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

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HIGHLIGHTS

- Birds eat sewage-contaminated prey containing antidepressants such as fluoxetine.
- Male starlings sang less to fluoxetine-treated females than to control females.
- Increased male aggression towards fluoxetine-treated females.
- First evidence of fluoxetine-induced courtship disruption in a songbird.
- Antidepressants in the environment alter fitness-related traits in wildlife.

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ABSTRACT

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

1. Introduction

Chemical contaminants are a driver of global biodiversity loss, representing an additional stressor to wildlife already under pressure from factors such as habitat loss and climate change (Novacek and Cleland, 2001). In recent years, pharmaceuticals that contaminate the environment have been identified as a potential risk to wildlife, including birds (Shore et al., 2014; Arnold et al., 2014). An extreme example of this threat was demonstrated by the deaths in India of *Gyps* vultures from diclofenac residues in cattle carcasses, which led to local population collapse (Oaks et al., 2004). Direct mortality as a result of exposure to pharmaceuticals at environmental concentrations is apparently rare, yet such contaminants can instead exert sublethal effects on wildlife (Shore et al., 2014).

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Psychotropic pharmaceuticals, such as antidepressants, are designed to alter behaviour at low doses and so have the potential to modulate wildlife behaviours, with implications for individual fitness and even population persistence (Brodin et al., 2014). To date, few studies have explored the behavioural effects of exposure to psychotropic pharmaceuticals, such as antidepressants, in wild terrestrial vertebrates, including birds (Bean et al., 2014).

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

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

Sexual behaviour, or courtship, and sex hormones have been well studied in both free-living and captive birds (Eens et al., 1991; Pinxten et al., 2003; Dawson, 2008), albeit rarely in the context of ecotoxicology, although see (Markman et al., 2008). In songbirds, male song is known to vary according to environmental stressors such as food availability (Ritschard and Brumm, 2012) and has previously proven a sensitive endpoint for studying the effects of certain contaminants (Markman et al., 2008) and anthropogenic disturbances (Kempenaers et al., 2010). Male song is under strong sexual selection pressure and is a signal of male quality that females use to make mate choice decisions (Eens et al., 1991). However, some degree of mutual mate choice is predicted in species with biparental care (Edward and Chapman, 2011), such as the Eurasian starling (*Sturnus vulgaris*). Male starlings are selective in their mate choice and can choose females based on factors such as plumage iridescence or age (Komdeur et al., 2005). Males exercising mate choice might be expected to invest less time singing to less attractive or lower quality females; as observed in Bengalese finches (*Lonchura striata domestica*) (Heinig et al., 2014). If fluoxetine alters female reproductive behaviour or physiology in starlings and thereby alters female attractiveness, this could alter signalling of individual quality (Markman et al., 2008) by females, with associated consequences for male courtship responses and male mate choice. This could impact on individual fitness by reducing reproductive success, with predicted negative impacts at the local population level (Brodin et al., 2014).

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

2. Methods

2.1. Ethics statement

This work was carried out under a Home Office Licence (PPL 60/

4213) and approved by ethics committees at both the University of York and at the Animal and Plant Health Agency. The birds were captured under licences from Natural England and the British Trust for Ornithology.

2.2. Capture and husbandry

In October 2015, we captured 24 wild Eurasian starlings (*Sturnus vulgaris*) in North Yorkshire, UK and moved them to our experimental facility. The birds were uniquely marked on arrival with a numbered leg ring (AC Hughes, Hampton Hill, UK). DNA sexing (Avian Biotech, Truro, UK) confirmed that there were 16 females and 8 males. The birds were given four weeks to acclimate to captivity, which also allowed excretion of non-persistent contaminants. See electronic supplementary material for husbandry details.

2.3. Experimental treatment

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

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

2.4. Courtship experiment

We conducted a courtship experiment, based on a design that has been previously used to study Eurasian starling courtship (Markman et al., 2008; Eens et al., 1993), over two weeks from 29th April 2016, reflecting the UK breeding season (Pinxten et al., 2003; Markman et al., 2008). Each male (eight in total; four fluoxetine-treated and four control) participated in two replicate trials (one per week): one trial with a control female, the other with a fluoxetine-treated female. The primary objective of these trials was to assess the effect of female treatment and phenotype on male courtship responses, under the assumption that female phenotype was a driver of male behaviour. The order of female presentation and the order in which each male was tested in the first week were randomised. Each male underwent two trials, one in each of two identical test arenas. Each female (8 per treatment) was paired with only one male. We allocated birds randomly to pairs, within the constraint that pairs comprised visually unfamiliar individuals from non-neighbouring home aviaries.

Two visually, but not aurally, occluded outdoor courtship arenas were used, each containing a wooden nest box with an attached perch from which males could sing and two swinging perches in close proximity to the nest box (see electronic supplementary material, Fig. S1). We affixed a hidden condenser shotgun microphone (RØDE NTG2; RØDE Microphones, London, UK) to the top of the nest box in each arena. Nesting materials were provided on the floor and there was *ad libitum* access to food and water. A window into the arena allowed behavioural observation.

Each trial began at 14:00 on the first day and ended at 11:00 on the second day. Two pairs were tested simultaneously (one pair per arena). Before a trial started, the focal individuals were weighed (to the nearest 0.1 g) and introduced to the arena. The pair was given 15 min to settle before sound recording began. After an additional 15 min the observational period began, with 30 min of observational data collected per pair. After 2.5 h of sound recording, the microphones were removed and the pairs remained in the arenas overnight. The following morning, the microphones were reinstated, and the song and behavioural data collection protocol repeated. After the 2.5 h recording period, the focal birds were captured and immediately blood sampled by jugular venepuncture for quantification of testosterone and oestradiol in plasma, before being returned to their home aviaries.

To assess the effect of fluoxetine on female attractiveness, male singing directed at fluoxetine-treated and control females was compared. Song was recorded using one solid state recorder per microphone (Marantz PMD 660; Marantz Europe, Eindhoven, The Netherlands). We used a sound analysis package Raven Pro: Interactive So (2011) and followed protocols used previously (Markman et al., 2008) to analyse the recordings for number of male song bouts and total male time singing (in seconds). To be classed as song, each bout had to be longer than 5 s and contain complex or composite phrases, as opposed to simple calls. Separate bouts were defined as being at least 1.5 s apart.

To compare female aggressive and courtship behaviours between fluoxetine-treated and control females, and to compare male aggression and courtship behaviours directed to females of different treatments, we counted occurrences of certain behaviours during each 30 min observation window. Aggressive behaviour per individual was defined as the sum count of the following behaviours: displacement, chasing, tugging of feathers, pecking, clawing. Courtship behaviour per individual was defined as the sum count of the following behaviours: approaches (within two body lengths), perching on nest box, entering nest box, carrying of nesting material; plus singing and displaying for males only.

2.5. Physiological measures

Plasma samples (collected at the end of each courtship trial) were analysed for testosterone by radioimmunoassay (RIA), following the assay protocol described in (Pottinger and Pickering, 1985). The intra-assay coefficient of variation was 8.96% and the assay detection limit was 62.5 $pg\, mL^{-1}$. Circulating oestradiol was determined from plasma samples using an enzyme-linked immunosorbent assay (ELISA) kit (DKO003 Estradiol ELISA; DiaMetra, Milan, Italy). The intra-assay coefficient of variation for the

oestradiol assay was 17.48%, which is rather poor; probably due to limited sample volume. As such, our oestradiol data should be regarded as approximate rather than absolute. See electronic supplementary material for hormone analysis methods.

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

2.6. Statistical analyses

All statistical analyses were performed using the software R, version 3.3.1 (R Core Team, 2016). Using R package lme4 (Bates et al., 2015), we constructed generalised linear mixed models (GLMMs) to assess the effect of treatment and other predictors on the following response variables: number (count) of male song bouts, count of male aggressive behaviours, count of male courtship behaviours, count of female aggressive behaviours, count of female courtship behaviours. We included male or female ID as a random factor, depending on whether the response variable pertained to male or female focal birds respectively. We initially included the following fixed effects in each model: treatment (both sexes), day of experimental period (as ordered factor in ascending order; also controlled for changing photoperiod), trial phase (i.e. first or second day of trial), body condition index (both sexes), circulating testosterone (focal sex only), circulating oestradiol (focal sex only), female treatment*trial phase interaction, male treatment*female treatment interaction. For each response variable, we fitted a GLMM with either Poisson or (if overdispersed) negative binomial error structure (see electronic supplementary material) with a log link function. The model was reduced by iteratively removing the least significant term (based on Wald's Z test (Bolker et al., 2009)). Corrected Aikake's Information Criterion (AIC_c) (Burnham and Anderson, 2004) was used to select the minimum adequate model. To check whether the data met the assumptions of the models, diagnostic tests were conducted using R package DHARMa (Hartig, 2017).

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

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

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

3. Results

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

3.1. Male singing behaviour

There was a significant interaction between female treatment and trial phase (i.e. whether day one or two of the trial) on both the number of male song bouts (Fig. 1a and Table 1a) and male total

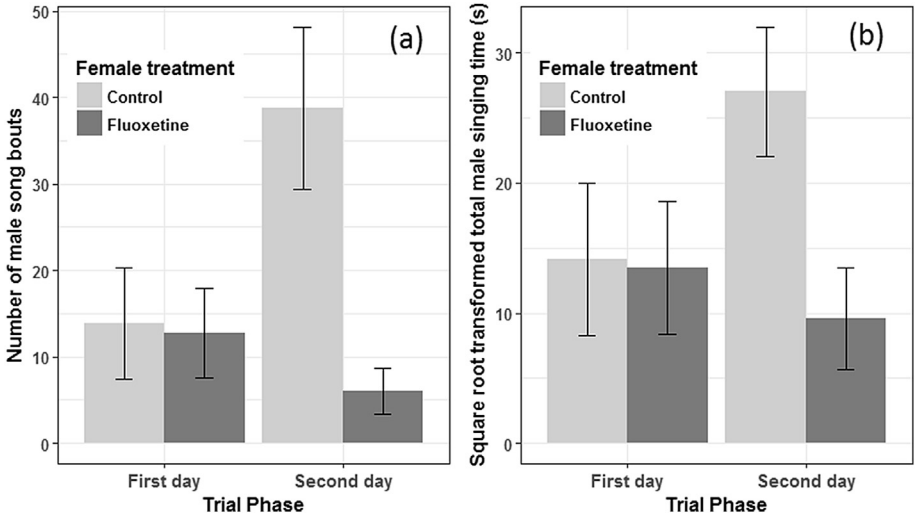

Fig. 1. Male song behaviour shown as mean ± S.E.: (a) number of male song bouts and (b) square root transformed total male time singing (s), by female treatment and trial phase ($n = 8$ observations per bar).

Table 1
Summary of GLMM minimum adequate model outputs for the response variables: a) number of male song bouts; b) count of male aggressive behaviours; c) count of male courtship behaviours; d) count of female aggressive behaviours, e) count of female courtship behaviours. $n = 32$ observations per model. Table shows: coefficient estimates (β), standard errors (β), Wald's z-score ($= \beta$ /SE(β)) and significance level p. GLMMs had negative binomial error distributions except for d), which had Poisson.

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

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

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

time singing (Fig. 1b and Table 2). During the second day of the trial, males sang significantly more to control than fluoxetine females, both in terms of number of song bouts (Fig. 1a and Table 1a) and total male time singing (Fig. 1b and Table 2). Males also spent significantly more time singing during the second day than the first day of the trial to control females (Fig. 1b and Table 2).

When we tested whether female behavioural and physiological traits explained variation in male song, we found no effect of any predictor on number of male song bouts or total male time singing ($p > 0.2$ in all cases). Full minimum adequate model outputs are reported in the electronic supplementary material, Tables S1 and S2.

3.2. Male aggressive and courtship behaviours

Males displayed significantly more aggressive behaviours towards fluoxetine-treated females than controls (Fig. 2a and Table 1b) and displayed more aggressive behaviours with increasing calendar date (Table 1b). Males displayed more courtship behaviours on the second compared to the first day (Table 1c). However, we found no effect of female treatment on male courtship behaviours.

Variation in male aggressive behaviours were not explained by any female behavioural or physiological traits, although male aggressive behaviour had a borderline significant positive relationship with female aggressive behaviour ($\beta = 0.17$, SE(β) = 0.09, $z = 1.90$, $p = 0.06$). Male courtship behaviours were explained by some female traits independent of experimental treatment; males directed more courtship behaviour at females who also expressed high levels of courtship behaviour compared with females that courted less ($\beta = 0.14$, SE(β) = 0.05, $z = 2.98$, $p = 0.003$). Complete minimum adequate model outputs are reported in the electronic supplementary material, Tables S1 and S2.

3.3. Female aggressive and courtship behaviours

There was a significant interaction between female treatment and trial phase (i.e. whether day one or day two of the trial) on female aggression (Fig. 2b and Table 1d), as fluoxetine-treated females were more aggressive during the second day of the trial compared to the first, whilst control females displayed intermediate levels of aggression throughout. There was a significant positive relationship between circulating oestradiol and female courtship (Table 1e), irrespective of female treatment. No other female traits explained variation in female behaviours.

3.4. Physiological measures

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

4. Discussion

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

One important function of male song is to attract females (Eens et al., 1991). Copulation attempts by male starlings are typically preceded by song (Eens and Pinxten, 1990), therefore the higher number of song bouts and longer time spent singing towards control females suggests that males found control-treated females more attractive than fluoxetine-treated females. Male starlings have previously been shown to increase their song rate to females

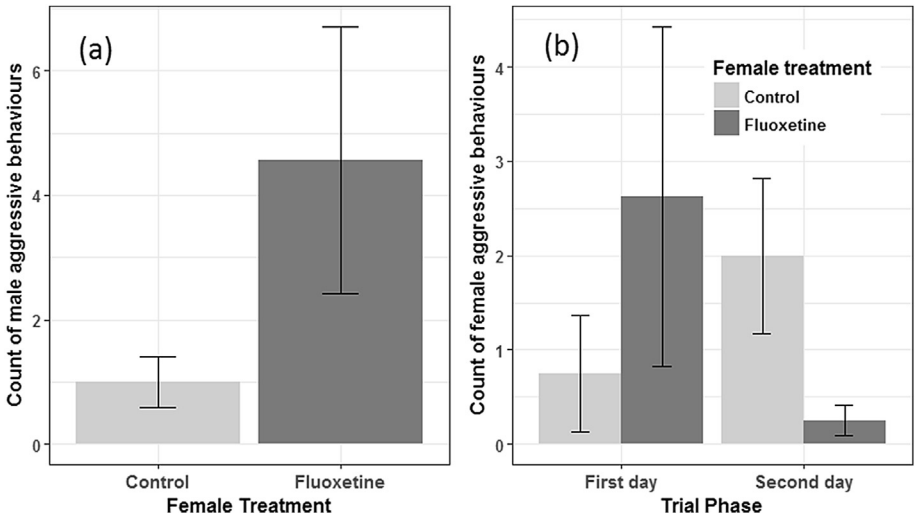

Fig. 2. Male and female courtship interactions shown as mean ± S.E.: (a) count of male aggressive behaviours, (b) count of female aggressive behaviours. (a) is split only by female treatment, as there was no significant effect of trial phase on this response. In (a), $n = 16$ observations per bar; in (b), $n = 8$ observations per bar. NB. Different scaling on y-axes.

in the late morning compared to the evening (Pinxten and Eens, 1998) and we observed such an increase in singing when males were paired with control females but not when they were paired with fluoxetine-treated females. This again demonstrates reduced courtship activity towards the apparently unattractive fluoxetine-treated females.

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

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

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

During our courtship experiment, fluoxetine-treated females were initially aggressive but became less aggressive in the second compared to the first day of the trial. Generally, since there is intersexual conflict within the starling mating system, alterations to female aggression may have fitness costs (Sandell, 1998). The offspring of polygynous males receive less parental care than the offspring of monogamous males (Sandell et al., 1996). Therefore, displaying high levels of female-female aggression during the breeding season could enable a female to maintain a monogamous status (Sandell, 1998). However, the observed disruption to fluoxetine-treated female aggression levels was not associated with changes in testosterone levels or an effect of treatment on female testosterone, or indeed on oestradiol. This contrasts with female rats, where there is some evidence that sexual dysfunction from fluoxetine treatment results from disruption of the neuroendocrine axis (Matuszczyk et al., 1998; Uphouse et al., 2006; Sarkar et al., 2008), although the doses used in these studies were several orders of magnitude higher than our dose. Environmentally relevant concentrations of fluoxetine administered subchronically have been reported to cause endocrine disruption in fish (Mennigen et al., 2017) but since our study involved a chronic rather than subchronic exposure, direct comparison with these studies is difficult. Since we observed no endocrine effects of chronic fluoxetine treatment in females, the reduction in attractiveness could have been mediated instead by altered neurotransmission (Higgins et al., 2010). 5-HT_{1A} receptors have been suggested to play a role in fluoxetine-induced sexual dysfunction

in female rats (Guptarak et al., 2010). Birds possess 5-HT_{1A} receptors (Dennis et al., 2013), presenting the possibility that female birds exposed to fluoxetine could likewise experience inhibition of sexual behaviour. In mammals, sexual dysfunction can occur following even an acute or subchronic dose (Sarkar et al., 2008; Guptarak et al., 2010), indicating a need to assess the effects of shorter exposures in passerines. In general, further work in this area should now focus on elucidating the mechanism, in terms of alterations to neurotransmission in fluoxetine-exposed females, that results in reduced attractiveness. Such work should again collect behavioural courtship data, but should also investigate whether key mode of action related targets, such as serotonin transporter (SERT) and relevant serotonin receptors (e.g. 5-HT_{1A}), are differentially expressed in fluoxetine-treated compared to control female brain tissue during the breeding season. Finally, generating a dose-response curve, ranging from low environmental concentrations through to high human dose equivalent concentrations, could be beneficial in furthering the current level of understanding of the effects of fluoxetine on behaviour and other ecologically relevant traits, and the implications of exposure in the environment. However, determining traditional threshold concentrations at which effects become apparent could be challenging for two reasons. Firstly, fluoxetine has already been shown to exhibit a non-monotonic dose-response relationship at environmental concentrations in other vertebrates (Martin et al., 2017; Saaristo et al., 2017). Secondly, a trait such as 'courtship' consists of different behaviours with different underlying mechanisms and responses are likely to be context dependent. Thus, the utility of a dose response curve in defining 'safe' environmental concentrations is likely to be limited for contaminants with sublethal effects.

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

Conflicts of interest

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

Authors contributions

SEW carried out experimental work, ran statistical analyses and drafted the manuscript, MGP supported laboratory work, RFS edited manuscript drafts, JL co-ordinated the aviary experiment. KEA conceived of the study and edited manuscript drafts. All authors participated in the study design, contributed to manuscript preparation and gave final approval for publication.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.07.074.

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