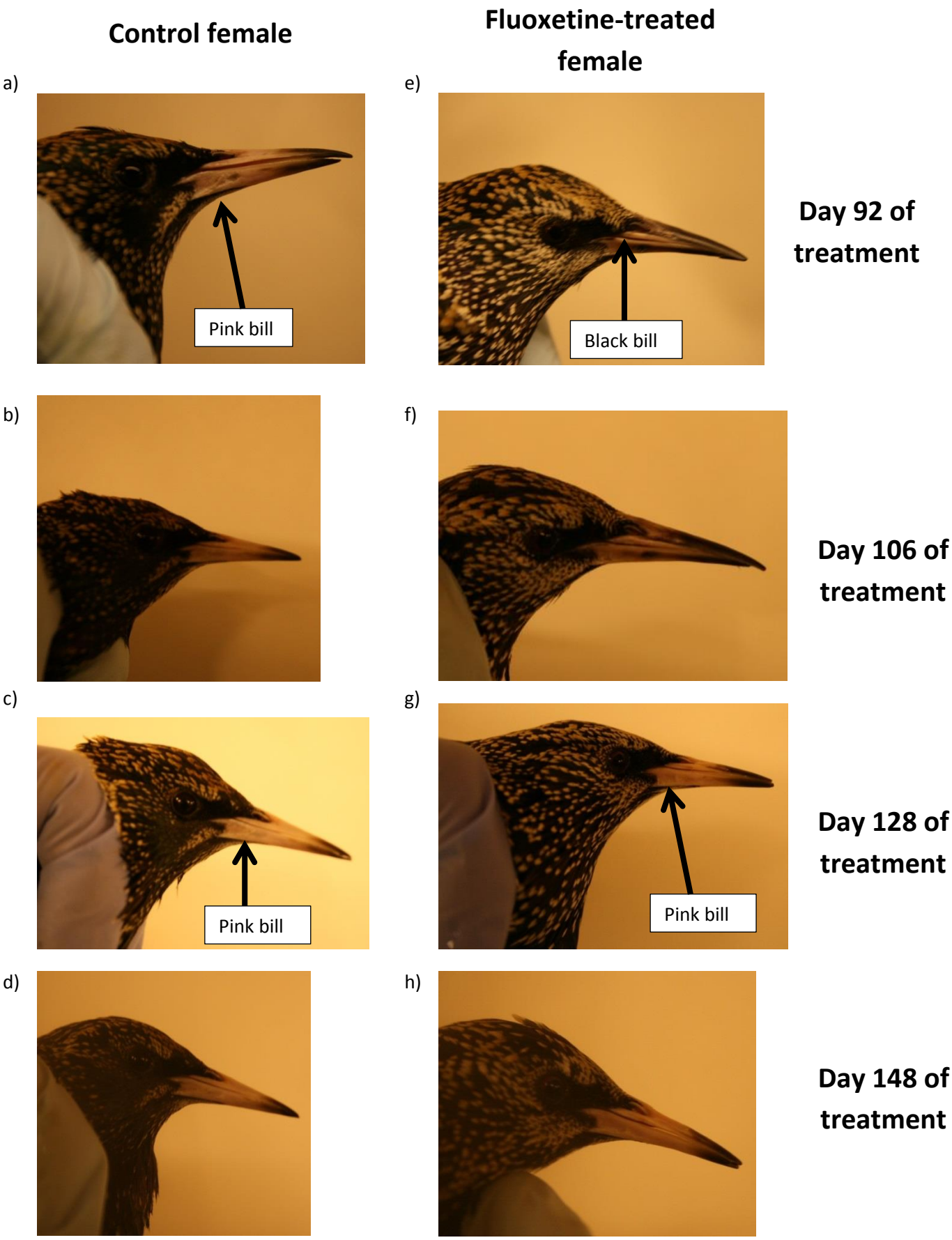



**Figure 4.5: Female bill colour change from black to pink for control (a-d) and fluoxetine (e-f) treated females on days 92, 106, 128 and 148 of treatment. The delay in colour change for the fluoxetine female is clearly shown by comparing figures 4.5 a and e on Day 92 of treatment. Miller (1969) also observed that the starling bill did not turn yellow in captive starlings.**

## 4.4 Discussion

There was evidence to suggest that fluoxetine-treated females were less likely to spend time associating with a male than control females, particularly the fluoxetine-treated males. There was no overall difference in male attractiveness and none of the physiological (gonad size, body mass and bill colour) or social behaviour measures (monopoliser and leader-follower rank) that compared between the male pair choice were found to significantly affect female association time with males, or differ in the way they affected association time between treatment groups. Although these physiological and behavioural variables were found not to differ between treatment groups for either sex, a more detailed analysis of the rate of bill colour change over the course of the breeding season (rather than simply looking at the colour at the time of the mate choice trials) found fluoxetine-treated females to significantly lag behind the controls in the rate that their bill changed from over-winter condition to breeding condition.

A possible explanation for the lack of female interest in males is that fluoxetine caused the females to have reduced libido, as is commonly observed in humans (Shen and Hsu 1995), mammals (Cantor et al. 1999; Matuszczyk et al. 1998; Uphouse et al. 2006) and aquatic species (Mennigen et al. 2008). Small adjustment to the serotonergic system initiates a range of neuro-adaptive mechanisms (Lesch et al. 1991). Axons from the raphe nuclei, the area of the brain containing the majority of the serotonergic system, form a neurotransmitter system that connects to nearly all areas of the brain. This neurotransmitter system includes neurons in the hypothalamus (see Figure 1.7 in Chapter 1), which control the release of a variety of hormones, including those responsible for libido such as oxytocin (Serres et al. 2000; Uphouse et al. 2006). In theory, the neuro-adaptations in response to chronic exposure to fluoxetine eventually desensitizes the post synaptic serotonin receptors (Lesch et al. 1991), which in turn leads to decreased activation of the neurons in the hypothalamus, which leads to decreased release of oxytocin (Raap and Van de Kar 1999). However, the exact relationship between fluoxetine, serotonin, oxytocin and sexual dysfunction remains incompletely understood at present (Cantor et al. 1999). A potential indicator of neuro-endocrine changes that could result in reduced libido are phenotypic changes indicating a change from winter to breeding condition (Bennett et al. 1997) which are driven by androgens (Yadav 2008). The androgen system is also known to be closely related to the serotonin system (Bethea et al. 2014). Therefore, the delay in female bill colour change in the early breeding season provides a potential signal of the underlying reason. It is not possible to be certain that mate-choice *per se* drove the observed effects in the mate choice trial. Previously, no treatment effect on female activity levels was found (see Figure C1 in Appendix C2). Repeat experiments, an investigation of the concentrations of serotonin and a measure of the levels of sex hormones (androgens) (Raap and Van de Kar 1999) could go some way

to confirming whether bill colour change is a plausible marker for changes in the neuro-endocrine system (i.e. side effects of fluoxetine). This biomarker could then be used as an indicator of the behavioural effects I observed on reduced male interest (see Chapter 6).

Interpreting the measures of male (and female quality) can be challenging. For example, the body mass a dominant individual is not necessarily a heavier one as is found more in terrestrial species (Krebs and Davies 1993). Optimum body mass is a function of both predation pressure and environmental conditions (Brodin 2001) and it is difficult, without taking measurements of fat score (Houston and McNamara 1993), to interpret whether a heavier bird is a dominant or a subordinate male. Behavioural measures can provide a better indication of dominance than body-mass alone. Starlings are a gregarious species (Apfelbeck and Raess 2008; Feare 1984); social interactions such as flocking behaviour enable each individual within the social group to reduce the amount of time they allocate to predator evasion and increase the time that can be spent foraging (Powell 1974).

There was no other evidence to suggest that the reason for the fluoxetine–treated females showing a lack of interest in males had anything to do with the quality of males physically or socially. Therefore any other explanation must be to do with the females. For the females, at the time of the mate choice study, there was no significant difference in social ranks (monopoliser or leader-follower), bill colour, body-mass or ovaries size. The only difference found between fluoxetine and control females was the delayed development of breeding condition, indicated by the rate of bill colour change had a lasting effect on female reproductive or social behaviours.

Although there was evidence to suggest that the fluoxetine-treated females did lag behind control females in the rate of bill colour change, no birds' bill turned entirely from winter condition to breeding condition before the experiment was terminated, possibly due to captive conditions and or isolation from the opposite sex during the exposure period. At the time of the mate choice study, the proportion of the fluoxetine-treated females' bill that had changed colour had caught up with the control females. It is plausible that treatment with fluoxetine could have caused the observed difference in rate of bill colour change in the fluoxetine-treated females as reduced sociality and sexual dysfunction are common side effect of therapeutic use of SSRIs such as fluoxetine in both sexes in human and mammalian subjects (Cheeta et al. 2001; Montgomery et al. 2002).

It is suggested that fluoxetine exposure in wild starlings may have an impact on female interest in males and or sociality, but cautiously, as a lack of courtship displays from either group of males, e.g. bouts of song or wing waving (Feare 1984) may suggest that the captive environment was not conducive to male courtship display. Indeed, previous studies looking at courtship behaviours in starlings have either presented males with nest boxes (Gentner and Hulse 2000) or played male song

in front of different perches (Markman et al. 2008) to create a mating context. Nevertheless, despite this limitation, longer term effects on female behaviour were found here than in a study dosing rats with 10 mg/kg/day for six weeks, in which fluoxetine only decreased male association time after the first week (Matuszczyk et al. 1998).

My results are in contradiction with studies in aquatic species such as Siamese fighting fish (*Betta splendens*) (Dzieweczynski and Hebert 2012) although recent work by Weinberber and Klaper in fathead minnow (*Pimephalas promelas)* supports the idea that fluoxetine can alter sexually related traits (Weinberger and Klaper 2014). Mennigen et al. (2011) provide support for the view that fluoxetine can act as an endocrine disruptor in non-target species with their work in Goldfish (*Carassius auratus*). Differences in uptake between aquatic and terrestrial species make it difficult to extrapolate between the terrestrial and aquatic environments (Dzieweczynski and Hebert 2012); nevertheless, my findings used a dose that is expected to be within the ecologically relevant range for avian exposure (see section 2.3.1). For applied taxonomic comparisons, comparisons of the effects of ecologically relevant doses is a more relevant approach than assessing toxicity *per se* with a dose unlikely to be encountered by an environmental species. Having said this, it is worth noting that some species are particular sensitive to the effects of pharmaceuticals at low doses. For example *Gyps* vulture species suffered severe population declines due to ingestion of diclofenac contaminated carcases as they did not possess the necessary detoxification enzymes, pharmaceuticals (Oaks et al. 2004; Swan et al. 2006a). Comparing metabolic processes between species could provide a useful complementary tool for identifying species likely to be particularly sensitive to adverse effects from pharmaceutical contamination e.g. (Hutchinson et al. 2014). It is also important to remember that fluoxetine is one of many antidepressants to be detected in the environment (Calisto and Esteves 2009), therefore it is plausible that the combined dose of these drugs could be sufficient to cause mode of action related effects through additive or synergistic interactions (Backhaus 2014).

Research into the effects of fluoxetine on mate choice in birds provides an interesting area for future research. Although it is difficult to extrapolate effects from captive birds to wild populations it is plausible that delayed reproductive development could have negative impacts, especially in a gregarious species that breeds in the temperate zone like European starlings (Boogert et al. 2008; Boogert et al. 2006; Feare 1984). Delayed reproductive development may lead to females showing reduced interest in males and could place individuals at a competitive disadvantage when it comes to selecting the highest quality mates. Moreover reduced female interest in males could lead to individuals missing out on reproducing altogether. If these effects on reproductive success were to occur in the wild, the ecological implications are potentially severe at a population level, particularly so in a species that has seen declines of over 84% since the 1979 (RSPB 2014). Given that in Chapter

3 I found further evidence that fluoxetine can alter fitness related traits at a body mass and dose corrected accumulation level in the brain approximately 31 times less than humans (Chapter 2), it is important to clarify both the potential for pharmaceuticals in the environment to alter behavioural endpoints and to improve our understanding of the relevance of these behavioural endpoints to wild populations.

# Chapter 5:

# An *in-vitro* assessment of differences in bioaccessibility of an antidepressant between humans and birds


## Abstract

There is increasing interest in utilising data collected in model species to establish the risks posed by chemical contaminants to wildlife species (read-across). However, one of the factors that this approach does not account for is differences in internal exposure. Physiologically based extraction tests (Pbets), an *in-vitro* method for quantifying bioaccessibility, have been developed for inorganic contaminants but to date the approach has not been extended to organics. In a two-compartment *in-vitro* gastrointestinal tract model, gastric conditions were initially simulated to compare the bioaccessibility of the antidepressant fluoxetine between human and avian Pbets. The contaminated 'prey' inserted into the Pbets were earthworms which had been exposed to fluoxetine in contaminated soil for 21 days. Samples of gastric phase and intestinal phase digestive juice were obtained along with residual solid material 'faeces', extracted with solvent and analysed by High Performance Liquid Chromatography (HPLC). For both humans and birds, a range of digestive tract conditions was simulated to account for within species variation. Overall, the avian Pbet with the most acidic gizzard was found to have lower bioaccesibilty (after the intestinal phase) than all three human Pbets and the less acidic avian gizzard Pbet. The area of the gastro intestinal tract where fluoxetine was mobilised was also significantly different between Pbets, with the exception of the less acidic avian Pbet, and the average human conditions Pbet. Additionally, the *in-vitro* results for the percentage recovered in the faeces (humans: 14.1-15.8%, birds = 18.7%) agree well with *in-vivo* data (humans: 5-15%, starlings *Sturnus vulgaris* = 19.3 %). Given that pharmaceuticals are well studied in terms of clinical and pre-clinical data, there is potential to extend the scope of read-across to include wildlife species in risk assessments, using the Pbet approach to adjust for differences in bioaccessibility between species.


## 5.1 Introduction

Over the last 15 years there has been increased attention on the potential for active pharmaceutical ingredients (APIs) to enter the natural environment and to adversely affect non-target species

(Monteiro and Boxall 2010; Arnold et al. 2014; Shore et al. 2014). Due to the extensive number of clinical and pre-clinical tests conducted on active pharmaceutical ingredients, these substances are amongst the most studied compounds in terms of their impact on humans and other mammals (Boxall et al. 2012). There is therefore increasing interest in using the existing data from API clinical and pre-clinical tests to read-across to species in the natural environment (Huggett et al. 2003; Boxall et al. 2012; Rand-Weaver et al. 2014; Margiotta-Casaluci et al. 2014). There are several issues with extrapolating effects across species, including assumptions that the sensitivity of receptors, metabolism and bioaccessibility (internal exposure) are equivalent (Gunnarson et al. 2008). Bioaccessibility is the percentage of the pharmaceutical that goes from the ingested matrix (e.g. food or soil) into the digestive juice. While bioaccessibility does not necessarily equal bioavailability (the percentage of the dose that reaches systemic circulation), an understanding of how much of the ingested contaminant is available for uptake is an important stepping stone towards predicting internal concentrations.

One of the most important factors likely to affect bioaccessibility is likely to be the pH of the digestive tract of the organism as the behaviour of pharmaceuticals, which are typically ionisable substances, can be very sensitive to changes in pH (Brooks 2014; Nakamura et al 2008; Chasseaud 1970). Even within species, factors such as the presence of food (Ruby et al. 1996) and the geochemistry of grit in the avian gizzard (Best and Gionfriddo 1991) will affect pH and subsequently bioaccessibility. Therefore the pKa of the contaminant, i.e. the pH at which 50% is present in the ionised form, and the gastrointestinal tract pH of the organism, are both likely to be important factors for predicting bioaccessibility of pharmaceuticals in different species. However, the digestive systems of different species also vary in terms of temperature, enzymatic composition, gastrointestinal tract transit time and physical breakdown (Li and Zhang 2013; Martinez-Haro et al. 2009; Ruby et al. 1996). These factors are likely to be additional factors to pH and pKa that influence the rate and extent to which a contaminant is mobilised from food in the gastrointestinal tract.

It is known that there is considerable variability in the physiology of the digestive systems of different species. For example, birds have a muscular gizzard, which often contains grit to grind food, while mammals' have teeth do the grinding role and so they only have a glandular stomach (Kaufman et al. 2007; Martinez-Haro et al. 2009; Moriarty et al. 2012; Ruby et al. 1993). To add further complexity, it has also been demonstrated that bioaccessibility of a chemical from different matrices (e.g. soil, plant, fish and meat) can vary considerably (Laird et al. 2009; Ollson et al. 2009; Ruby et al. 1999). Understanding differences in bioaccessibility is therefore clearly important if we are to be able to read-across from existing *in-vivo* data, for a test species and standard matrix to other species and

matrices (Saunders et al. 2011). This study assesses whether the bioaccessibility of a pharmaceutical from the same matrix varies both within and between species.

In the past decade an *in-vitro* test system, a Physiologically based extraction test (Pbets), that simulates gastro-intestinal conditions in a two phase *in-vitro* model, has been used in a variety applications including testing the safety of children's toys containing metals (Ruby 2004), in the assessment of contaminated land (Button et al. 2009; Collins et al. 2013) and to assess the risks presented by lead shot to wild birds (Martinez-Haro et al. 2009). Pbets have already been developed and validated to assess the bioaccessibility of trace metals for several organisms including humans, (Bruce et al. 2007; Li and Zhang 2013; Oomen et al. 2002; Ruby et al. 1993; Ruby et al. 1996; Ruby et al. 1992; Ruby et al. 1999; Tilston et al. 2011; Turner and Ip 2007), small mammals (Kaufman et al. 2007; Moriarty et al. 2012) and birds (Kimball and Munir 1971; Levengood and Skowron 2001; Martinez-Haro et al. 2009). It has been suggested that Pbets could play a wider role in the assessments of risks of chemicals to mammals and birds by helping to adjust for differences in bioaccessibility (Saunders et al. 2011). By using data collected in model species as part of clinical and pre-clinical trials, it would be possible to quantify differences in bioaccessibility between species to make Environmental Risk Assessments more applicable to wildlife.

The potential risks of pharmaceuticals in the environment to wild bird and mammal populations are only recently starting to be studied (e.g. Shore et al. 2014; Cuthbert et al. 2014; Zorilla et al. 2014; Lazarus et al. 2014). Indeed, the need to improve our knowledge about the risks that pharmaceuticals in the environment pose to species of birds, mammals, reptiles and amphibians was one of the key questions for future research on pharmaceuticals in the environment, identified at a recent workshops of international experts (Arnold et al. 2013; Boxall et al. 2012). By combining Pbet-derived data on relative bioaccessibility for different species, with information on expected daily doses for wildlife and effects data in model species, it would be possible to assess the risks posed to wildlife with greater certainty. For ionisable substances, such as pharmaceuticals, an assessment of how the compound behaves across cell membranes would also be required to see how bioaccessibility relates to bioavailability. In the human system, oral bioavailabilities of pharmaceuticals are well defined (e.g. Drugbank 2009), but little is known about bioavailability of organics, or how to quantify this in non-mammalian systems.

As the first study to look at internal exposure for organic chemical contaminants, the *in-vitro* methods that have been developed and validated for inorganics were applied to organics. The use of a human gastro-intestinal Pbet system, and an avian gizzard-intestinal Pbet system for establishing the relative bioaccessibility of the selective serotonin reuptake inhibitor (SSRI) antidepressant

fluoxetine from earthworm prey, is demonstrated. Fluoxetine is the active ingredient in products including Prozac, which are used to treat anxiety related conditions such as depression in humans (Eli Lilly 2009). SSRI anti-depressants have been shown to occur in the environment, e.g. in effluent dominated surface waters (Brooks et al. 2005), trickling filter invertebrates at WWTPs (Bean et al., 2014 Chapter 2 and Appendix A1) and sewage sludge (McClellan and Halden 2010). Earthworms have been shown experimentally to bioaccumulate fluoxetine to levels up to 35 times higher than in the soil (Carter et al. 2014b). To maintain ecological relevancy earthworms were exposed to fluoxetine in the soil and left to accumulate fluoxetine for 21 days. Then one intact earthworm plus any adhered soil (to mimic a bird eating a worm in the wild) was inserted into each Pbet replicate. By conducting a range of human and avian Pbets, both with the same food type inserted, I generated a comparative assessment of how much of the ingested contaminant is made available for absorption. This assessment of relative bioaccessibility will enable read-across between species to happen with greater accuracy, e.g. by improving the understanding of whether lower fluoxetine internal concentrations in birds than expected, based on human data, could be due to differences in bioaccessibility (see Chapter 2 for details of why internal concentrations are important in read-across). This assessment could not be achieved if the contaminant was introduced to the human system in tablet form (a realistic scenario for humans) and a worm for birds; doing so introduces matrix type as a confounding variable, which will not enable a fair comparison of the solubility of fluoxetine between species.

*In-vivo* validations are a challenging but important aspect to developing *in-vitro* gastrointestinal tract models for new contaminants or species. While inorganics have been validated *in-vivo* using x-ray techniques to assess corrosion rates of lead shot in birds (Kimball and Munir 1971), this is not possible for organics; theoretically collecting and extracting the digestive juices from the gastrointestinal tract would be needed, but sampling these presents many challenges. Therefore, an *in-vivo* evaluation based on the percentage of the dose excreted unchanged in the faeces of captive European starlings (the data presented in Chapter 2) was used to assess the performance of the avian Pbets.

## 5.2 Materials and Methods

### 5.2.1 Test chemicals and soil

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

A sandy loam (pH 6.47) was collected from an unpolluted site (SE 56647 51563) for use in the earthworm exposures. Roots and stones were first removed by hand. The soil was air dried for 24 hours before passing through a 2 mm sieve. Details of how moisture content and maximum water holding capacity were determined are given in Appendix D1.

### 5.2.2 Preparation of earthworms

This experiment investigated the bioaccessibility of fluoxetine from earthworm prey. A colony of *Eisenia fetida* were obtained from The Food and Environment Research Agency (FERA York, UK) and maintained at optimal conditions (Carter et al. 2014b), until a sufficient number of individuals weighed 0.5 g ± 0.1 g. After three weeks, when earthworms were of sufficient size for the experiment, individuals were removed from the colony, soil, which had adhered to the earthworm, was removed, by holding the earthworm with a pair of blunt ended forceps and pipetting deionised water. Earthworms were dabbed dry on paper towels prior to inserting into their individual exposure jars.

To expose individual earthworms to fluoxetine, 50 g of moist soil was weighed out into individual glass jars (approximate volume of jars was 100 mL). Soil was either spiked with 1 mL of fluoxetine solution, (30 mg/mL fluoxetine dissolved in methanol) to give an expected fluoxetine concentration of 600 µg/g soil. A fluoxetine concentration of 0.37 µg/g is an environmentally relevant concentration in treated sludge (Monteiro and Boxall 2010). The spiking concentration, was based on previous work on uptake of fluoxetine into earthworms (Carter et al. 2014b), and was selected to yield a concentration of fluoxetine in earthworms of at least 60 µg per earthworm, i.e. above the limits of detection using HPLC (see Chemical analyses section 5.2.6). Control soils were spiked with 1 mL of methanol. After spiking, the soil was left for two hours before stirring with a spatula. In total soil jars were left for 48 hours prior to adding a single earthworm to each jar.

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

To control for the effect of soil moisture content on bioaccumulation, the soil moisture content was maintained at 60% MWHC over the 21 day exposure period. This was achieved by adding the required mass of deionised water daily to return the jar to its starting mass (OECD 2010). A small amount of food was re-applied as necessary, approximately every other day. The experiment was

staggered so that the Pbets were carried out on nine separate days. Throughout the 21 day exposure periods, earthworms were kept in a Controlled Environment room (20°C, 70% humidity, 16 hours light and 8 hours dark).

### 5.2.3 Physiologically based extraction tests

Relative bioaccessibilities for the human and avian digestive systems were quantified by inserting earthworms (*Eisenia fetida*), along with soil which had adhered to the worm, into the human and avian Pbets. The earthworms were not cleaned first in order to mimic foraging conditions in the wild. From each Pbet, samples of stomach (human) or gizzard (bird) and intestinal digestive juice were obtained. Digestive juice samples were centrifuged and extracted with solvent, passed through a 0.2 µm polytetrafluoroethylene (PTFE) syringe filter and analysed by HPLC with fluorescence (see section 5.2.6). The solid material remaining at the bottom of the intestinal digestive juice sample was also extracted and taken to represent the 'faeces' as in (Martinez-Haro et al. 2009). The percentage of fluoxetine recovered at the end of the intestinal phase (relative to the total recovery, which is the percentage recovered at the end of the intestinal phase plus the percentage recovered in the faecal pellet) was compared within, and between, species to assess total bioaccessibility. Total bioaccessibility was given by the sum of the percentage recovered in the stomach (or gizzard) and the additional amount extracted in the intestinal compartment of the model (see Figure 5.2). The percentage recovered in the faecal pellet was used to represent the percentage of the dose excreted unchanged. This was compared and contrasted with *in-vivo* data to evaluate the performance of the Pbets.

In total five Pbets were conducted: two avian and three human. These Pbets were designed to cover the broad range in digestive tract conditions for birds and humans. To simulate uptake of fluoxetine by wild birds, I inserted earthworms, which had been exposed to fluoxetine in the soil, directly into each Pbet along with any soil adhered to the earthworm. The mean total weight of earthworm and soil was 0.49 g ± 0.11 g for earthworms exposed to fluoxetine (N = 20), and 0.70 g ± 0.13 g for control earthworms (N = 20), and the mean mass of adhered soil was 0.155 g ± 0.042 g (determined by rinsing the soil from four earthworms that did not go into a Pbet). It is worth noting that although the fluoxetine and control earthworms had similar masses before the 21 day exposure period (fluoxetine = 0.534 g, RSD = 6.6%, N=24; control = 0.518 g, RSD = 7.2%, N = 24), the control earthworms gained a significant amount of weight over the 21 days while the fluoxetine group lost mass. To humanely kill the earthworms, the tubes containing the earthworm were placed in a -20°C freezer and brought back up to room temperature prior to digestion in the simulated gastrointestinal tract.

In addition to the fluoxetine and control earth earthworms, I also ran blank Pbets containing no food. All simulations were performed in triplicate as is widely used in Pbets (e.g. (Laird et al. 2009; Li and Zhang 2013; Martinez-Haro et al. 2009; Ruby et al. 1996) (see Appendix D2 for details of the structure of this experiment).

### 5.2.4 Human Pbets

The human Pbets were adapted from the methods of Li and Zhang (2013) and Ruby et al. (1996) (see Figure 5.1a). Human Pbets were carried out in 50 mL centrifuge tubes using stomach digestive juice at three different pHs, to account for the effect of time since the last meal on stomach pH. Stomach digestive juices were prepared in conical flasks by adding pepsin (1.25 g $L^{-1}$), malate, citrate (both 0.5 g $L^{-1}$), lactic acid (420 µL $L^{-1}$) and acetic acid (500 µL $L^{-1}$) to deionised water. Stomach digestive juice was adjusted to one of three pHs using concentrated (35%) HCl: pH 1.3 was used to represent fasted conditions, pH 2.5 represented average gastric conditions and pH 4 recently fed conditions (Ruby et al. 1996) and then warmed to 37°C in a water bath.

To begin the Pbet simulation, whole earthworms and any adhered soil were added to tubes intact along with 20 mL of stomach digestive juice. The head space was purged with $N_2$ to create a low oxygen environment. Tubes were placed in the shaking incubator at 37°C but were not shaken for 10 minutes as in (Ruby et al. 1996), after which tubes were shaken at 250 rpm for one hour in total. At three time points (every 20 minutes) during this hour, shaking was stopped and a 1.5 mL aliquot of gastric juice was taken from each tube and, and replaced with a fresh 1.5 mL of gastric solution. The gastric juice samples were transferred to a 1.5 mL sealed microcentrifuge tube and immediately centrifuged (10 mins at 11,000 × g). The supernatant decanted into a fresh tube and stored at -20°C.

Once the gastric simulation was complete, the digestive juice in each simulation tube was adjusted to pH 7 with $NaHCO_3$ powder (intestinal pH) (Ruby et al. 1993). For the pH 1.3 simulation, the worm had completely disintegrated after 1 hour, pH 2.5 had partially disintegrated but at pH 4 the worm could clearly still be seen intact prior to the intestinal simulation. Once pH had been adjusted, 52.5 mg of bile extract and 15 mg of pancreatin were added to each replicate (Li and Zhang 2013) before returning to the shaker. The 50 mL centrifuge tubes were shaken on their sides at 100 rpm to mimic the slow intestinal passage of food. After 1 hour (data not presented here) and after 3.5 hours of intestinal incubation, 1.5 mL aliquots were taken from each centrifuge tube. Unlike the stomach phase, the intestinal 1.5 mL aliquots were not replaced after sampling. The intestinal samples were centrifuged and stored at -20°C. The remaining solid material was considered to be faeces.

### 5.2.5 The Avian Pbet

To capture the complexity of the avian digestive tract, a dynamic avian gizzard-intestine system was simulated following the methods of Martinez-Haro *et al.* (2009), which is summarised in Figure 5.1b. The avian gizzard digestive juice was prepared in a 1 N NaCl solution, as in (Martinez-Haro et al. 2009), and contained either 2 g of siliceous (Si grit) or 2g of calcareous grit (Ca grit) (obtained from a local pet shop), as in (Martinez-Haro et al. 2009), to account for the distinct geo-chemistries and the influence on gastrointestinal tract pH experienced by wild birds (Best and Gionfriddo 1991). The intestinal digestive juice was made up in deionised water containing bile extract (3.5%) and pancreatin (0.35%), as in (Martinez-Haro et al. 2009). To begin the simulation, 12 mL of gizzard digestive juice (Martinez-Haro et al. 2009) was added to a 50 mL centrifuge tube containing grit and an earthworm. Tubes were placed in the shaking incubator on their sides for a total of three hours (350 rpm, 42°C) following a Pbet validated for waterfowl (Martinez-Haro et al. 2009). Gizzard digestive juice changes took place as in the human Pbets, but at 36 minute intervals to give a total of five changes in the three hour gizzard simulation. At each change of digestive juice two 1.5 mL aliquots were removed and replaced with 3 mL of fresh digestive juice. The first aliquot (the gizzard sample) was centrifuged; the supernatant was transferred to a fresh tube and stored at -20°C.

The second aliquot (which became the intestinal simulation) went into a separate 1.5 mL centrifuge tube. The pH was adjusted to 6.2 using a $NaHCO_3$ solution (9 g/100 mL deionised water) as outlined in (Martinez-Haro et al. 2009), however I encountered a number of issues with their approach. Firstly, when adjusting the pH of the gizzard digestive juice samples to intestinal pH (6.2), the initial pH of the siliceous grit samples was approximately pH 3.6 for the first gizzard digestive juice change, and had fallen to pH 2.8 by the fifth change, meaning that a stronger base was required to raise the pH to 6.2. I added 10 µL of a 5% NaOH solution to each, and made the remaining adjustments to pH with the $NaHCO_3$ solution (approximately 15-25 µL). For the Calcareous grit, the initial pH was actually above 6.2 (approximately 6.7); I still added 10 µL of $NaHCO_3$ and made the residual change with 0.2M HCl. An issue I experienced was that the method of Martinez-Haro et al. (2009) used triplicates for each grit simulation and a fourth replicate was used to monitor pH change (to speed up the procedure). Martinez-Haro et al. (2009) noted down the amount of $NaHCO_3$ added to the fourth replicate and then added the same amount to the other three samples so that pH only had to be measured once for each digestive juice change time point (instead of three times). However when I was validating the methods, I found that using the approach of Martnez-Haro to speed up digestive juice changes led to variable pHs in the triplicates, which I suggest could be due to differences in earthworm weight added to each Pbet. Subsequently, the pH of each replicate was changed individually.

Once the pH of had been adjusted to 6.2, the intestinal simulations were shaken on their side (150 rpm, 42°C), as an end over end shaker as used in Martinez-Haro et al. (2009) was unavailable. After three hours of the intestinal simulation, samples were removed from the shaking incubator and processed as in the human Pbets.

### 5.2.6 Chemical analyses

Gizzard, stomach and intestinal samples were defrosted, vortex mixed and then a 500 µL aliquot was taken and combined with 500 µL methanol. Faecal samples were extracted into 1 mL of methanol using sonication for 3 minutes. Sample/methanol mixtures were centrifuged for 10 minutes at 11,000 × g and filtered using a 0.2 µm filter (no further chemical clean-up was required in order to exceed the limits of quantification of the HPLC method used).

Extracts were analysed by HPLC with fluorescence (excitation = 230 nm, emission = 305 nm), using a gradient at 1 mL $min^{-1}$ consisting of mobile phase of A) water containing 0.1% $H_3PO_4$ and B) methanol. The mobile phase was degassed in an ultra-sonication bath for 20 minutes. Separation was achieved using a gradient ranging from 90% to 10% 'A' on a C-18 column (Kinetex 5 µm C18 150 × 4.6 mm, Phenomenex, Macclesfield UK). The run time was 23 minutes with a retention time of 11.7 to 11.8 minutes.

### 5.2.7 Validation of chemical analyses

Using blank digestive juice samples that had been through the Pbet procedures for each of the five Pbets, we spiked in 0, 1, 10, 50, 100 and 200% of the fluoxetine concentration, which was found to be extracted in each compartment of the model (stomach or gizzard, intestine and faeces) during a pilot study. To extract fluoxetine from samples, 500 µL aliquot was taken and extracted with 500 µL methanol. Samples were vortex mixed for 5 secs, centrifuged for 10 minutes at 11,000 × g before passing through a 0.2 µm PTFE filter and analysing by HPLC (see Chemical analyses in section 5.2.6). The percentage recoveries of the methanol extraction are presented in Table 5.1 along with the limits of quantification. The limits of detection were below the levels detected in all of the real samples.

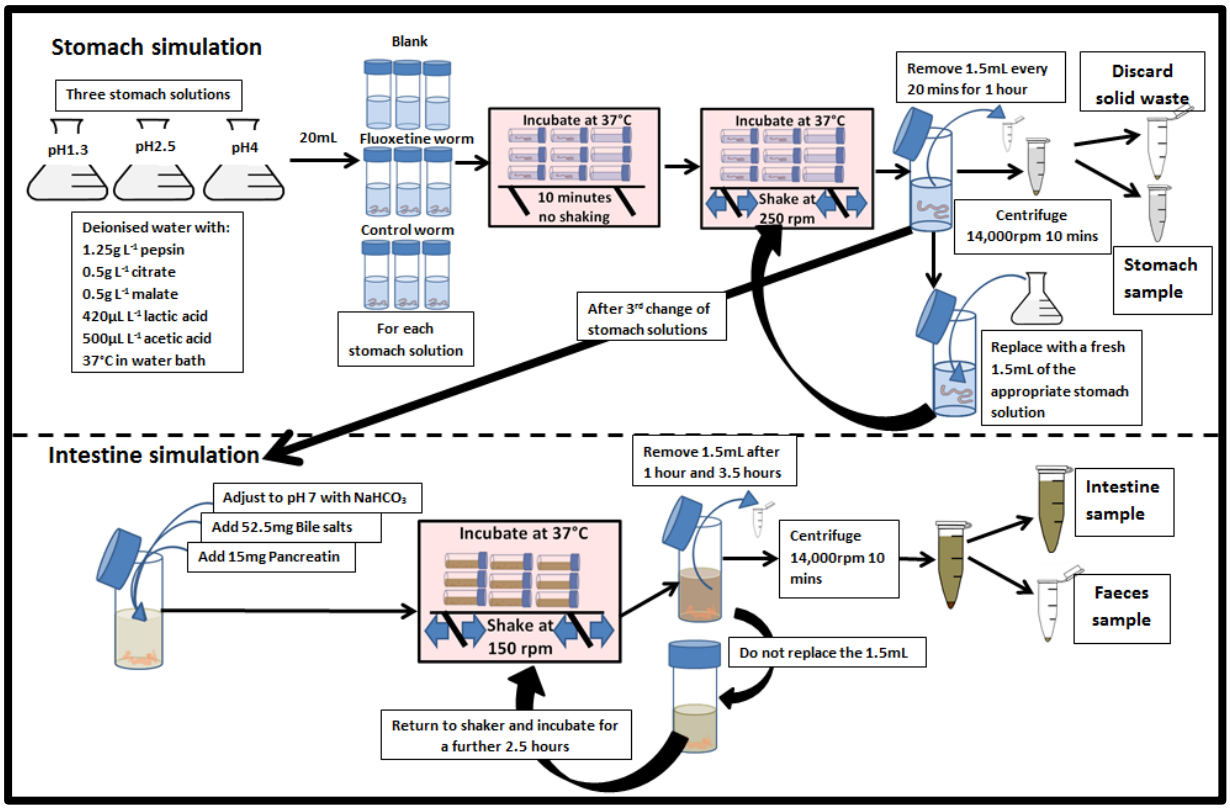
### 5.2.8 Quality Assurance and Quality Control

To maintain environmental relevance, the earthworms inserted to Pbets accumulated fluoxetine from soil, rather than being spiked with a known amount. As a result, the exact concentration of fluoxetine in each earthworm and adhered soil was unknown. I needed to determine the expected amount of fluoxetine determined in each Pbet based on the mass of worm and soil added; this required me to assay concentrations in the fluoxetine spiked soil and the worms that had been

exposed for 21 days. To assay concentrations in earthworms, additional fluoxetine and control earthworms (N = 4 for each) were exposed under the same conditions as the earthworms that were inserted into the Pbets, and extracted with solvent and analysed by HPLC to determine a mean concentration in earthworm tissue (μg $g^{-1}$ wet weight). Additionally, I quantified the concentration in soil and the mean mass of soil that adhered to earthworms, so that it was possible to estimate the amount of fluoxetine inserted into Pbets, using the mean concentration of fluoxetine in earthworms and soil and correcting for mass of the earthworm. The mass of earthworm (± 0.01g) and soil was determined before rinsing soil away with deionised water and dabbing dry on paper towels. The mean mass of adhered soil was determined by taking the difference between earthworm plus soil and earthworm alone.

In the QA/QC, fluoxetine was extracted from earthworms by homogenisation (Turrax) with 6 mL of methanol followed by ultra-sonication for 10 minutes. The extract was centrifuged for 10 minutes at 4500 × g before passing an aliquot of the supernatant through a 0.2 μm PTFE for analysis by HPLC. To validate the earthworm extraction, additional earthworms, which were taken straight from the colony, rinsed of deionised water and dabbed dry, were spiked at 0, 1, 10, 50, 100 and 200% of the expected 60 μg $earthworm^{-1}$ in triplicate (mean recovery 75.6%, Limits of quantification 0.6 μg/mL, RSD between triplicates = 10.0%). Five grams of soil (at 60% Maximum water holding capacity) were taken from each jar of the QA/QC earthworms and placed into a 50 mL centrifuge tube. Soil was extracted twice using 10 mL of 70:30 acetonitrile: water. Samples were shaken on their side at 420 rpm before ultrasonicating and centrifuging (4500 × g, 20°C) for 10 minutes as in (Carter et al. 2014b).

a)




b)

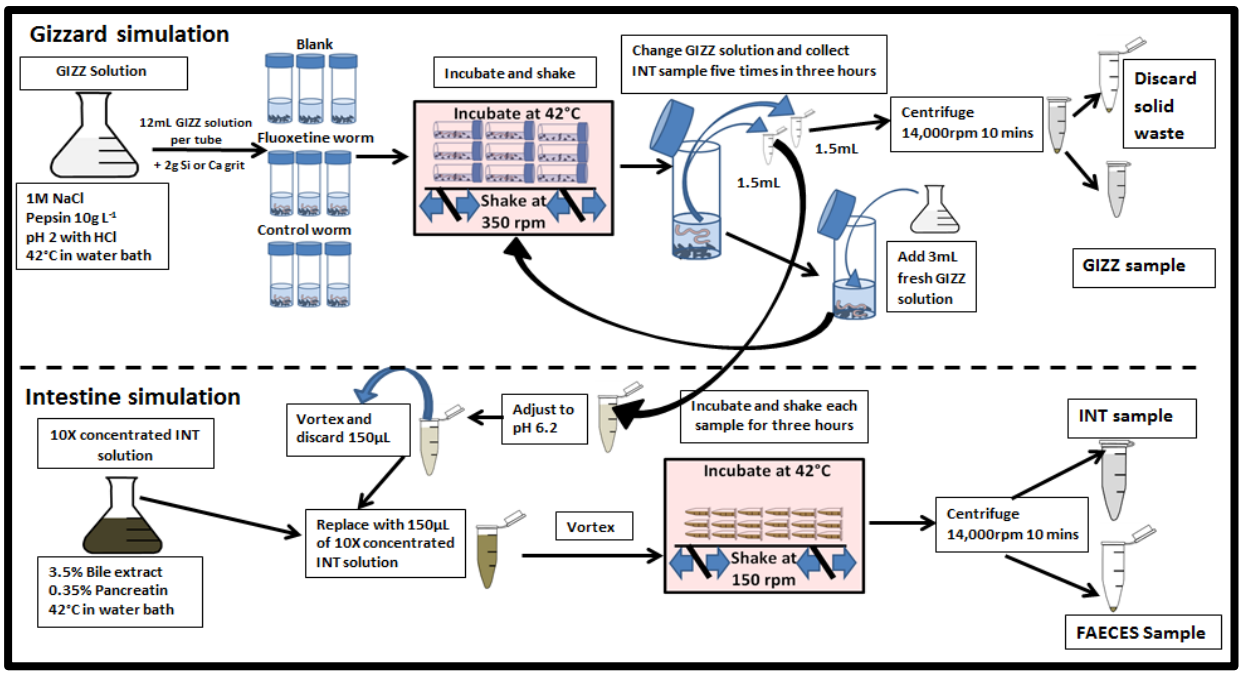



**Figure 5.1: Schematics of the Pbets for a) humans and b) birds. Whole earthworms containing fluoxetine or control (earthworms without fluoxetine) plus adhered soil were inserted initially into the stomach (a) or gizzard (b) along with digestive juice at low pH. For the stomach or gizzard simulations took place in 50 mL centrifuge tubes. In a) digestive juice samples were taken and replaced with fresh digestive juice three times and in b) five times to simulate the dynamic nature of the gastrointestinal tract. The intestinal phase was simulated by taking the samples from the stomach or gizzard simulation and raising the pH to 7 in (a) and 6.2 in (b) before adding bile extract and pancreatin. The intestine simulation in a) took place in a 50 mL centrifuge, in b) it was in a 1.5 mL microcentrifuge tube. Digestive juice samples were centrifuged and the solid material at the bottom of the intestinal sample was taken to represent the faeces.**

**Table 5.1: Limits of Quantification (LOQ) of the High Performance Liquid Chromatography (HPLC) method (µg/mL), mean percentage recoveries and percentage Relative Standard Deviations using methanol to extract fluoxetine spiked into blank digestive juice and faecal samples.**

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

The extraction of fluoxetine from soil was validated by spiking 5 g of soil at (60% MWHC) with 0, 1, 10, 50, 100 and 200% of the expected 600 μg/g concentration in triplicate and following the above extraction method. Mean percentage recovery was 77.2%, percentage Relative standard deviation (RSD) = 5.2).

### 5.2.9 Data analysis and statistics

In order to accurately estimate the amount of fluoxetine added to the Pbets in the earthworms, data was collected on: 1) the combined mass of earthworm plus soil (see section, 5.2.8), 2) the average mass of soil which adhered to an earthworm, so that the mass of the earthworm without soil could be estimated (section 5.2.8) and 3) the concentration of fluoxetine in earthworm and soil (also section 5.2.8). Using these three pieces of information, I then determined the mass of each earthworm by subtracting the mean mass of adhered soil. The mass of fluoxetine inserted into each Pbet was determined from the mean concentration in earthworm (537.1 μg $g^{-1}$, RSD = 17.6 %) and soil (572.7 μg $g^{-1}$, RSD = 3.3 %) while correcting for mass. The mean mass of adhered soil was 0.155 g, i.e. this soil contained on average 0.88 μg of fluoxetine.

To calculate the percentage of fluoxetine that was bioaccessible in each Pbet, the method of Yu et al. (2012; 2013) for calculating bioaccessibility was applied (see Figure 5.2). The combined percentage of the fluoxetine that was added to the Pbet, based on the mass of earthworm and soil, which was recovered in the intestinal digestive juice and the faecal pellet was assumed to be 100%. The percentage recovered at the different phases of the Pbets was then back calculated, based on how the concentrations in the sample related to the total amount of fluoxetine recovered (see Figure 5.2).

Statistical analysis was performed using a MANOVA to look at the percentage of fluoxetine recovered in the gastric phase, the intestinal phase minus the gastric phase, at the end of the intestinal phase (total bioaccessibility), the faecal sample remaining at the end of the intestinal phase and the residual fluoxetine not recovered using a MANOVA. As I obtained an overall effect of treatment, ANOVA's were used individual to assess where the differences between Pbet compartments lay. Finally, where there were significant differences in the individual ANOVAs, Post-Hoc Tukey's HSD tests (all on 4 degrees of freedom) were used to identify where the significant differences were. All statistical analysis was performed in SPSS (Version 19) and significance was tested for at the 5% level. As the percentage recovered in the faeces was simple 1 – the amount recovered at the end of the intestine, the statistics would be the same as the total bioaccessible percentage, i.e. total recoveries would always equal 100% as fluoxetine could only be in solution or remain to be extracted in the faecal pellet (see discussion).

### 5.2.10 *In-vivo* evaluation of the avian Pbet

The avian Pbet I used was designed and validated for waterfowl (Martinez-Haro et al. 2009). Here, the Pbet was evaluated for passerines, specifically the European starling; a species that forages on invertebrates at wastewater treatment works and on fields fertilised with sewage sludge throughout the year (Gough et al. 2003; Feare 1984; Fuller and Glue 1978; Fuller and Glue 1980; Markman et al. 2011). Wild caught starlings were dosed in captivity (Bean *et al* 2014, Chapter 2) with lesser wax earthworms spiked with fluoxetine (1300 ng/day five days a week, i.e. average dose of 928 ng/day) or carrier medium (n = 12 per treatment group). After 22 weeks of dosing, three birds from each treatment group were placed into individual test cages lined with paper for two hours after the final dose. For each bird, all faeces was collected after the two hours, (as gastrointestinal tract transit time for starlings eating invertebrates is as fast as 12 minutes (Levey and Karasov 1989)), and placed in a 1.5 mL sealed tube. The mean dry weight of faeces was (0.169 g ± 0.022). Faecal samples were processed as in (Chapter 2, section 2.2.6d) before extracting with 1 mL methanol, shaking for 30 minutes at 420 rpm, ultrasonicating for 5 minutes in an ultrasonication water bath, centrifuging for 10 minutes at 11,000 × g. The supernatant was blown dry under a steady stream of nitrogen at 45°C before reconstituting to 1 mL in 50:50 methanol:water. The percentage of the dose excreted unchanged was determined using LCMS/MS (methods presented in Appendix D6 or Chapter 2 section 2.3.6e). The percentage of the dose excreted unchanged was compared between *in-vivo* and *in-vitro* data to provide an indication as to how well the avian Pbet predicted the percentage excretion of fluoxetine by passerines. I compared the *in-vivo* data with the Ca grit model as the *in-vivo* starling diet included a calcium supplement.

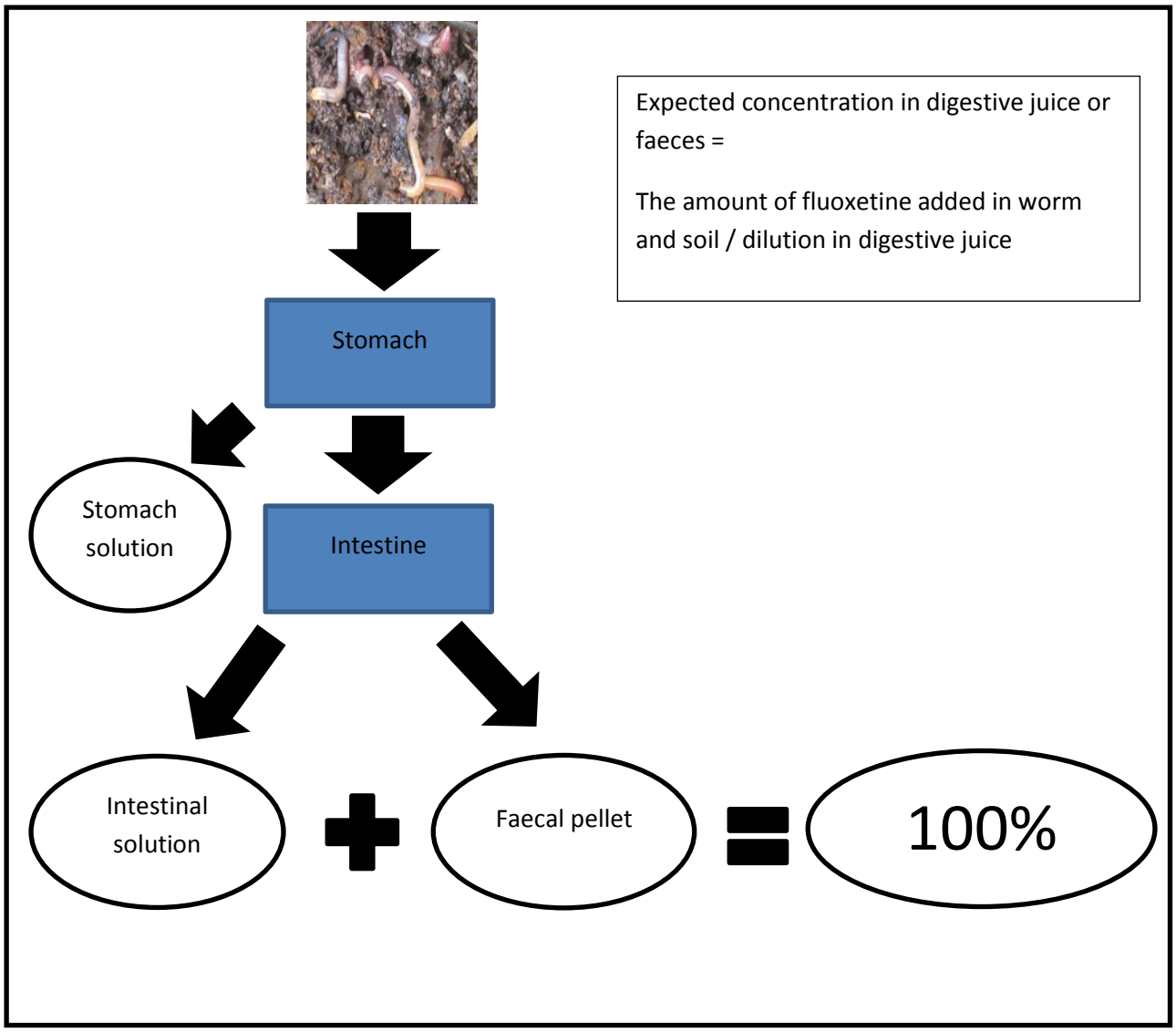



**Figure 5.2: Schematic illustrating how the percentages that were bioaccessible and excreted unchanged (faecal pellet) were calculated. Blue boxes represent the phases of the Pbet, white ovals with a black outline are the samples collected. The concentration in solution at the end of the intestinal phase plus the concentration in the faecal pellet was related to the expected concentration to work out the total percentage recovery. The expected concentration was calculated based on the mass of the worm and soil added. Whatever the sum of the percentage recovered in the intestinal solution and the faecal pellet were this was assumed to be all of the fluoxetine added and so by definition had to equal 100%. The percentage recovered in the different stages of the Pbet i.e. the stomach or gizzard, intestine – stomach or gizzard, total bioaccessibility (in solution at the end of the intestine phase) and the proportion in the faeces was then back calculated.**

## 5.3 Results

Concentrations of fluoxetine in samples from the control earthworms and blank treatments were below the limit of detection (< 0.2 µg/mL) so no blank correction was applied. There were significant differences between the Pbets in the amount of fluoxetine recovered in the different phases (MANOVA: Pillai's score = 1.77, $F = 19.65$, $p < 0.001$). The tests of between subjects effects showed

that there were significant differences between Pbets for total bioaccessibility (and faeces, see 5.2.11) (df = 4, F = 13.08, p = 0.001), the stomach or gizzard phase (df = 4, F = 104.82, p < 0.001), the intestine-(stomach or gizzard) phase (df = 4, F=107.01, p < 0.001). Table 5.2 contains Post-Hoc Tukey's HSD tests to assess which Pbets were significantly different from which.

### 5.3.1 Differences between the Pbets

In terms of total bioaccessibility, the Bird Si grit model (73.3%) had significantly lower bioaccessibility than all three human Pbets and the Ca grit bird Pbet (Figure 5.3, Table 5.2). There were no significant differences in total bioaccessibility between the Ca grit Pbet (81.3%) and any of the human Pbets (pH 1.3 = 85.9%, pH 2.5 = 84.2% and pH 4 = 84.3%).

As the percentages for total bioaccesibility and faeces were compositional data, the significant differences found for total bioaccessibility were the reverse for the faeces. For faeces, fluoxetine recovery was significantly higher in the bird Si grit Pbet than all other Pbets with 26.7% recovered in the faeces. The bird Ca grit Pbet was not significantly different to any of the human Pbets with 18.7% (the percentages in the faeces for the human Pbets were: pH 1.3 = 14.1%, pH 2.5 = 15.8% and pH 4 = 15.7%).

For the stomach or gizzard, the more acidic Pbets for both species (pH 1.3 for humans and Si grit for birds respectively) mobilised significantly more fluoxetine, 75.2 and 78.6% respectively, than the less acidic Pbets from the other species (Ca grit bird = 40.1%, pH 2.5 = 45.0% and pH 4 = 23.0%). The gizzard of the bird Ca grit model extracted significantly more fluoxetine than the human pH 4 model but was not significantly different from the human pH 2.5 model.

For the additional percentage of fluoxetine extracted in the intestinal phase of the model (additional to the stomach or gizzard), the less acidic Pbets mobilised more fluoxetine from the worms than the more acidic Pbets. The intestine of the Human pH 4 Pbet extracted significantly more fluoxetine than all other Pbets (61.3% compared with bird Ca grit 40.4%, bird Si grit -5.3%, Human pH 1.3 = 10.7% and Human pH 2.5 =39.2%); human pH 2.5 was significantly higher than Human pH 1.3 and Bird Si grit but not significantly different to Bird Ca grit; Bird Ca grit was significantly higher than human pH 1.3 and Bird Si grit and Human pH 1.3 was significantly higher than Bird Si grit. The percentage of fluoxetine that was bioaccessible in the intestine of the bird Si grit actually went down slightly compared with what had been mobilised at the end of the gizzard phase. Adsorption to grit is suggested as the potential explanation (see section 5.4 and Appendix D5.5).

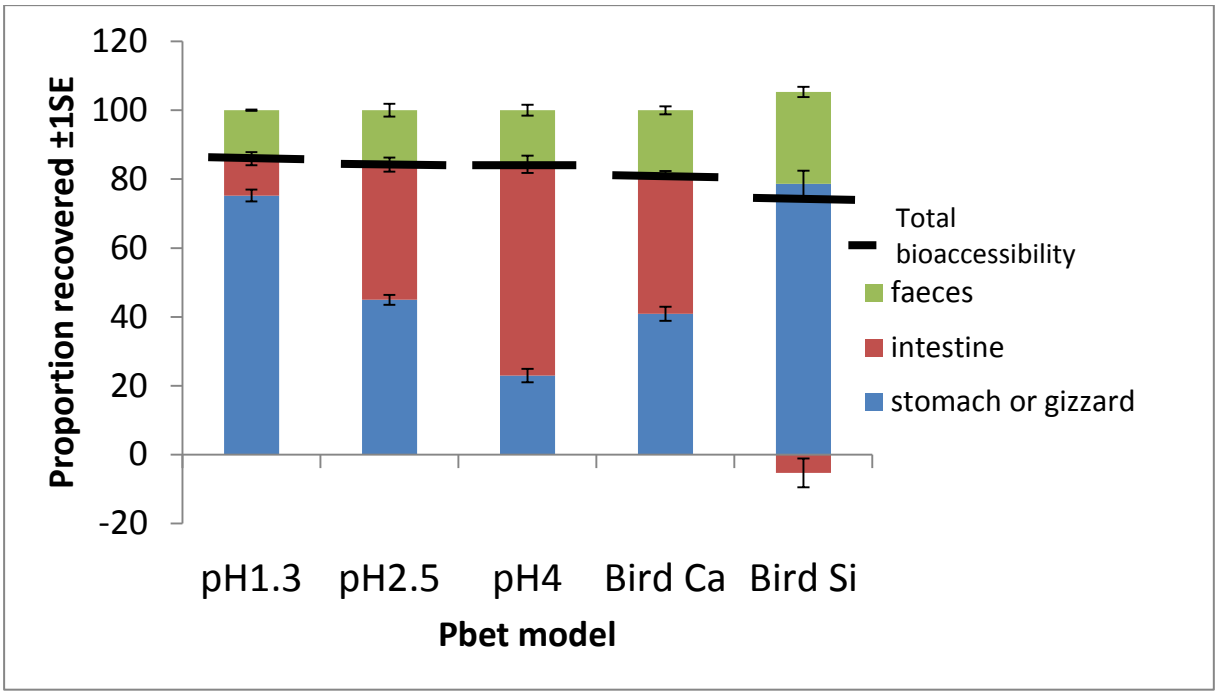



**Figure 5.3: For each Pbet model, the mean percentage of the fluoxetine (±1 Standard Error) mobilised in the different phases is shown: Stomach or Gizzard (Blue), Intestine (red), Faeces (Green). The bold black line at the top of the red bar (except for the bird Si grit where it is slightly below the top of the blue bar) shows the bioaccessible proportion. For the bird Si grit Pbet bioaccessibility went down slightly from gizzard to intestine which is why there is a negative percentage for the intestine. The actual percentage recovered at the end of the intestinal phase for bird Si grit was 73.3% i.e. 5% lower than it was at the end of the gizzard phase. This loss of fluoxetine from solution in the intestine is believed to be due to sorption to the grit (see Appendix D5).**

**Table 5.2: Post-hoc Tukey's HSD tests between Pbets for the percentage of fluoxetine mobilised in the stomach (or gizzard), the additional amount recovered in the intestine and the total bioaccessible percentage which is 100-the percentage in the faeces) All tests are on 4 degrees of freedom. Each Pbet has been compared with all other Pbets. The bird models are Ca and Si (i.e. the grit type used in the gizzard) and the human models are 1.3, 2.5 and 4 which refers to the pH of the stomach phase. μ (A-B) is the mean difference between the Pbets, p= the probability of A-B being outside the confidence interval. *significant at the 5% level**

| Pbet | | Stomach or Gizzard | | Intestine (-stomach or Gizzard) | | Total bioaccessibility (P value also applies for Faeces) | |
|---|---|---|---|---|---|---|---|
| A | B | Mean % diff (A-B) | p | Mean % diff (A-B) | p | Mean % diff (A-B) | p |
| **Ca** | **1.3** | -34.3 | <0.001* | 29.8 | <0.001* | -4.6 | 0.21 |
| **Ca** | **2.5** | -4.1 | 0.73 | 1.2 | 0.997 | -2.9 | 0.610 |
| **Ca** | **4** | 17.9 | 0.002* | -20.9 | <0.001* | -3.0 | 0.57 |
| **Ca** | **Si** | -37.7 | <0.001* | 44.7 | <0.001* | 8.0 | 0.015* |
| **Si** | **1.3** | 3.4 | 0.84 | -16.0 | 0.009* | -12.6 | 0.001* |
| **Si** | **2.5** | 33.7 | <0.001* | -44.5 | <0.001* | -10.9 | 0.002* |
| **Si** | **4** | 55.7 | <0.001* | 66.7 | <0.001* | -11.0 | 0.002* |
| **1.3** | **2.5** | 30.3 | <0.001* | -28.6 | <0.001* | 1.7 | 0.90 |
| **1.3** | **4** | 52.3 | <0.001* | -50.7 | <0.001* | 1.6 | 0.92 |
| **2.5** | **4** | 22.0 | <0.001* | -22.1 | 0.001* | -0.1 | >0.99 |

### 5.3.2 *In-vivo* evaluation

In the Ca grit avian Pbet, 18.7% ± 1.2% of the fluoxetine inserted to the Pbet was recovered in the faeces. Therefore, for the avian system, the *in-vitro* data collected here were in good agreement with the *in-vivo* data (mean concentration 1487 ng $g^{-1}$) presented in Chapter 2. The mean fluoxetine concentration in starling faeces was equivalent to 19.3%±15.9% of the dose (n = 3). However, the *in-vivo* data were highly variable with a standard error equivalent to 15.9% of the dose.

For the *in-vitro* human system, the percentage recovered in the faeces for the human Pbets was 14.1-15.8% ± 1.9%, this falls within the range of values quoted in the literature for the percentage excreted unchanged *in-vivo* in the faeces 5-15% (5-26% urine and faeces combined) see (Jjemba 2006; Lienert et al. 2007).

## 5.4 Discussion

Overall, total bioaccessibility (the percentage extracted by the end of the intestinal phase) was significantly lower in the bird Si grit Pbet (73.3%) than all other Pbets (81.3-85.9%). The area of the digestive tract where fluoxetine was mobilised was also found to be significantly different between Pbets both within and between species, the only exception being bird Ca grit and human pH 2.5 (average human gastrointestinal tract conditions). Therefore, the amount of fluoxetine excreted unchanged was significantly higher for the bird Si grit model than all other models. This was possibly due to this being the most acidic avian Pbet (lower total recovery than less acidic models) and also containing a greater amount of solid material than the human models (i.e. grit forms faeces, see Appendix D). The percentage of the dose recovered in the faeces for the human models was similar to the values quoted in the literature (Lienert et al. 2007) while the percentage recovered in the faeces from the avian Pbet was a good match with my *in-vivo* data for a model passerine. This good match between avian *in-vitro* and *in-vivo* data was despite a difference in gastrointestinal tract transit time of over five hours between the waterfowl model used for the Pbets (Martinez-Haro et al. 2009) and the gastrointestinal tract transit time for starlings (the species used for the *in-vivo* evaluation), which is quoted in the literature as being as little as 12 minutes (Levey and Karasov 1989).

The data I have collected suggest that the bioaccessibility of fluoxetine for birds is actually very similar to the bioaccessibility in the human system. The statistically significantly lower bioaccessibility in one of the avian Pbets (relative to Humans) is noteworthy but in the wrong order of magnitude to be able to state that bioaccessibility was a big contributor to the differences in accumulation observed between starlings and humans in Chapter 2. There is a possibility that my *in-vitro* measurements of bioaccessibility may overestimate bioaccessibility for some passerines due to the longer gut transit time in the waterfowl model used than is observed in passerines (Martinez-Haro et al. 2009; Levey and Karasov 1989). However, the good match between percentage of the fluoxetine dose found in the faecal pellet in the *in-vitro* Ca grit bird Pbet and the *in-vivo* proportion excreted unchanged (also presented in Chapter 2) suggests that gastrointestinal tract transit time may not be such an important factor on internal uptake after all.

One factor that could significantly affect internal uptake is the area of the gastrointestinal tract where fluoxetine is mobilised from the food item and enters the solution of the digestive juices (i.e. becomes bioaccessible). In addition to significant differences between species in total bioaccessibility, I also found the significant differences within and between species in the area of the gastrointestinal tract where fluoxetine is mobilised from the earthworm. Although the majority of drugs are

absorbed from the intestine, some uptake from the stomach is possible (Oh 2002). All of the information on fluoxetine in the literature (e.g. Altamura et al. 1994; Drugbank 2014; Hiemke and Hartter 2000) says that fluoxetine is well absorbed from the gastrointestinal tract but none specify from which part of the gastrointestinal tract. Uptake from the intestine is known to be more efficient than it is from the stomach (or gizzard) due to the presence of microvilli. These microvilli increase surface area (up to one-thousand times greater than the stomach) available for absorption and greater blood flow, but that does not mean to say that fluoxetine that is bioaccessible in the stomach or gizzard is not bioavailable (Oh 2002). Therefore, the measurement of fluoxetine's solubility in the stomach or gizzard was simply to see at what stage the fluoxetine was mobilised, rather than trying to make assumptions about where it is taken up. As fluoxetine would have to pass through the stomach or gizzard before being absorbed from the intestine, it is the concentration at the end of the intestinal phase that was used to determine total bioaccessibility.

I found the more acidic the stomach or gizzard phase, the greater the percentage of fluoxetine that was extracted in this compartment of the Pbet. Early extraction in the stomach or gizzard reflects the fact that pH 2 is the optimum pH for pepsin, the main enzyme in the stomach or gizzard phase (Degara et al. 1986). In the stomach or gizzard, the lower pH of the human pH 1.3 and Bird Si grit models helped to breakdown the worm quicker (60 minutes and 30 minutes respectively) than in the pH 2.5 and pH 4 models (worm did not break down until the intestinal simulation) and the Ca grit model (≈ 3 hours of the gizzard simulation). The higher bioaccessibility in the stomach at lower pH also fits in with what is expected based on the pKa of fluoxetine (10.06 Brooks et al. 2003); i.e. at a low pH fluoxetine is increasingly present in the ionised form which is more soluble in an aqueous solution than the non-ionised form.

In the Si grit intestine, the percentage of fluoxetine in solution (and so that which is bioaccessible) was actually 5.3% lower than it had been in the gizzard compartment. This 5.3% loss from solution is unlikely to be due to destruction by hydrolysis as fluoxetine is very resistant to hydrolysis under acidic conditions (USEPA 2007). A more likely explanation for the 5.3% loss from solution in the intestine is adsorption to solid material i.e. grit (see Appendix D5.5). Adsorption of fluoxetine to grit was found to occur for both the siliceous grit and the calcareous grit. The fact that fluoxetine was in solution for longer in the Si grit model than the Ca grit model could help to explain why a small proportion adsorbed to the grit and was removed from solution in the faecal pellet.

Although data from the literature suggest fluoxetine has high bioavailbility (60-80% of the oral dose) in the human system (e.g. Hiemke and Hartter 2000), little is known about the bioavailability of pharmaceuticals in the in the avian gastrointestinal tract. It is important now to consider approaches

for assessing the behaviour of bioaccessible fluoxetine in cell membranes, to assess the extent to which bioaccessibility equals bioavailability (e.g. Vasiluk 2006).

The results I obtained seem to tie in with the literature (e.g. Eli Lilly 2009) in that food does not affect the percentage of the dose that is bioavailable (bioaccessibility not measured); it just delays the time to peak plasma concentration. In the simulations with the higher gastric or gizzard pH (equivalent to the neutralising effect food has) less fluoxetine was soluble in the stomach than in the more acid simulations (typical of an empty stomach). However once the stomach (or gizzard) contents had been transferred to the intestinal phase, the initial pH did not affect total bioaccessibility. Differences in the region of the digestive tract where an organic contaminant is extracted from the food item could be an important factor when predicting risk due to the differences in pH and absorption efficiency. The uncertainty surrounding how bioavailability is affected by the region of the gastro-intestinal tract, where an organic contaminant becomes bioaccessible, highlights an area for future research. In order for Pbets to realise their full potential in terms of the role they could play in environmental risk assessments, collaboration of experts from a variety of fields including the fields of gastrointestinal tract physiology, bioaccessibility and ecotoxicology will be required.

Expanding the Pbet approach from inorganic to organic chemicals is highly relevant to the needs of ecotoxicology to help quantify differences, both between species and food types, in internal exposure (Saunders et al. 2011; Boxall et al. 2012). In terms of the wider application of Pbets in risk assessment, one of the biggest challenges is to develop and validate *in-vitro* gastrointestinal tract models for a wider range of species and contaminants. Pbets have already been developed and validated to provide bioaccessibility assessments for a few species. These validated Pbets are almost exclusively for trace metals (Kaufman et al. 2007; Martinez-Haro et al. 2009; Moriarty et al. 2012; Ruby et al. 1993; 1996). It is now important to extend these *in-vivo* validations to both a wider range of species and organic contaminants, if Pbets are to play a role in environmental risk assessments.

*In-vivo* validations of Pbets are logistically challenging to conduct, as they usually take the form of running a contaminant containing matrix through both the test organism and the *in-vitro* simulation and then correlating the data (Bruce et al. 2007; Oomen et al. 2002; Ruby et al. 1996). To that extent it is encouraging that my simple *in-vivo* evaluation of fluoxetine, recovered from avian faecal samples, produced good agreement with the *in-vitro* data. However, to properly validate the Pbet, samples of digestive juice from the bird would have to be collected after the drug has been administered. Not only would digestive juice be challenging to sample, there is limited information on how much digestive juice (and the timing) is secreted by different species and so it would be difficult to relate the concentration in digestive juice to a percentage of the administered dose. Inorganic techniques

for evaluation of Pbets have used x-rays to look at the dissolution of lead shot *in-vivo*, an option that cannot be expanded to organics (Kimball and Munir 1971). Collecting blood plasma is one option for organics, but this sort of evaluation relies on 1) the assumption that the bioaccessible contaminant is 100% bioavailable (or an assessment of the behaviour in cell membranes e.g. Vasiluk 2006) and 2) knowledge of the timing of peak plasma concentrations for different contaminants which will vary between species (Chiou et al. 1998). Developing a standard protocol for *in-vivo* validations of Pbets for new species and contaminants is a top priority for taking this approach forwards; it is suggested that comparing the percentage of the parent compound recovered in faeces between *in-vivo* assessments and *in-vitro* models may provide a useful evaluation tool until such a time.

Once Pbet methodologies have been validated *in-vivo* for a wider range of contaminants and species, it would then be possible to read-across from existing *in-vivo* data between species with greater accuracy than is currently available. Simultaneously, a greater number of species could be included in Environmental Risk Assessments whilst fewer new *in-vivo* toxicity studies would be required. Pbets could also play an important role in reducing the number of animals used in chemical safety tests. For example Pbets could be used to read-across between an old-formulation (previously tested *in-vivo*) and a new formulation, to assess whether bioaccessibility is equivalent between formulations. Further *in-vivo* assessments would only be justified if the bioaccessibility of the new formulation is significantly greater than that of the old formulation.

Expanding the Pbet approach faces many challenges, for example the difficulty in obtaining licences for *in-vivo* validations and gaining access to sensitive data from pre-clinical and clinical drug trials. Additionally, data on gastrointestinal tract physiology for different species is rarely complete for wildlife. For example the pH of the intestine of one species may have been studied but the gastrointestinal tract transit time may not be known. Nevertheless, Pbets are both economically feasible (relatively cheap and quick) and ethically viable compared to the equivalent *in-vivo* assessments. The collaboration of experts in gastrointestinal tract physiology, bioaccessibility and ecotoxicology, as well as making partnerships with industry, are required to generate the data necessary to successfully embed Pbets into environmental risk assessments for wildlife.

# Chapter 6

# General discussion and recommendations

## 6.1 Rationale and Aim

This thesis has explored the uptake, distribution and effects of an environmentally relevant concentration of fluoxetine on a model species of bird, the European starling. The study focussed on assessing whether fluoxetine could cause effects in starlings from the macromolecular (changes in Corticosterone) through to changes in behaviour and physiology that could affect the survival of individuals if they were to occur in the wild. The data collected in starlings were compared with data in the literature from model mammals and humans to evaluate the extent to which pharmacology and toxicology data can be read-across to predict the likelihood of effects of pharmaceuticals in birds. In this final Chapter, the major findings from the work are brought together and discussed in light of the project's Objectives and the implications for Environmental Risk Assessment and wildlife conservation are discussed. Finally, recommendations for further work are presented.

## 6.2 Progress against Objectives

### 6.2.1 Objective 1: To predict and validate an environmentally relevant daily dose of fluoxetine for starlings

The dosing levels for the study were predicted based on data on the usage of fluoxetine, patient metabolism, water usage and bioconcentration data for earthworms in soils. A small-scale field monitoring study was done towards the end of the project indicated that my predicted dose was within the range of uptake that could occur in reality.

### 6.2.2 Objectives 2 and 3: To quantify Accumulation, Distribution, Metabolism and Elimination (ADME) data for fluoxetine in starlings and compare it with ADME data for fluoxetine in model mammals and humans.

After 22 weeks of feeding starlings with the environmentally relevant dose, accumulation of fluoxetine and norfluoxetine in starling brain, liver, kidney and muscle were found to be one to two orders of magnitude lower than is seen in humans (when differences in dose and body mass were accounted for, see Table 6.1). Nevertheless, fluoxetine could still be detected in the starling brain, which is the key site for the pharmacological activity, and so effects could occur if receptors were more sensitive than those of humans. The ratios of fluoxetine to norfluoxtine were found to be very

different for starlings compared to what has been found in humans (Table 6.1) and no metabolites ,other than norfluoxetine, could be detected in starling samples. The proportion of the dose excreted unchanged by starlings was at the upper end of the range quoted for humans (Lienert et al. 2007) and higher than is found for dogs (EMEA 2008). In human faeces, significant quantities of six metabolites other than norfluoxetine, are also detected (Lienert et al. 2007; Drugbank 2014). The fact that none of these were detected in starling faeces indicates different metabolism between humans and starlings. A major proportion of the enzymes responsible for fluoxetine metabolism in humans have not been evolutionarily conserved in birds (Watanabe et al. 2013), rats or mice, (Martignoni et al. 2006) which points to a different metabolic pathway than for humans. Finally, the elimination half-lives for fluoxetine and norfluoxetine were much quicker in starlings than in humans (Hiemke and Hartter 2000), but were similar to levels found in rodents (Caccia et al. 1990; Holladay et al. 1998; Mukherjee et al. 1998) see Table 6.1. Basal metabolic rate (BMR) may also be an important factor when selecting a model mammal for read-across to birds, see Table 6.1.

### 6.2.3 Objective 4: To assess the likelihood of mode of action related effects occurring in starlings by administering the environmentally relevant dose to birds over an ecologically relevant period of time

Contrary to expectations, I found that after 16-weeks of treatment with fluoxetine there were no treatment effects on the anxiety related behaviours assessed. However, fluoxetine can also cause a number of side effects (Visser et al. 1993) including changes to appetite (Broberger and Hokfelt 2001; Guimaraes et al. 2002), levels of activity, and libido (Clayton 2002; Matuszczyk et al. 1998; Shen and Hsu 1995; Uphouse et al. 2006). I found no effect on activity *per se* but did find the fluoxetine-treated birds made less visits to the food tray than controls, particularly at key times for energy replenishment.

Additionally, I found the fluoxetine-treated birds did not show the expected relationship between high levels of stress hormones and mass loss in a situation of chronic stress (Apfelbeck and Raess 2008), while the control birds did. My finding could be indicative of a disruption to Corticosterone production in the fluoxetine-treated birds, which may influence the way birds respond to environmental stimuli. It is plausible that if the lack of response to stimuli and altered foraging behaviours were to occur in free-living birds then both starvation and predation risk would be increased, but this remains to be tested.

In terms of decreased libido, fluoxetine-treated females were found to be less interested in males than the control females were (Table 6.1). There appeared to be no influence of male quality on the

preferences of females, however, there was some evidence to suggest that fluoxetine caused a delay in the rate at which females came into breeding condition. I suggest that fluoxetine acted as an endocrine disruptor in female starlings, as has been observed in aquatic species e.g. (Mennigen et al. 2010), which served to reduce their interest in courtship.

The data I presented in Chapters 3 and 4 suggest fluoxetine has the potential to alter fitness related traits in wild birds. It is now important to establish the extent to which the effects observed experimentally could occur in wild populations (see 6.4.4).

### 6.2.3 Objectives 5 and 6: To quantify and compare the bioaccessibility of fluoxetine from invertebrate prey for birds and humans

In read-across, bioaccessibility is often assumed to be equivalent between species and food items and is not usually quantified. When the bioaccessibility of fluoxetine from earthworms, that had accumulated fluoxetine from contaminated soil, was assessed using human and avian physiologically based extraction tests (Pbets), bioaccessibility was found to be significantly lower (73.3% of the added fluoxetine was bioaccessible) in one of the avian Pbets (containing siliceous grit) than the other avian and human Pbets (range 81.3-85.9%). Nevertheless, the difference in bioaccessibility (maximum 13%) between humans and birds is not sufficient to explain why accumulation in bird tissues was one to two orders of magnitude lower than in humans when corrected for differences in body mass (Chapter 2). The proportion recovered in the faecal pellet from these *in-vitro* models was in good agreement with the *in-vivo* data presented in the literature for humans (Jjemba 2006, Hiemke and Hartter 2000; Lienert 2007) and collected by this study for starlings (see Table 6.1). To take this work forward, it is important to assess how much of the bioaccessibile fluoxetine is bioavailable by assessing behaviour in cell membranes (Vasiluk 2006).

## 6.3 Implications for Environmental Risk Assessment

### 6.3.1 The use of behaviour as an endpoint

The absence of an effect on anxiety related behaviours could have been for many reasons. Firstly, it could have been an artefact of conducting the wrong behavioural assays (boldness and high-risk exploration) to assess anxiety. Adaptations of standard anxiety tests used in rats such as mazes and light/dark exploration, or even response to a starling alarm call, may have yielded different results to those obtained here (File et al. 2004; 2005). The absence of an effect on anxiety related behaviours highlights a bigger issue; the importance of, but difficulty in, interpreting non-standard sub-lethal endpoints. Many effects and toxicity studies use standard (lethal) endpoints (e.g. OECD 48 hr mortality in fish) (Luft and Bode 2002). Standardised ecotoxicological tests (e.g. OECD protocols) do not properly consider ecologically important behaviours, which can cause changes to ecosystem

processes, food-web dynamics and the way an ecosystem functions (Brodin et al. 2014). Behavioural endpoints are of particular relevance to both pharmaceuticals and wildlife (Brodin et al. 2014; Clotfelter et al. 2004; Scott and Sloman 2004; Zala and Penn 2004). Behaviour provides an important biomarker for both exposure and understanding how a chemical contaminant, especially an anti-depressant such as fluoxetine, which is designed to regulate behaviour (Bossus et al. 2014), can impact an organism (Clotfelter et al. 2004; Zala and Penn 2004). Despite, the important role behaviour has to play in toxicology, the two fields are currently evolving largely independently (Brodin et al. 2014). As pharmaceuticals are biologically active molecules, designed to bind to, and interact with, a receptor, many of which have been evolutionarily conserved in wildlife species (Gunnarson et al. 2008; Brown et al. 2014), they have the potential to affect organisms at levels that are well below more traditional toxicological endpoints (e.g. the lethal dose at which 50% of the organisms die). Behavioural endpoints can provide a useful biomarker for changes that could impact fitness related traits, e.g. predator avoidance, feeding rate and reproductive success. Standardising these behavioural endpoints would help improve our understanding of how environmentally relevant concentrations of pharmaceuticals affect wildlife, as this would enable better read-across between studies.

### 6.3.2 The use of read-across

The read-across hypothesis as proposed by Huggett et al. (2003) is based on the assumption that if a drug target is evolutionarily conserved in a non-target species, then measurements of internal concentrations can compared between non-target and model species (or humans) to assess the likelihood of a Mode of Action (MOA) related effect occurring. Drug targets in domestic chickens (Gunnarson et al. 2008) are similar to humans and the serotonergic system is known to be conserved in birds, so it is believed that fluoxetine has the potential to have MOA related effects in starlings (e.g. Sperry et al. 2003; 2005; Challet et al. 1996).

In terms of internal concentrations, many studies report plasma and not tissue concentrations for pharmaceuticals e.g. (Pawluski et al. 2014). Therefore it was unfortunate that the ELISA kit did not work well for quantifying levels of fluoxetine in starling plasma. Analysis by highly sensitive LCMS/MS, as I will do for the paper from Chapter 2, is a better option for future studies.

For the pharmacokinetic variables calculated based on the starling tissue concentrations, I found that starling to rodent read-across has the potential for use in Environmental Risk Assessments (ERA), but there was not a good match for human to avian read-across. A recent study by Margiotta-Casaluci et al. (2014) looking at human to fish read-across for fluoxetine found that anxiety related effects in

fish were only observed at plasma concentrations that exceeded the human therapeutic plasma concentration. I observed lower than therapeutic accumulation in tissues of birds, particularly in the brain, but still observed effects on fitness related traits, and did not observe any effect on anxiety. The long exposure period for the starlings (18 weeks longer than Margiotta-Casaluci et al. (2014)) may have enabled the low levels of fluoxetine that accumulated in the brain to cause subtle changes to neurophysiology (Hiemke and Hartter 2000; Lesch et al. 1991). Higher receptor sensitivity in birds (Hutchinson et al. 2014, Sperry et al. 2005), or differences in metabolism (Hutchinson et al. 2014; Watanabe et al. 2013), are suggested as potential explanations for the effects being observed in starlings at low internal concentrations (see section 6.4.2).

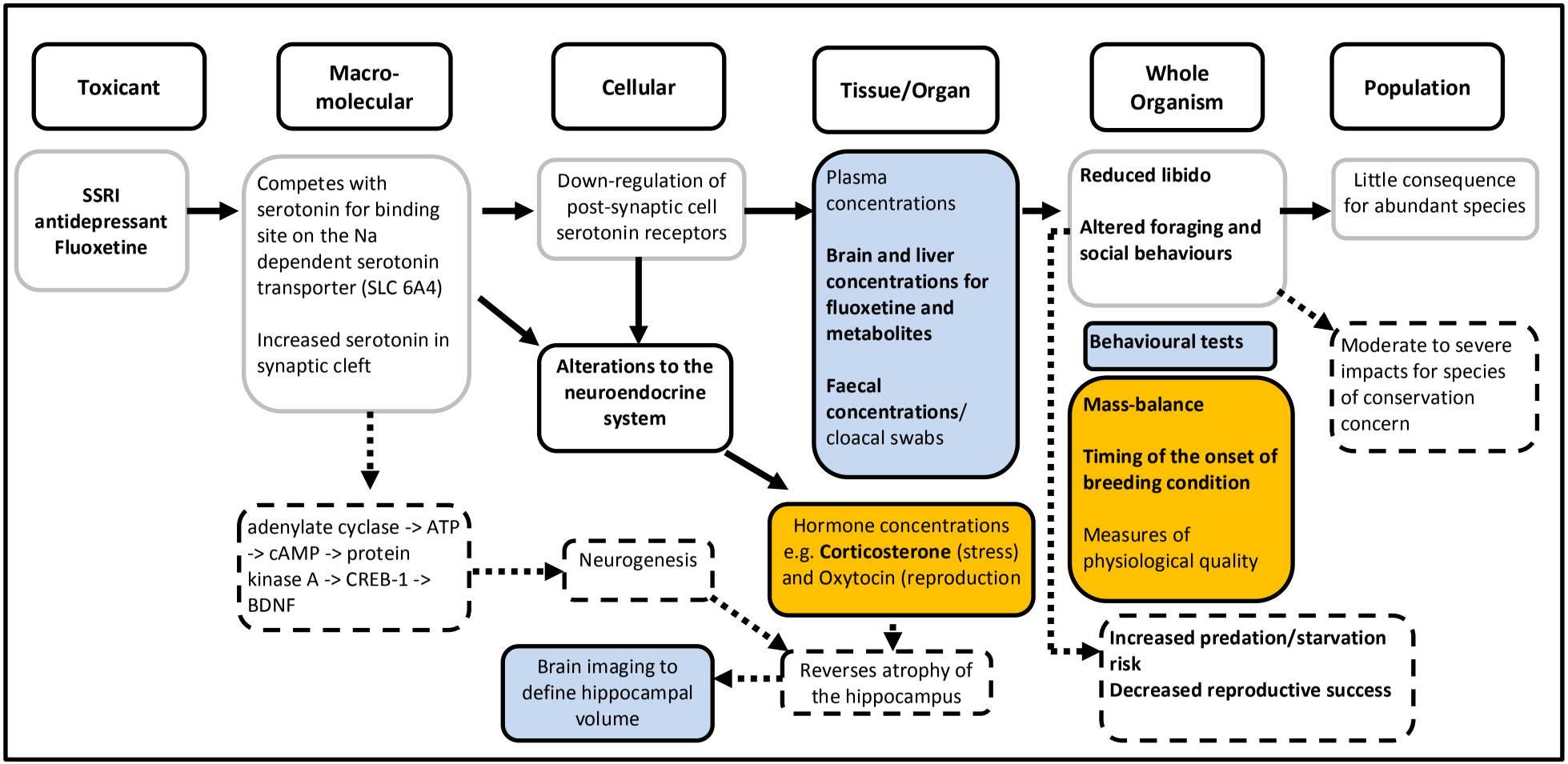



**Figure 6.1: Adverse Outcome Pathway for fluoxetine in wild birds, updated with the findings from this study (text in bold). The AOP is a framework that conceptualises knowledge on the pharmacology and effects of fluoxetine in humans and model mammals to relate a molecular interaction of a toxicant with a receptor through various levels of biological organisation finishing with population level effects.**

**Table 6.1: A summary of the effects and pharmacokinetics of fluoxetine in starlings, humans and model mammals (rats, mice and dogs); data collected by my PhD thesis are given in bold. * indicative only as I could not quantify levels in plasma**

| | Starlings | Model species |
|---|---|---|
| Drug target | Serotonin system | Serotonin system |
| Therapeutic mode of action effects ( anxiety related behaviours) | **No evidence of anxiety related effects** | Reduction of depression, successful in the treatment of major depressive disorders (2) (Humans, Rats, Mice) |
| Other mode of action related effects (side effects) | **Altered foraging behaviours, mass balance in response to isolation stress, decreased female interest in males, delayed development of breeding season bill colour** | Decreased libido, altered appetite and body mass, lethargy (2) (Humans) |
| Basal Metabolic Rate (kJ/h/kg body mass) | 43.0 (1) | ≈ 3.6 (3)( Humans)<br>≈33.6 (1) (Mice) |
| Body Mass | **mean = 79 g, range = 69 - 91 g** | ≈ 70 kg (3) (Humans)<br>≈ 250-300 g (4) (Rats)<br>≈ 15-30 g (5)(Mice) |
| Accumulation in plasma<br><br><br>Accumulation in tissue | **Predicted plasma = 24 ng/mL (dose 1300 ng) ELISA did not work for detecting fluoxetine in plasma**<br><br>**16-78% of dose/g in brain, kidney, liver and muscle** | 89-100 ng/mL (6) (Humans)<br><br>93-1050% of dose/g (7) (Human brain, kidney, liver and muscle)<br><br>157% of dose/g (5) (Mouse brain) |
| Distribution into Brain | **≈ 6 times plasma concentration (assuming plasma conc. to be half LOD 1ng/mL)*** | ≈ 15-20 times plasma concentration (6) (Humans)<br><br>3-10 time serum conc. (8) (Rats)<br>23-26.5 times serum conc. (5) (Mice) |
| Metabolism (ratio fluoxetine:norfluoxetine) | **Brain = 19.63**<br>**Kidney = 2.27**<br>**Liver = 0.58**<br>**Muscle = 0.22**<br>**Six other inactive metabolites below LOD** | Brain = 0.65 (7) (All Human data)<br>Kidney = 0.85 (7)<br>Liver = 0.67 (7)<br>Muscle = 1.14 (7)<br>**Six other inactive metabolites similar to levels of parent compound (9)** |
| Elimination | **Range of means = 3.2-10.8 h (tissue)** | 24-96 h (10) (Human plasma)<br>5 h (Rat plasma) (4)<br>12.9 h (5) (Mouse plasma) |
| Excreted unchanged | **19.3% ± 15.9% (±1SE) *in-vivo***<br>**18.7% (16.8-29.4%) of dose recovered in avian faeces for *in-vitro* model** | 5-26% *in-vivo* (9, 11) (Humans)<br>**14.1-15.8% *in-vitro* (humans)**<br>10.9% (12) (Dogs) |
| Bioaccessibility from Pbet (*in-vitro* model) | **73.3-81.3% ±3.8 % (±1SE)** | **84.2-85.9% ± 2.0% (±1SE) (Humans)** |

1. Koteja (1991); 2. Eli Lilly (2009); 3. Livestrong (2014); 4. Caccia et al. (1990); 5. Holladay et al. (1998); 6. Brunswick et al. (2002); 7. Lewis et al. (2007); 8. Mukherjee et al. (1998); 9. Lienert et al. (2007); 10. Hiemke and Hartter (2000); 11. Jjemba (2006); 12. EMEA (2008).

### 6.3.3 The Adverse Outcome Pathway concept

The Adverse Outcome Pathway (AOP) from Chapter 1 (Figure 1.5) has been updated in Figure 6.1 so that the knowledge gaps filled by this study are included (bold text). The AOP concept relates a molecular interaction between a toxicant and a receptor through four levels of biological organisation, finishing with population level effects (Ankley et al. 2010). In the AOP concept, biomarkers or diagnostic tools can be used to make established linkages between different levels of biological organisation. The text in bold in Figure 6.1 shows the main knowledge gaps that this study has filled, which are:

1) I have estimated and validated an environmentally relevant concentration of fluoxetine for wild birds feeding on invertebrates living on the trickling filters of WWTPs.

2) I have found evidence that fluoxetine can cause changes to the neuro-endocrine system, e.g. effects on the Hypothalamic-pituitary-adrenal (HPA) axis appear to have caused alterations to the levels of Corticosterone, a hormone associated with stress response.

3) I have developed and validated methods for the extraction of fluoxetine and norfluoxetine from starling tissue and faeces.

4) ADME data has been collected which could be related to internal fluoxetine concentrations in wild birds to assess the likelihood of the effects I observed occurring.

5) I have found effects of fluoxetine on foraging, mass balance, the development of female breeding condition and female behaviour in mate choice trials.

6) Suggestions for potential mechanisms of population level effects based on individual level impacts have been made.

The updated AOP in Figure 6.1 will be valuable information for designing future studies. However, several knowledge gaps regarding the specific effects of antidepressants on birds remain including:

1) Whether wild starlings ingest the quantity of contaminated invertebrates that I assumed in the dosing calculation based data presented in the literature;

2) The sensitivity of receptors in passerine birds;

3) The extent to which fluoxetine can cause alterations to the avian neuroendocrine system e.g. oxytocin;

4) Whether or not fluoxetine can cause neurogenesis in avian brains;

5) Understanding how effects in the laboratory could translate to effects in free living birds;

6) The likelihood and severity of population level effects and how these effects could be changed by interactions of fluoxetine with other pharmaceuticals and stressors.

These knowledge gaps are divided into five broad themes in section 6.4:

1) The predicted dose

2) Receptor sensitivity and neurogenesis

3) Interpretation of sub-lethal endpoints

4) Going from the laboratory to the field

5) The bigger picture

## 6.4 Remaining knowledge gaps and recommendations for future studies

### 6.4.1 The predicted daily dose of fluoxetine for wild birds

Although the predicted concentration in invertebrates was found to be within the range of measured values, the predicted bird daily dose for birds is a factor of both the concentration in invertebrates and the assumed feeding rate. For starlings, it was assumed that they take 50% of their invertebrate prey from trickling filters in winter and the breeding season following the work of Markman et al. (2008; 2011). Field observations and an extensive electronic tag network could be used to monitor starlings foraging on trickling filter beds to evaluate the extent to which the same individuals forage there.

It will also be important to consider ingestion of different invertebrate species in future studies, as Carter at al. (2014a) and Brodin et al. (2014) have demonstrated that there is variability in bioconcentration of pharmaceuticals including fluoxetine into different species of invertebrates. Field

monitoring of pharmaceutical concentrations contained within these invertebrates will help improve the accuracy with which predicted bird daily doses can be estimated. The composition of the different invertebrate species in starling diet could be quantified by mist-netting on WWTPs and using next generation sequencing of avian faeces; this would enable spatial and temporal differences in invertebrate species consumed by starlings to be identified (e.g. Pompanon et al. 2012). Additionally, bioaccessibility is known to vary between food types for inorganic contaminants (Laird et al. 2009) and this will probably also be the case for organics. To date, Pbets have only been developed and validated for inorganics, extending the approach to organics is needed as this will help improve the understanding of the internal level of a contaminant within an organism.

### 6.4.2 Receptor sensitivity and neurogenesis

Further work is needed to identify the drug receptors (if they exist) that bind to specific pharmaceuticals, including fluoxetine, and to determine how the sensitivity of birds' receptors differ to those of humans. Neither a histopathological analysis to assess whether fluoxetine caused neurogenesis, nor a measurement of receptor sensitivity (e.g. Lillesaar 2011) was conducted prior to fluoxetine being extracted from starling brains. A follow up project to assess neurogenesis in the avian brain in response to treatment with fluoxetine has already been discussed with potential collaborators. This project would help to understand the potential mechanisms by which fluoxetine could cause subtle changes to fitness related traits at low doses.

### 6.4.3 Interpretation of sub-lethal endpoints

As the experiment aimed to be environmentally relevant, birds were administered with a dose several orders of magnitude lower than what has been used in previous studies on fluoxetine in birds (Sperry et al. 2003). The low dose has the advantage that only evidence of ecologically relevant treatment effects are measured but the disadvantage that changes to behaviour and physiology can be more challenging to interpret than acute endpoints such as mortality.

High variability between individuals is one issue with sub-lethal endpoints. The low dose used here may not have been sufficient to override individual personality, the effects of which are further exacerbated by the genetic variability (Brown et al. 2009) and the relatively small sample size (relative to some behavioural tests e.g. (Minderman et al. 2009; 2010), but not for others e.g. (Herborn et al. 2011) or ecotoxicology e.g. (Rattner et al. 2008). Robust experimental design with well justified, statistically validated sample sizes and appropriate treatments and numbers of treatment groups are, therefore, vital.

Sub-lethal endpoints are particularly challenging to interpret for an antidepressant such as fluoxetine, which even draws variable responses in human patients suffering with depression. For example 32-

72% of patients successfully respond to treatment with fluoxetine (Cook et al. 1999). It would be worth assessing different anxiety related behaviours, possibly adapting some of the standard anxiety tests used in rodents (e.g. File et al. 2004; 2005), but as I was not measuring effects in depressed birds it is perhaps not surprising that data were variable; the effects on anxiety related behaviours require further investigation.

Although, the data I collected in the laboratory suggest that fluoxetine has the potential to alter fitness related traits, it remains uncertain how the effects observed would translate to wild populations. The areas where I identified treatment effects certainly merit further investigation. For the faecal Corticosterone analysis, it would be worth repeating the experiment and using plasma Corticosterone instead to assess habituation to isolation stress (see Goyman 2005). For the investigation of sexual dysfunction there are numerous possible areas for further investigation. As the males did not display any courtship behaviours (Feare 1984), it is possible that the females' choices had nothing to do with mate choice; particularly given the fact that wild animals brought into captivity often do not breed in the first season due to stress (Miller 1969). Other factors that could have contributed to the absence of courtship displays and lack of breeding condition are dietary deficiency (Miller 1969) and the effect of lack of exercise in captivity on hormones (Rees et al. 1985). It would be useful to reverse the female mate choice trials and assess male preferences for females and also repeat the experiment I did with a different set of females. The influence of the colour leg bands on mate preference in starlings as the male colour leg bands were not controlled for in this experiment (see Burley 1986; Burley et al. 1982). Measurements of androgens may also provide a useful indicator of the impact of fluoxetine on the development of breeding condition (Markman et al. 2008). More environmentally relevant studies, especially in the terrestrial environment, are urgently needed to assess the risks posed by pharmaceuticals in the environment. The generation of a larger data set will enable effects to become clearer.

### 6.4.4 Going from the laboratory to the field

The rapid elimination rates of fluoxetine and norfluoxetine from the starling system I observed in captivity means that if a free living starling ingested the expected amount on one day but did not return to the WWTP for more than 24 hours, then levels in their brain would be negligible by that point. In humans and model mammals, it takes continued treatment for several weeks for therapeutic effects from fluoxetine to result from the neurological adaptations that fluoxetine induces in the brain; additionally, in humans, these neuro-adaptations seem to outlast the period of fluoxetine treatment (Warner-Shcmidt and Durman 2006; Lesch et al. 1991; Raap et al. 1999; Uphouse et al. 2006). Therefore, it is hypothesised that maintaining fluoxetine within the avian system will also be important to cause MOA related effects in birds.

Monitoring internal concentrations of fluoxetine in wild birds is required to assess how my findings in the laboratory apply to free-living birds. Internal concentrations in wild birds could then be compared with the internal concentrations I presented in Chapter 2. One potential approach for sampling internal fluoxetine concentrations in wild birds would be to take samples from wild birds mist-netted near to trickling filter beds. Collecting blood plasma and comparing pharmaceutical concentrations with the captive dosed birds' plasma levels would be one approach that may work, if a sufficiently large blood sample can be obtained and funding for highly sensitive LCMS/MS analysis is available e.g. (Lazarus et al. 2014). My data suggest blood plasma may not work well for small paserines (i.e. small blood sample so it is difficult to achieve high levels of sample concentration) and lipophilic pharmaceuticals with a high volume of distribution such as fluoxetine (Hiemke and Hartter 2000). Fluoxetine concentrations in human plasma are typically 9-60 times lower (plasma 2.2 times lower than muscle) than concentrations found in various tissue types (Lewis et al. 2007). Given the trace levels detected here in tissues samples, alternative approaches to plasma analysis are perhaps better for quantifying levels of pharmaceuticals in wild birds.

A more logistically challenging approach would be to euthanize the mist netted birds immediately and transport back the laboratory for post-mortem dissection to remove tissue samples. This approach does have the disadvantage that there will be a lag time between death and dissection, meaning that some post-mortem metabolism may take place (Robertson and Drummer 1995).

Analysis of tissues from carcasses of dead birds, for example those submitted to monitoring schemes (Shore et al. 2014), has the advantage over the previous approach of not causing further mortality or suffering to wild birds. The main disadvantages with using such carcasses are the uncertainty over factors such as time of death, and whether the bird had ever fed on a contaminated source. Therefore, the sample size required to produce a meaningful data set would have to be much larger than samples collected from live birds using a targeted monitoring approach.

Non-invasive approaches to sample collection are also worth investigating. One option that remains to be validated is to swab the cloaca to collect un-metabolised parent compounds. This has the advantage of being a non-invasive method, but has the disadvantage of being highly sensitive to gastrointestinal tract transit times and elimination rates of pharmaceuticals in passerines are likely to be fast (typically < 20 minutes) (Levey and Karasov 1989). Extracting pharmaceuticals from fur has previously been used to detect non-steroidal anti-inflammatories in otter hair (*Lutra lutra*) (Richards et al. 2011), thus there may be merit in developing and validating methods to extract pharmaceutical residues from feathers and coming up with a means of relating levels in feathers to internal concentrations (Smith et al. 2003).

### 6.4.5 The bigger picture: Population level effects, mixtures of pharmaceuticals and multiple stressors

I have found in the laboratory that a predicted environmentally relevant concentration of fluoxetine has the potential to alter fitness related traits in starlings. This is a potential conservation concern. However, feeding behaviour and receptor sensitivity must be quantified in future work given the low level accumulation and rapid elimination. With the exception of the well-known population declines suffered by vultures in India, at the hands of diclofenac, little is known about how pharmaceuticals in the environment can affect wild-birds at the individual and population level. Recently Zorilla et al. (2014) presented the first example outside of Asia of a wild bird suffering mortality from ingesting pharmaceutically contaminated meat. Also, Lazarus et al. (2014) detected diltiazem in wild osprey plasma but did not measure effects.

I have found fluoxetine has the potential to alter fitness related traits in starlings. In the UK, the starling's numbers have declined by 84% since 1979 (RSPB 2014). This trend of population decline is common to many other species of bird in the UK with 26 other species suffering declines of over 50% in the last 31-44 years (Baillie et al. 2014). Starlings are just one of the 215 species of breeding birds in the UK (Gregory et al. 2004) and fluoxetine is just one of the 3,000 pharmaceuticals licenced for use in Europe (Boxall et al. 2012; Ternes et al. 2009). Many of these other pharmaceuticals have been detected in the environment e.g. (Grabic et al. 2012; Kasprzyk-Hordern et al. 2010; McClellan and Halden 2010; Walters et al. 2010) which potentially exposes a whole range of species of bird to pharmaceuticals (e.g. Chapter 1, Table 1.1). To expand upon my work on fluoxetine in starlings, it is important now that the effects of pharmaceuticals from different therapeutic classes in wild birds are investigated. Table 6.2 outlines the potential MOA effects that a selection of some of the most commonly used pharmaceuticals could have in wild birds (NHS 2013). The experiments suggested in 6.4.1-6.4.4 would help with prioritisation of pharmaceuticals, while knowledge of the MOA and pharmacology could be used to come up with an AOP (e.g. Figures 1.5 and 6.1) to help with designing experiments.

As pharmaceuticals in the environment do not occur as single compounds, it is also important to consider the implications exposure to pharmaceutical mixtures could have for wildlife conservation. Although antagonistic effects are most common for mixtures, additive and synergistic interactions can occur to increase toxicity (Backhaus 2014). Fluoxetine is not the only antidepressant detected in the environment (Calisto and Esteves 2009) and many of the antidepressants detected have similar modes of action (Hiemke and Hartter 2000), which together may combine additively to increase the likelihood of a MOA related effect occurring (Backhaus 2014). Assessing the effects of pharmaceutical mixtures will help to increase the environmental relevance of studies.

**Table 6.2: Potential Mode of Action (MOA) related effects and suggested impacts on fitness-related traits in wild birds of commonly used pharmaceuticals. Common side effects, i.e. they occur in >1% of human patients are given in brackets. Data from NHS (2013) www.patient.co.uk**

| Pharmaceutical | Therapeutic class | Mode of Action (MOA) related effects | Potential impact on fitness related traits |
|---|---|---|---|
| **Atenolol** | Beta-blocker | Blocks the activity of adrenaline to reduce activity of heart (dizziness, tiredness, blurred vision) | Decreased motivation and ability to evade predators |
| **Carbamazepine** | Anti-epilepsy | Stops seizures, pain relief and muscle relaxant (dizziness) | Decreased flight performance and predator evasion |
| **Codeine** | Opiate | Pain relief (drowsiness, constipation, addiction) | Inappropriate response to injury, delayed reaction to predation, effects on mass balance. |
| **Cyclophosphamide** | Anti-cancer | Cytotoxic, induces death of T-cells (immunosuppression, haemorrhagic cystitis) | Necrosis of cells, increased susceptibility to disease |
| **Dantron** | Stimulant laxative | Diarrhoea and possible carcinogen | Weight-loss and increased starvation risk. Potential long-term toxic effects. |
| **Ibuprofen** | Non-steroidal Anti-inflammatory | Pain relief, liver damage | Potential acute toxic effect e.g. mortality as it is a NSAID like diclofenac which causes liver failure and eventually mortality in *Gyps* vultures |
| **Orlistat** | Anti-obesity | Lipase inhibitor, prevents absorption of fats (oily diarrhoea) | Weight loss, soiled plumage, effects on thermo-regulation, increased starvation risk. |
| **Simvastatin** | Statin (blood pressure) | Controls cholesterol levels (interacts with other drugs to cause muscle damage) | Decreased flight performance and predator evasion. Reduced egg production through changes to cholesterol levels. |
| **Sulfamethoxazole** | Sulfonamide Antibiotic | Toxicity to bacterial cells (gastro-intestinal problems) | Mass loss |
| **Tamoxifen** | Antineoplastic | Antagonist of the receptor cells in breast tissues (Cancers) | Reproductive success, mortality |

Finally, there are a multitude of anthropogenic induced stressors impacting upon wild bird populations in the 21st century. My work (and that of others e.g. Lazarus et al. 2014, Zorilla et al. 2014) suggests pharmaceuticals in the environment could be another one of these stressors. The

main driver of the anthropogenic pressures on the environment is rapid human population growth (WHO 2014). Population growth is supplemented by the increasing living standards demanded by society, which places greater pressure on the environment in terms of energy consumption and food production. Additionally, higher living standards come hand in hand with increased demand on access to healthcare and an ageing population (Arnold et al. 2014; Kookana et al. 2014). In 2014, 54% of the global population live in urban areas (WHO 2014). The growth of large urban centres and increasing reliance upon the private car has led to increasing levels of anthropogenic pollution in cities (e.g. greenhouse gasses, heavy metals, PAHs and NOx) some of which are known to contribute to increased levels of oxidative stress and susceptibility of birds to the development of disease (Isaksson 2010; Rodriguez-Estival et al. 2010). Increased energy usage is widely believed to have led to anthropogenic induced climate change (Vineis 2014). Climate change has caused alterations to hydrology and phenology that ultimately affect the availability of habitat and food resources have knock on consequences for survival and reproduction in wild bird populations (Ausden 2014; Harris et al. 2014; Shore et al. 2014). The increased demand of the human population for food has led to intensification of agricultural production. Intensive agriculture causes habitat destruction, a loss of connectivity between habitat patches and a direct loss of food (for example insect pests, which birds would feed on, are killed off by insecticides) (Ovenden et al. 1998).

Finally, it is important now to conduct multiple stressor studies to assess, for example, how the risks posed to wild birds by pharmaceuticals in the environment are affected by other anthropogenic induced stressors such as habitat loss and climate change induced changes in phenology.

## 6.5 Overall conclusion

This thesis has demonstrated experimentally the potential for an environmentally relevant concentration of a pharmaceutical to cause subtle effects to fitness related traits in wild-birds from the macromolecular level up to the individual organism (Figure 6.1). Individually, these effects may have limited impact on free-living individuals, but collectively and when combined with the challenges posed by factors such as climate change, there is potential for population level effects to occur. This project has managed to fill some of the knowledge gaps presented in the initial AOP (Figure 6.1) but the challenge is now to go to the next scale. By going beyond the laboratory out into the field and going from the organism level of the AOP to the population level it is now important to determine how fluoxetine and pharmaceuticals more generally, interact with other anthropogenic and environmental stressors and impact upon free-living birds in a changing environment.

# Appendix A

## A1 Bioconcentration and bioaccumulation of fluoxetine and norfluoxetine

**Table A1: Mean ± 1SE (N=12) bioconcentration (BCF) and bioaccumulation factors (BAF) for fluoxetine and norfluoxetine from influent wastewater into biofilm and earthworms.**

| | BCF Influent to biofilm | BAF Biofilm to earthworm | BCF Influent to earthworm |
|---|---|---|---|
| Fluoxetine | 5.18 ± 0.61 | 5.78 ± 1.31 | 26.27 ± 5.10 |
| Norfluoxetine | 9.67 ± 1.76 | 4.00 ± 1.31 | 30.14 ± 7.04 |

In the laboratory, Carter et al. (2014a) found a mean Bioconcentration factor (BCF) of 30.8 for fluoxetine into *Eisenia fetida* from soil and pore-water (range 25.4-35.8). The BCF I obtained from influent wastewater into earthworms was 26.27. This shows good agreement with the data of a Carter et al. (2014a) even though they did a lot more replication than I did (this is because determining the BCF of fluoxetine in earthworms was not the primary aim of my study).

## A2 Injected invertebrates experimentally fed to starlings

In order to confirm that the fluoxetine injected wax worms contained the expected concentration of fluoxetine, I carried out a QA/QC study.

### A2.1 Sample preparation

The extraction and solid Phase Extraction (SPE) method was adapted from that presented by Chu and Metcalfe (2007) as follows. Whole wax worms weighing 0.15-0.3 g were extracted with 2 mL of methanol. Samples were homogenised for approximately 10-30 seconds using an (Turax) homogeniser. Samples were then diluted with 4 mL of 0.05 M HCl in water which was added in two steps, after each of which the sample was homogenised for a further 10-30 seconds. After homogenisation each sample was then vortex mixed briefly, ultrasonicated for 10 minutes and then centrifuged for 10 minutes (4500 × g, 20°C). For muscle samples, it was necessary to pass samples through a 5 µm PTFE filter prior to the SPE.

MCX (Cation exchange and reversed phase) cartridges (Oasis 3cc, 60 mg) were conditioned with 1 mL of methanol and then equilibrated with 1 mL of water. A 2.4 mL aliquot was taken for each sample and placed in a glass vial, samples were loaded to the cartridge and then vials were rinsed with 0.6mL methanol. Cartridges were then washed with 1 mL methanol followed by 1 mL of dichloromethane. Cartridges were eluted with 2.8 mL of 95% methanol 5% ammonium hydroxide (made from a 35%

ammonium hydroxide solution) into a glass vial containing 100 µL of 'keeper solution' (9:1 Methanol:ethylacetate). Glass tubes were transferred to a Turbovap set at 45°C and blown until dry under a steady flow of nitrogen (typically 5-10 psi); the keeper solution was used to prevent the analytes from sticking to the glass. To reconstitute the samples, 0.5 mL of methanol was added first. Samples were vortex mixed for a few seconds and then a further 0.5 mL of deionised water was added to make samples up to 1 mL. Each sample was then passed through a 0.2 µm PTFE filter into a 2mL vial sealed with a crimp cap containing a PTFE septum.

### A2.2 HPLC analysis of wax worm extracts

Wax worm extracts were analysed by High Performance Liquid Chromatography (HPLC) with fluorescence (230 nm, 305 nm). I used a gradient mobile phase (1 mL $min^{-1}$) which ran from 10-90% aqueous (0.1% $H_3PO_4$) with the remaining percentage made up of HPLC methanol. All solvent used was HPLC fluorescence grade (Fisher, Loughborough UK). I used a C-18 column (Kinetex 5 µm C18 150 × 4.6mm, Phenomenex, Macclesfield UK). Run time was 23 minutes per sample with the fluoxetine peak typically coming off at 11.7-11.8 minutes.

### A2.3 Results

The mean fluoxetine concentration per spiked wax worm was 1580 ng/worm, N = 8, percentage RSD = 13, compared with the planned concentration of 1300 ng/worm. Therefore the actual dose was around 20% higher than the desired dose of 1300 ng/worm; nevertheless 1580 ng is still within the range of concentrations measured in the environment. Using the maximum concentration in the environment (i.e. 53.8 ng/g in the environment and 23.5 g of worms per day), the concentration in wax worms could be 1770 ng/worm and still be environmentally relevant in a worst case scenario.

## A3 Chromatograms from LCMS/MS analysis

The concentrations of fluoxetine and norfluoxetine in tissue and faecal samples were determined using liquid chromatography coupled to a triple quadrupole mass spectrometer. Liquid Chromatography was performed using a Dionex Acclaim® RSLC C18 Polar Advantage II column (2.2 µm 120A 2.1 × 100 mm). The triple quadrupole mass spectrometer was an Applied Biosystems/MDS Sciex API 3000 triple quadrupole in positive ion mode for LC-MS/MS analyses. Analysis Figures A1a-f shows a series of extracted ion-chromatograms (norfluoxetine, fluoxetine and fluoxetine-d5 internal standard) for a liver sample taken from a fluoxetine treated bird and a control bird that was euthanized 2 hours after the final dose. Peaks can be seen at 5.5 minutes in Figs. A1a and b showing norfluoxetine and fluoxetine in the fluoxetine-treated bird, however there are no equivalent peaks in

the equivalent chromatograms for the control bird (Figs A1 d and e). Figs A1c and A1f show the internal standard at 1 ng/mL that were spiked into both fluoxetine-treated and control samples.

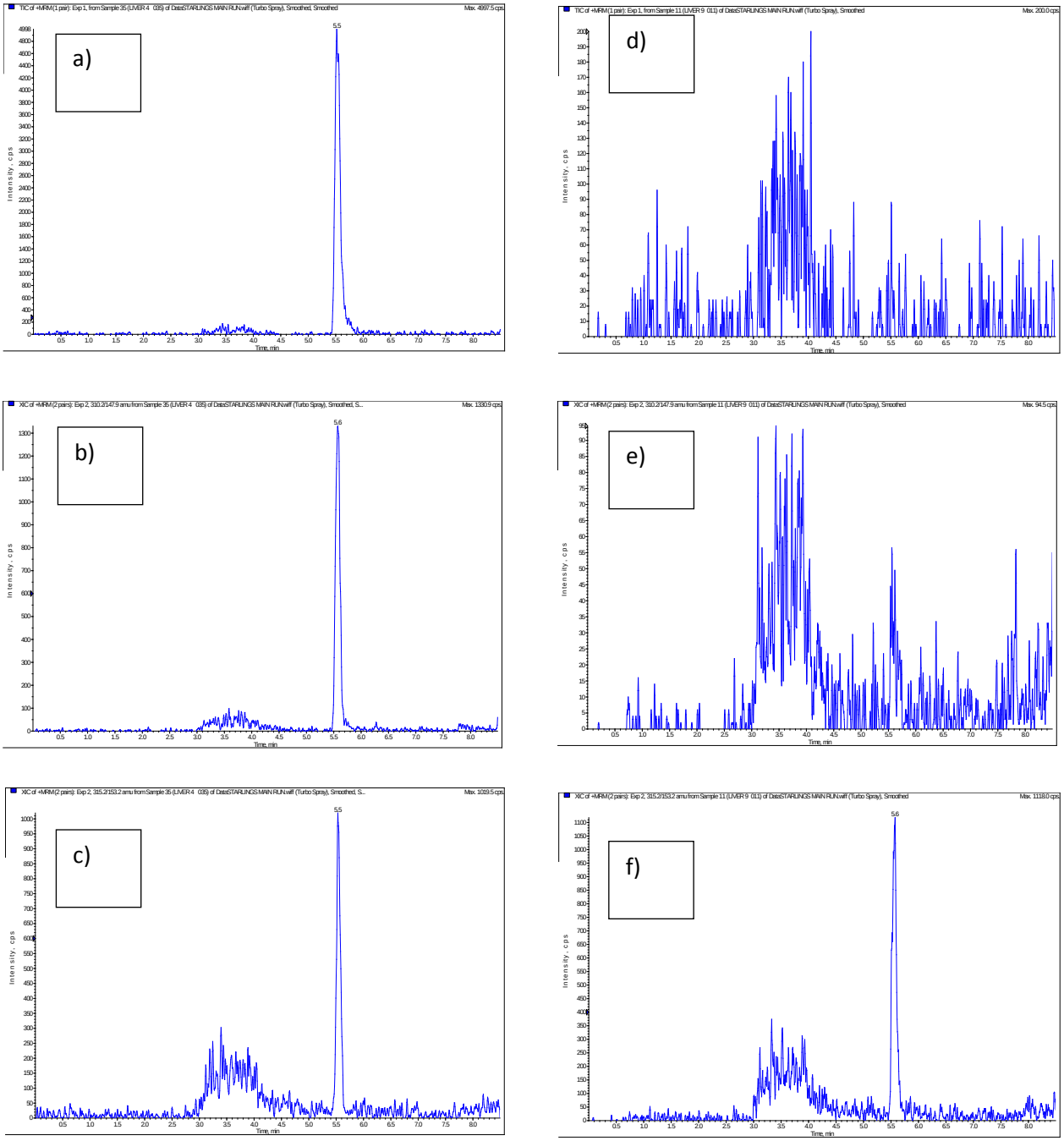



**Figure A1: Extracted Ion Chromatograms from liver samples taken from a fluoxetine-treated bird (a) norfluoxetine, b) fluoxetine, c) fluoxetine-d5 internal standard and a control bird (d) norfluoxetine, e) fluoxetine and f) fluoxetine-d5 internal standard. Note the different y-axis scales (signal). Retention time of both analytes and internal standard were identical at 5.5 minutes.**

## A4 Quality control and quality assurance

The mean (±1SE) masses of tissue samples extracted were: Brain = 0.65 ± 0.044; Liver = 0.40 ± 0.031 kidney = 0.13 ± 0.014 and muscle = 0.27 ± 0.032.

### A4.1 Calibrations

Table A2 contains the $R^2$ values for the matrix specific standards used to calibrate the LC-MS/MS. The $R^2$ value was obtained by fitting a linear trend-line for signal detected by the LC-MS/MS (y-axis) against the concentration spiked into the sample (x-axis). All values are above 0.9 which means that the matrix specific calibrations were reasonably linear.

**Table A2: $R^2$ value for calibration series from matrix specific standards used to calibrate the LCMS. These $R^2$ values show that the linear regression line fitted through the calibration series was a good fit for the values plotted.**

| | Brain | Kidney | Liver | Muscle | Faeces | Earthworm | Biofilm | Influent |
|---|---|---|---|---|---|---|---|---|
| Fluoxetine | 0.9967 | 0.9985 | 0.9971 | 0.9945 | 0.9290 | 0.9983 | 0.9733 | 0.9962 |
| Norfluoxetine | 0.9955 | 0.9980 | 0.9961 | 0.9927 | 0.9743 | 0.9915 | 0.9964 | 0.9770 |

## A5 Inactive metabolites of fluoxetine in humans

**Table A3: Mono-isotopic molecular weights and Molecular formulas for the six metabolites searched for using Fourier Transform Mass Spectrometry (Drugbank 2014)**

| Metabolite | Monoisotopic molecular weight | Molecular formula |
|---|---|---|
| Para-Trifluoromethylphenol | 162.029249397 | $C_7H_5F_3O$ |
| Hippuric Acid | 179.058243159 | $C_9H_9NO_3$ |
| Fluoxetine Glucuronide | 485.166136806 | $C_{23}H_{26}F_3NO_7$ |
| Norfluoxetine Glucuronide | 487.145401364 | $C_{22}H_{24}F_3NO_8$ |
| Norfluoxetine Alcohol | 296.102414339 | $C_{16}H_{15}F_3O_2$ |
| Norfluoxetine Acid | 310.081678897 | $C_{16}H_{13}F_3O_3$ |

# Appendix B

## B1 Baseline Results (before treatment with fluoxetine)

The data used in these analyses were collected prior to treatment in November and December 2011; Table B1 shows that there were no significant differences prior to treatment.

**Table B1: Effects of treatment and day on behaviour (Exploration, Activity and Boldness), CORT and mass measured pre-treatment (baseline). Wald statistics from repeated measures GEE compared to a $\chi^2$ distribution with 1 degree of freedom. The $\chi^2$ value along with P> $\chi$ are reported for each of the explanatory variables.**

| Endpoint | Treatment | | Day | | Interaction | |
|---|---|---|---|---|---|---|
| | $\chi^2$ | P | $\chi^2$ | P | $\chi^2$ | P |
| **Exploration** | 0.032 | 0.86 | 1.02 | 0.31 | 1.70 | 0.19 |
| **Activity** | 2.14 | 0.14 | 0.39 | 0.53 | 0.80 | 0.37 |
| **Boldness** | 0.57 | 0.45 | 3.39 | 0.066 | 1.60 | 0.21 |
| **Corticosterone metabolites** | 1.11 | 0.29 | 3.37 | 0.067 | 0.80 | 0.37 |
| **Body Mass** | 2.20 | 0.14 | 78.13 | <0.01 | 0.13 | 0.72 |

# Appendix C

## C1 Levene's Tests for assumption of equal variances

The data in Table C1 were used to test the assumption of equal variances between the treatment groups for the independent samples t-tests presented in Table 4.2. As the p-values in Table C1 are all greater than 0.05, then the assumption of equal variances is reasonable (although the value was marginally significant for male monopoliser rank: fluoxetine treated birds more variable than controls).

**Table C1: F-statistics and p-value (the significance of the test) for Levene's test for homogeneity of variance for fluoxetine and control males and females. The variables assessed were gonad size relative to body mas, the arcsine square root transformed proportion of the bill that had turned from winter condition to breeding condition, body mass, the rank based on time spent monopolising the food resource and the rank based on the order birds came down to feed in.**

| | Males | | Females | |
|---|---|---|---|---|
| | F | p | F | p |
| Relative Gonad size | 0.89 | 0.37 | 3.40 | 0.10 |
| Bill colour at time of mate choice | <0.01 | 0.98 | 0.12 | 0.73 |
| Body mass | <0.01 | 0.97 | <0.01 | 0.98 |
| Monopoliser Rank | 4.80 | 0.053 | <0.01 | 0.95 |
| Leader-follower rank | 0.18 | 0.68 | 1.60 | 0.24 |

## C2 Female Activity levels

One possible explanation for the fluoxetine-treated birds spending more time in front of neither male is that they were less active than controls and so just remained perched on the middle divide. Independent of the mate choice study, I assessed activity levels during two 10 minute exploration trials on consecutive days after 16 weeks of treatment (see Bean et al., 2014 or Chapter 3). Activity levels were marginally repeatable across the two days ($F_{11,12} = 2.31$, $r = 0.40$, $p = 0.083$), so a mean

was created and differences between the treatment groups tested using an independent samples t-tests (equal variances (Levene's: F = 2.9, p = 0.12; T-test, df = 10, t = -1.75, p = 0.11). Therefore there was no significant difference in the mean activity levels of the females (Figure C1). Thus the differences observed in mate choice and male association time are unlikely to be due to differences in activity.

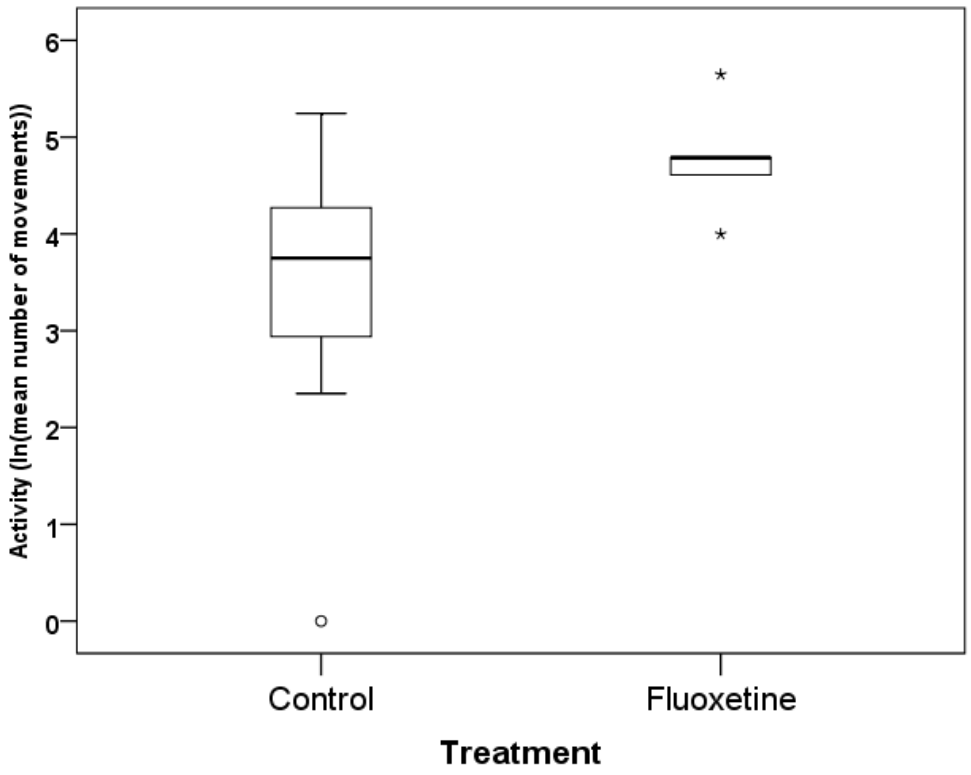



**Figure C1 Median activity levels (natural log of the mean number of movements in two ten minute exploration trials) for control and fluoxetine-treated Females. Mean activity taken over two trials on consecutive days. The boxes represent the upper and lower quartiles and points beyond the end of the whiskers are outliers as defined by Tukey (1977).**

# Appendix D

## D1 Preparation of food for Pbets: Earthworm exposures

The uptake of fluoxetine into earthworms followed OECD Guideline 317 "Bioaccumulation in Terrestrial Oligochaetes" (OECD 2010).

### D1.1 Determination of Soil moisture content

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

**Equation D1**

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

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

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

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

***Cont***= mass of empty container (g)

### D1.2 Determination of Soil Maximum Water Holding Capacity

A square of muslin was fixed to the bottom of each of five separate plastic rings (volume = 40.707 $cm^3$) with elastic bands. The weight of rings, muslin and elastic band were recorded for each of the five replicates. Soil was then packed into each of the five rings, tapping the rings carefully during the process to ensure that the packing density fell in the range of 0.76-1 g $cm^{-3}$ (Equations D2 and D3).

**Equation D2**

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

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

***RS***= mass of ring and moist soil (g)

***R***= mass of empty ring (g)

**Equation D3** ***Den = Dsoil /RingVol***

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

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

The rings containing the soil were weighed and if the packing density (Equation D3) fell in the range 0.76-1 g $cm^{-3}$ then it was acceptable, if the packing density was outside these limits then it was necessary to re-pack and re-weigh until the packing density fell within the acceptable range. The soil rings were then placed into a glass tray which was then slowly filled to a depth of approximately 3 mm with deionised water. The soil rings were left for three hours to saturate with the water level checked every hour and topped up as necessary.

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

After three hours saturation time, the soil rings were placed muslin side down on the sand bath for 24 hours to drain. After which the soil rings were removed and the soil was scraped out from the ring into individual pre-weighed foil dishes and re-weighed. The foil dishes containing saturated soil were oven dried at 105°C for 24 hours and reweighed. The maximum water holding capacity is the moisture content of the saturated soil (Equation D3).

### D1.3 Determination of mass of moist soil for a certain dry mass content

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

Firstly, the mass of water in 50 g of moist soil was determined using Equation D4.

**Equation D4**

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

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

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

Secondly, I determined the mass of water required in order for the soil to be at MWHC using Equation D5.

**Equation D5**

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

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

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

Finally, the mass of water to be added to soil to make up to 60% of MWHC was calculated using Equation D6.

**Equation D6**

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

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

For the soil used here, 4.75 g of water was added to the 50 g of moist soil to make it up to 60% of MWHC. Earthworms were then exposed for 21 days, maintaining the moisture content at 60% MWHC each day by adding deionised water, full details of the 21 day exposure are given in section 5.2.2.

## D2 Structure of the experiment

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

## D3 The Human Pbet

The human gastro-intestinal system is very different from that of birds with gastric pH varying from a basal value of pH 1 to 2 to between 4 and 6 after a recent meal (Ruby et al. 1993). After one to two hours, the gastric contents are emptied into the small intestine. Bicarbonate ions are secreted with the pancreatic juices, which on combination with the intestinal chyme reaches a pH of approximately seven in the small intestine (Murthy et al. 1980). Human body temperature is 37°C and total gastrointestinal tract transit time is on average four to five hours, with approximately one hour in the stomach and three to four hours in the small intestine (Ruby et al. 1996). As with the avian system, the main gastric enzyme is pepsin but the concentration is eight times lower for humans than birds. Hydrochloric acid is also present along with malic acid, citric acid, lactic acid and acetic acid. The intestinal digestive juices for humans also contains bile salts and pancreatin, but as with the gastric digestive juices, their concentrations are less concentrated than in the avian system (Martinez-Haro et al. 2009; Ruby et al. 1996).

## D3.1 Human Pbet procedure

The human Pbets were adapted from the methods of Li and Zhang (2013) and Ruby et al. (1996) (See Fig. 5.1a). Human Pbets were run using digestive juice at three different timings since the consumption of food. In conical flasks, gastric digestive juice was prepared by adding Pepsin (1.25 g $L^{-1}$), Malate, Citrate (both 0.5 g $L^{-1}$), lactic acid (420 µL $L^{-1}$) and acetic acid (500 µL $L^{-1}$) to deionised water. Gastric juice was adjusted to the desired pH with concentrated HCl (pH 1.3 was used to represent fasted conditions, pH 2.5 represented average gastric conditions and pH 4 recently fed conditions (Ruby et al. 1996)) and then warmed to 37°C in a water bath.

To begin the Pbet simulation, earthworms and any adhered soil were added to tubes along with 20 mL of gastric digestive juice. The head space was purged with $N_2$ to create a low oxygen environment. Tubes were placed in the shaking incubator at 37°C but were not shaken for 10 minutes as in (Ruby

et al. 1996), after which tubes were shaken at 250 rpm for one hour in total. At three time points (every 20 minutes) during this hour, shaking was stopped and a 1.5 mL aliquot of gastric juice was taken from each tube and, and replaced with a fresh 1.5 mL of gastric solution. The gastric juice samples were transferred to a 1.5 mL sealed microcentrifuge tube and immediately centrifuged (10 mins at 11,000 × g). The supernatant decanted into a fresh tube and stored at -20°C.

Once the intestinal simulation was complete, the digestive juice in each simulation tube was adjusted to pH 7 with $NaHCO_3$ powder (intestinal pH) (Ruby et al. 1993). Once pH had been adjusted, 52.5 mg of bile extract and 15 mg of pancreatin were added to each replicate (Li and Zhang 2013) before returning to the shaker. Tubes were shaken on their sides at 100 rpm to mimic the slow intestinal passage. A 1.5 mL aliquot was taken from each sample and not replaced after 1 hour intestinal incubation with a final aliquot of 1.5 mL taken after a total of 3.5 hours of the intestinal incubation. The intestinal samples were centrifuged and stored as the gastric phase samples, the only exception being that the remaining solid material was considered to be faeces.

## D4 Calculation of concentration of fluoxetine inserted into Pbets

As a Quality assurance/control, I exposed eight additional earthworms for 21 days, four fluoxetine earthworms and four control earthworms. Exposure conditions exactly replicated that of the earthworms that were inserted to the Pbet. Prior to extraction, earthworms were weighed, rinsed of soil with deionised water using a pipette and paper towels and then re-weighed. The weight difference before and after rinsing was used to quantify the mass of soil adhered to the earthworms. I extracted the earthworms using the same method as above and analysed the extract using HPLC to quantify the mass of fluoxetine per gram of earthworm on a wet weight basis. The concentration in the earthworm extract was corrected for a 75.3% recovery (as found in the earthworm validation) and the mean concentration was found to be 537.1 μg $g^{-1}$, RSD = 17.6%. No fluoxetine was detected in the control earthworms.

## D5 Quality Assurance / Quality Control

### D5.1 Extraction of fluoxetine from earthworms as a QA/QC

To determine the amount of fluoxetine inserted to each Pbet replicate, I used the mean concentration per gram of earthworm as determined from the QA/QC and corrected for the mass of the earthworm. The four replicates of fluoxetine and control worms that were exposed under the same conditions as the earthworm samples were rinsed of soil with deionised water. Earthworms were then dabbed dry on paper towels. The weight was recorded (± 0.1g) and earthworms were extracted by homogenisation (Turrax) with 6 mL of methanol. Extracts were ultrasonicated in an

ultrasonication water bath for 10 minutes, centrifuged for 10 minutes at 4500 × g before analysing by HPLC.

### D5.2 Extraction efficiency of fluoxetine from earthworms

I extracted earthworms spiked with 0, 0.6, 6, 30, 60, 120 µg of fluoxetine. Earthworms were left for 2 hours to absorb the spiked fluoxetine prior to extraction. Earthworms were homogenised (Turrax, UK) in 6 mL methanol. Samples were ultrasonicated for ten minutes, centrifuged for 10 minutes (4500 × g, 20°C) before 0.2 µm filtering and analysing by HPLC (Recovery = 75.3%, LOQ = 0.6 µg $mL^{-1}$, RSD between triplicates = 10.0%).

### D5.3 Soil extractions

To determine the amount of fluoxetine contained in soil that adhered to earthworms, I needed to determine the concentration in the soil that earthworms were exposed in. Five grams of soil (at 60% Maximum water holding capacity) was taken from each jar of the QA/QC earthworms and placed into a 50 mL centrifuge tube. Soil was extracted twice using 10mL of 70:30 acetonitrile:water (HPLC fluorescence grade). Samples were shaken on their side at 420 rpm, they were then ultrasonicated for 10 minutes before centrifuging for 10 minutes (4500 × g, 20°C). The supernatant was decanted into a second 50 mL centrifuge tube before adding a fresh 10 mL of the 70:30 acetonitrile:water mixture and repeating the extraction process. After centrifuging for the second time, the two 10 mL aliquots were combined in the second falcon tube, vortex mixed before taking an aliquot and passing through a 0.2 µm PTFE filter into a vial and analysing by HPLC (See Analytical methods in Chapter 5).

### D5.4 Extraction efficiency of fluoxetine from soil

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

### D5.5 Sorption of fluoxetine to grit

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

I found sorption of fluoxetine to be particularly high with approximately 85% of fluoxetine adsorbing to Ca grit and 45% to Si grit at the concentration used in the experiment. Therefore sorption could

potentially explain why a greater percentage of fluoxetine was recovered in the faeces for birds than humans.

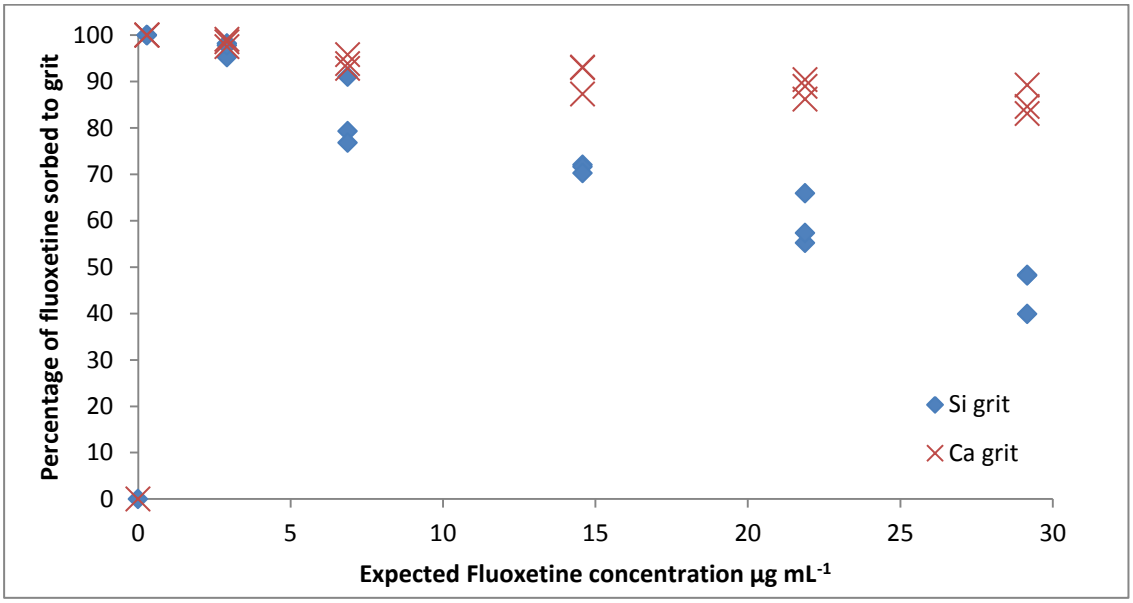



**Figure D1: Percentage of fluoxetine adsorbed to 2 g of Si grit (diamonds) and Ca grit (Crosses) between 0 and 30 µg $mL^{-1}$ fluoxetine after 48 hours shaking at 250 rpm.**

## D6 *In-vivo* evaluation: Excretion of fluoxetine by starlings

Three birds from each treatment group were placed in an individual test cage for 2 hours after the final dose. Immediately prior to euthanasia, a sample of faeces was collected and placed in a 1.5 mL microcentrifuge tube using a swab and placed on dry ice while post-mortem dissections took place. Approximately three hours later, samples were placed in the oven at 40°C until dry. Drying faecal samples were weighed daily until there was no further change in mass. Dry samples were then ground and stored in small glass vials at -20°C until analysis.

The dried sample was extracted with 1 mL of methanol, shaken on their sides at 420 rpm for 30 minutes, ultrasonicated for 5 minutes before centrifuging for 10 minutes in a microcentrifuge at 11,000 × g. The supernatant was transferred to a glass tube containing 100 µL of 9:1 Methanol:ethylacetate to prevent analytes from sticking to the glass during the concentration phase. Glass tubes were transferred to a Turbovap set at 45°C and blown until dry under a steady flow of nitrogen (typically 5-10 psi).

Samples were reconstituted first with 0.5 mL of methanol and vortex mixed for 10 seconds before adding 0.5 mL of deionised water and vortex mixing again. Samples were passed through a PTFE filter into a clear glass total recovery vial. Vials were sealed using a crimp cap containing a PTFE/Silica septa. Samples were analysed following the method outlined in Chapter 2.

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