

Research report

# The effects of environmental exposure to DDT on the brain of a songbird: Changes in structures associated with mating and song

Andrew N. Iwaniuk<sup>a,\*</sup>, Dallas T. Koperski<sup>a,b</sup>, Kimberly M. Cheng<sup>c</sup>, John E. Elliott<sup>d</sup>,  
Lori K. Smith<sup>e</sup>, Laurie K. Wilson<sup>f</sup>, Douglas R.W. Wylie<sup>a,b</sup>

<sup>a</sup> Department of Psychology, University of Alberta, Edmonton, Alta., Canada T6G 2E9

<sup>b</sup> Centre for Neuroscience, University of Alberta, Edmonton, Alta., Canada T6G 2E9

<sup>c</sup> Agroecology Group, Faculty of Agricultural Sciences, University of British Columbia, 2357 Main Mall, Vancouver, BC, Canada V6T 1Z4

<sup>d</sup> Science & Technology Branch, Environment Canada, Pacific Wildlife Research Centre, Delta, BC, Canada, V4K 3N2

<sup>e</sup> Canadian Centre for Behavioural Neuroscience, University of Lethbridge, Lethbridge, Alta., Canada T1K 3M4

<sup>f</sup> Canadian Wildlife Service, Environment Canada, Pacific Wildlife Research Centre, Delta, BC, Canada, V4K 3N2

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## Abstract

Dichlorodiphenyltrichloroethane (DDT) is a persistent organochlorine compound found worldwide that causes significant anatomical, physiological and behavioural abnormalities in humans and wildlife. However, little is known about whether environmental exposure to DDT affects the brain. Here, we show that environmental exposure to DDT alters the brains of American Robins (*Turdus migratorius*) in several ways. Increasing levels of DDT resulted in: (i) smaller brain and relative forebrain volumes; (ii) a reduction in the size of two song nuclei, nucleus robustus arcopallialis (RA) and HVC; and (iii) a drastic reduction in neuronal size and overall volume of nucleus intercollicularis (ICo), a structure that is critical for normal sexual behaviour. These changes likely result from stress, direct neurotoxicity and androgen receptor antagonism by the primary metabolite of DDT, *p,p'*-DDE and this is corroborated by analyses of brain region volumes and *p,p'*-DDE levels. Our results therefore demonstrate that environmental exposure to DDT is correlated with significant changes in the brain and specifically those structures related to mating and song. Given the magnitude of these changes in the brain and the fact that environmental DDT exposure was restricted to early development, we conclude that both humans and wildlife that live in DDT contaminated environments may be at risk of neurological damage.

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**Keywords:** Endocrine disruption; DDT; Song system; Songbird; Neurotoxicology

## 1. Introduction

DDT is a persistent, widespread environmental contaminant found in most regions of the world [71]. Chronic and acute exposure to DDT adversely affects the development, physiology, morphology and behaviour of both animals and humans [30,34,66,88]. DDT causes these effects primarily by disrupting normal endocrine function, such that the animal experiences an abnormal hormonal environment. Although the entire endocrine system is disrupted by DDT [34,61], the most prominent effects are those concerning reproduction because the metabolites of DDT possess varying degrees of andro-

genic, anti-androgenic, estrogenic and anti-estrogenic effects [44,50,51,73,75] and inhibit gonadotropin secretion [62].

Although all vertebrates are susceptible to the endocrine disrupting effects of DDT, birds appear to be particularly sensitive [34,62]. The effects of DDT-induced endocrine disruption on reproduction in birds are well documented and include infertility, reduced reproductive success and impaired reproductive behaviour and parental care [30,34,88]. Sexual differentiation of the brain and behaviour of birds is highly dependent on the hormonal environment experienced within the egg and significant alterations in this environment can result in complete behavioural sex reversal [13]. Due to the sensitivity of birds to changes in the egg's hormonal environment, they are extremely susceptible to endocrine disruption during early development and this has led to many of the behavioural abnormalities present in birds exposed to DDT [30,34,88]. As the brain is the

\* Corresponding author. Tel.: +1 780 492 7239; fax: +1 780 492 1768.  
E-mail address: [brainsize@yahoo.ca](mailto:brainsize@yahoo.ca) (A.N. Iwaniuk).

major site of hormone action for hormone-sensitive behaviours, these abnormal behaviours likely result from changes in the underlying neural structures. Neuroendocrine studies of songbirds and quail [5,7] have shown that changes in the hormonal environment are correlated not only with behaviour, but also the size of brain regions mediating those behaviours. Specifically, reducing the levels of steroid hormones, such as testosterone and 17 $\beta$ -estradiol, results in smaller brain regions. If the behavioural changes associated with DDT exposure are a result of endocrine disruption, then it is likely that there are changes in the brain, but this has remained largely unexplored [18].

In songbirds, there are several brain regions that could be affected by DDT, but the song system, the nucleus intercollicularis (ICo) and the septum are likely to be affected because they contain steroid receptors [32] and mediate behaviours that are impaired by DDT. The song system is composed of a discrete set of interconnected brain regions that mediate the learning, perception and production of learned vocalizations [7,79]. The song nuclei within the song system are sensitive to both estrogens and testosterone levels. Higher levels of testosterone and estrogens are both associated with larger song nuclei [7,80]. DDT-induced endocrine disruption could therefore affect the size of these nuclei. ICo also contains numerous androgen and estrogen receptors [32,56] and the size of ICo and its neurons are affected by circulating hormone levels [9,41,60]. Although the precise function of ICo remains unclear, it is involved in copulation, vocal displays and agonistic behaviour [3,9,15,16,55,84], all of which are impaired by DDT exposure [88]. Finally, the septum also contains steroid receptors [56] and plays a critical role in mediating social behaviour [36,37] as well as learning and memory [70], all of which are impaired by DDT exposure [88]. Given the antagonistic effects that DDT and its degradants can have on androgen and estrogen receptors [50,51], the behavioural abnormalities resulting from DDT exposure and the effects that varying hormone levels have on the sizes of brain regions, we predicted that the amount of DDT that songbirds were exposed to would be correlated with the size of the brain regions. Specifically, higher levels of DDT should be associated with smaller nuclei. Here, we test this hypothesis in American Robins (*Turdus migratorius*) that were naturally exposed to environmental levels of DDT *in ovo* and during early post-hatching development.

## 2. Methods

### 2.1. Contaminant levels

To determine the level of exposure to DDT and other persistent organic pollutants (POPs), eggs were collected from the nests of robins nesting in orchards of the Okanagan Valley (BC, Canada) in July 1997. The contents of the eggs were homogenized and contaminant levels analysed at the National Wildlife Research Centre (Hull, PQ, Canada) using a standard gas chromatography and mass spectrophotometry protocol [87]. In addition to DDT and all of its congeners and isomers, the levels of several other POPs were assayed, including polychlorinated biphenyl (PCB) isomers. As shown in Table 1, the levels of most POPs were below the assay's level of detection and DDT was present in the highest concentrations. The total level of DDT and its degradants ( $\sum$ DDT) in the eggs varied from 4.96 to 174.67  $\mu$ g/g (mean = 68.34  $\mu$ g/g). This level

Table 1

Means and ranges of organochlorine compounds and polychlorinated biphenyl's (PCB) found in American robin eggs ( $n=18$ ) collected from the Okanagan Valley

Chemical	Concentration ( $\mu$ g/g, wet weight)	
	Mean	Range
1,2,4,5-Tetrachlorobenzene	<0.001	
1,2,3,4-Tetrachlorobenzene	<0.001	
Pentachlorobenzene	<0.001	
$\alpha$ -Hexachlorocyclohexane	<0.001	
$\beta$ -Hexachlorocyclohexane	<0.001	
$\gamma$ -Hexachlorocyclohexane	<0.001	
Hexachlorobenzene	<0.001	
Octachlorostyrene	<0.001	
Heptachlor epoxide	<0.001	
Oxychlorane	<0.001	
<i>trans</i> -Chlordane	<0.001	
<i>cis</i> -Chlordane	<0.001	
<i>trans</i> -Nonachlor	0.002	0–0.016
<i>cis</i> -Nonachlor	0.003	0–0.022
Dieldrin	<0.001	
Photomirex	<0.001	
Mirex	<0.001	
Tris(4-chlorophenyl) methanol	0.050	0–0.233
<i>p,p'</i> -DDT	11.530	0.072–20.799
<i>o,p'</i> -DDT	0.069	0–0.245
<i>p,p'</i> -DDE	55.775	14.876–151.224
<i>o,p'</i> -DDE	0.006	0–0.012
<i>p,p'</i> -DDD	1.048	0.018–3.562
<i>o,p'</i> -DDD	0.008	0–0.016
Total ( $\sum$ ) DDT	68.408	14.956–174.671
Total ( $\sum$ ) PCBs	0.05800	0.0148–0.1570

of  $\sum$ DDT exposure is similar to that found in other species exposed to environmental levels of DDT [4,11,19] and in previous studies of robins from the same region [35,42]. Because there tends to be little variation in POP levels among eggs within a clutch [21,25,81], we used these levels as estimates of the exposure levels of nestlings taken from the same nests at a later date (see below).

### 2.2. Animals

Eighteen 10-day-old robin nestlings were collected from the same nests as the eggs. The nestlings were hand reared until they were self-feeding, after which they were provided with food and water *ad libitum* and released into outdoor or indoor aviaries fitted with windows and lighting that was timed to match the natural photoperiod. All of the birds were communally housed (one male and one female per aviary) in these aviaries. Over two breeding seasons (April–August) [67,72], the birds were observed in these aviaries as part of a related study on the effects of DDT exposure on growth, immune function and behaviour (for details, see [72]). Throughout both nesting seasons, both sexes sang, although the singing was far more frequent in males, and all of the birds successfully reproduced at least once. Because of the requirements of this behavioural study, the birds were sacrificed at the end of the second breeding season (first week of August 1999) by decapitation. The brains were then extracted and post-fixed in 10% buffered formalin.

### 2.3. Histology

The brains were gelatin-embedded and serially sectioned on a freezing stage microtome. Forty-micrometer coronal sections were collected in 0.1 M phosphate-buffered saline (pH 7.4), mounted onto gelatinised slides, stained with thionin, dehydrated through a graded ethanol series, cleared in Hemo-D and coverslipped with Permount.

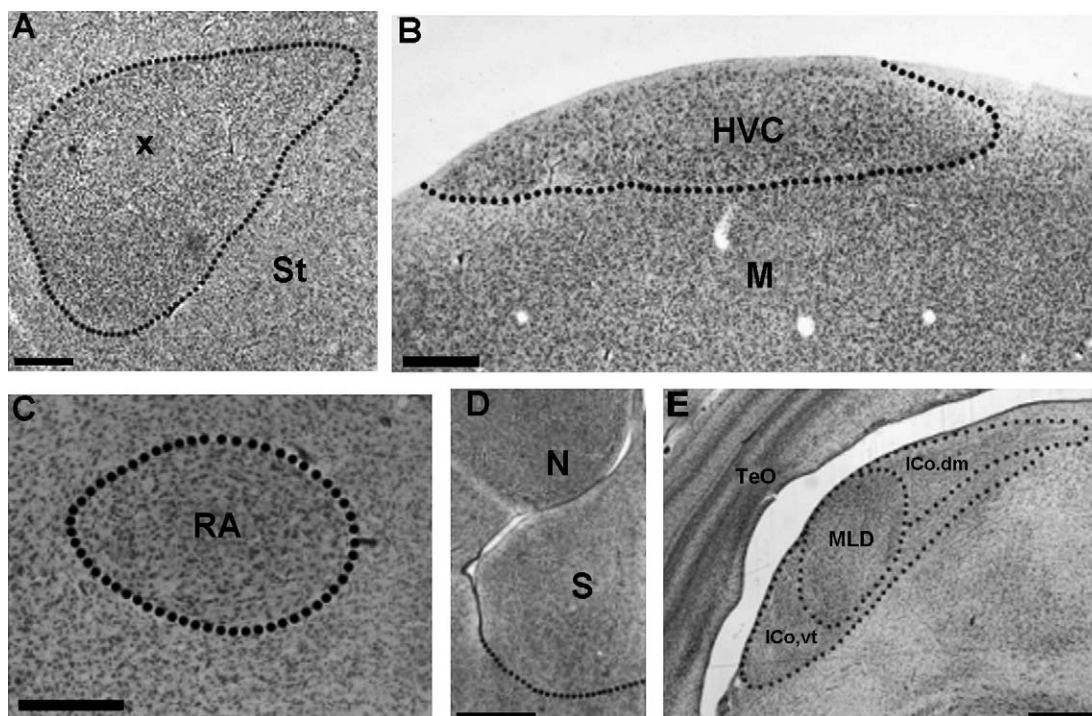


Fig. 1. Photomicrographs showing the three song nuclei measured, area X (A), HVC (B) and the robust nucleus of the arcopallium (C), as well as nucleus intercollicularis (D) and the septum (E). The dotted lines indicate the borders delineating each region. Abbreviations refer to the following: A—arcopallium; ICo, dm—dorsomedial nucleus intercollicularis; ICo, vl—ventrolateral nucleus intercollicularis; M—mesopallium; MLD—nucleus mesencephalicus lateralis, pars dorsalis; MSt—medial striatum; RA—robust nucleus of the arcopallium; S—septum; St—striatum; TeO—optic tectum; X—area X. Scale bars = 0.4 mm.

#### 2.4. Neuroanatomical measurements

For each specimen, a series of digital photos was taken throughout the rostro-caudal extent of each brain region. Measurements were made directly from the photos using the public domain NIH Image program (<http://rsb.info.nih.gov/ni-image/>). The three song nuclei measured were area X, HVC (used as its proper name) [63] and the robust nucleus of the arcopallium (RA). The song nuclei were all readily defined by darkly stained cells relative to surrounding tissue and distinct laminae surrounding each nucleus (Fig. 1A–C). All of the septal nuclei were included in our measurement of septum (Fig. 1D). The septomesencephalic tract, the anterior commissure and changes in cell density defined the ventral border of the septum [70]. ICo was defined by the presence of large, darkly stained cells relative to the adjacent central grey and separated from the nucleus mesencephalicus lateralis, pars dorsalis (MLd) by a lamina running along the ventro-lateral borders of MLd (Fig. 1E).

In addition to these regions, we also measured several brain regions where we did not expect to find a significant effect of DDT exposure. These nuclei are not involved in reproductive or parental behaviour and lack androgen and estrogen receptors. They included the ventral leaflet of the lateral geniculate nucleus (GLv), nucleus rotundus (Rt), MLd and olfactory bulbs (BO), and are hereafter referred to as control regions. The borders of these regions followed descriptions provided in the literature [20,26,52,54].

Because previous studies had found significant changes in the size of cells within RA [79] and ICo [9,60], we also measured soma size of cells within these regions. In RA, we measured cells from a section taken through the middle of the rostro-caudal extent of the nucleus for each individual. In ICo, we specifically measured only the larger neurons within dorsomedial ICo because these are the neurons that exhibit androgen-sensitive size changes [9,60]. As with RA, we measured these cells in a section taken through the middle of the dorsomedial ICo for each individual. Images were captured using the OPENLAB Imaging system (Improvision, Lexington, MA) with a Retiga EXi Fast monochrome digital camera mounted onto a Leica DMRE compound microscope. Between 30 and 40 neurons were measured from one section taken through the middle of both RA and dorsomedial ICo. The areas of the somata of these neurons were measured using the public domain NIH Image program.

#### 2.5. Statistical analysis

Prior to all statistical analyses, we assessed whether the volumes of the brain regions were affected by allometry. Allometric effects were assessed separately for each sex because of prominent sexual dimorphism in the volumes of several nuclei. We performed least-squares linear regressions on the volume of each brain region against the volume of the entire brain minus that of the brain region being tested [23,46,47]. Of all of the brain regions measured, only forebrain volume was significantly correlated with brain volume in males ( $F_{(1,8)} = 17.62$ ,  $P = 0.003$ ). That is, with the exception of forebrain volume, robins with larger brains did not necessarily have larger brain regions. We therefore compared DDT levels with the relative size of the forebrain (*i.e.* residuals from regression line) and the absolute size of the remaining brain regions.

Correlations between DDT and other POPs and both absolute and relative brain region size were conducted separately for each sex because of aforementioned sexual dimorphism in some regions and males were exposed to significantly higher DDT exposure levels than females (Wilcoxon  $Z_{(18)} = -2.93$ ,  $P = 0.003$ ). We had no *a priori* knowledge of whether a particular congener or isomer of DDT was going to exert a stronger effect than others, so we performed correlations on total DDT (the sum of all of the congeners and isomers of DDT =  $\sum$  DDT) as well as each of the six congeners and isomers identified (Table 1). The Kendall's tau ( $\tau$ ) test, a non-parametric correlation test that does not assume a linear relationship between two variables [74], was used to test for significant correlations. It should be noted, however, that the results were qualitatively similar using Pearson and Spearman correlations.

### 3. Results

#### 3.1. Correlations with $\sum$ DDT

In males, but not females, there was a significant reduction in brain (15%) and relative forebrain (13%) volume with increasing  $\sum$  DDT (Fig. 2A and B; Table 2). A negative trend in females was

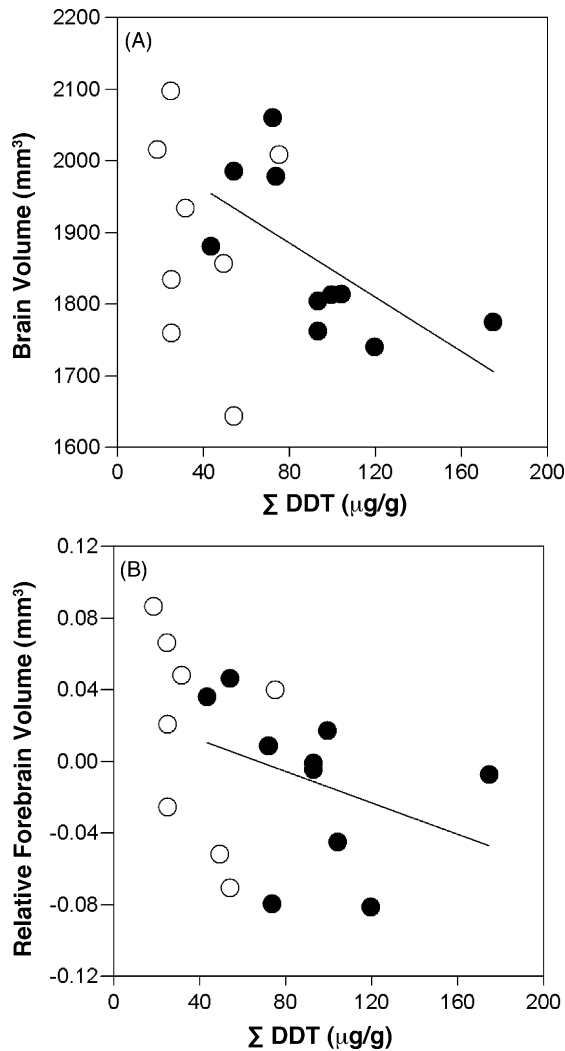


Fig. 2. Scatterplots of log-transformed volumes of (A) brain and (B) relative forebrain (see Section 2) plotted against the sum of all DDT isomers and congeners ( $\Sigma$ DDT in  $\mu\text{g/g}$ ). The open circles indicate the females and the closed circles indicate the males. The solid lines indicate significant correlations.

observed in the plot of relative forebrain volumes and  $\Sigma$ DDT (Fig. 2B) that just failed to reach significance ( $P=0.06$ ).

Although we expected significant reductions in all song nuclei, no significant change in area X volume was detected in either sex (Fig. 3A; Table 2). HVC exhibited a negative trend with  $\Sigma$ DDT levels in males (Fig. 3B), but this failed to reach significance ( $P=0.07$ , Table 2). However, there was a drastic reduction in the size of RA as a function of  $\Sigma$ DDT in males (Fig. 3C; Table 2). Males exposed to low  $\Sigma$ DDT had RA volumes much larger than females whereas one male exposed to high  $\Sigma$ DDT had an RA volume as small as that of the females (Fig. 3C). This amounts to approximately a 40% reduction in RA volume of males. This negative correlation was confirmed by the analysis of relative RA volume; higher  $\Sigma$ DDT levels were significantly correlated with relatively smaller RA volumes in males, but not females (Table 2). Despite this significant reduction in RA volumes of males, no significant correlation between  $\Sigma$ DDT and soma size was detected in males ( $\tau_{(10)}=-0.05$ ,  $P=0.85$ ) or females ( $\tau_{(8)}=-0.33$ ,  $P=0.26$ ).

Table 2

Kendall's  $\tau$  tests of the correlations between each of the brain regions measured and  $\Sigma$ DDT levels

Brain region	Males ( $n=10$ )	Females ( $n=8$ )
Whole brain	-0.49*	-0.25
Forebrain	-0.63*	-0.55
Song nuclei		
Area X	0.09	-0.11
HVC	-0.45	-0.59
Nucleus robustus arcopallialis (RA)	-0.54*	-0.11
Septum (S)	-0.08	0.30
Olfactory bulbs (BO)	-0.13	0.36
Nucleus rotundus (Rt)	0.04	0.06
Nucleus geniculatus lateralis, pars ventralis (GLv)	-0.18	0.10
Nucleus intercollicularis (ICo)	-0.49*	-0.91**
Nucleus mesencephalicus lateralis, pars dorsalis (MLd)	0.18	-0.11

Note that HVC could only be measured in seven of the females.

\*  $P<0.05$ .

\*\*  $P<0.01$ .

As expected from the high concentration of androgen and estrogen receptors in ICo [32], the volume of ICo was also negatively correlated with  $\Sigma$ DDT. In both males and females there was a dramatic reduction in absolute ICo volume, by as much as 30%, with increasing  $\Sigma$ DDT (Fig. 3D; Table 2). This significant decrease in the size of ICo reflected a decrease in soma size of ICo neurons (Fig. 4). In males, ICo neuron size decreased significantly with increasing  $\Sigma$ DDT ( $\tau_{(10)}=-0.58$ ,  $P=0.02$ ). A negative trend was also present in females, but did not achieve significance ( $\tau_{(8)}=-0.55$ ,  $P=0.16$ ).

Although we did expect a significant reduction in septum volume, no such correlation was observed (Table 2). We also did not detect any significant correlations between the volumes of any of the control regions and  $\Sigma$ DDT (Table 2). Thus, not all brain regions were affected in the same way as the song nuclei and ICo.

### 3.2. Correlations with DDT congeners and isomers

$p,p'$ -DDE comprised the largest portion of  $\Sigma$ DDT in the robins (Table 1) and was significantly and negatively correlated with relative forebrain and absolute HVC, RA and ICo volumes in males and ICo volume in females (Fig. 5; Table 3). Scatterplots of brain region volume against  $p,p'$ -DDE levels (Fig. 5) were remarkably similar to those of  $\Sigma$ DDT (Figs. 2B and 3B–D). No significant relationship was present between  $p,p'$ -DDE and RA neuron size in males ( $\tau_{(10)}=0.00$ ,  $P=0.99$ ) and females ( $\tau_{(8)}=-0.18$ ,  $P=0.53$ ). However, in both males ( $\tau_{(10)}=-0.63$ ,  $P=0.01$ ) and females ( $\tau_{(8)}=-0.62$ ,  $P=0.03$ ), higher levels of  $p,p'$ -DDE were also associated with smaller neurons in ICo.

Of the other five congeners and isomers (see Table 1) only  $p,p'$ -DDD was significantly and negatively correlated with ICo volume in males ( $\tau_{(10)}=-0.87$ ,  $P=0.001$ ). The remaining comparisons, including soma size of RA and ICo neurons, were all not significant (all  $P>0.08$ ).

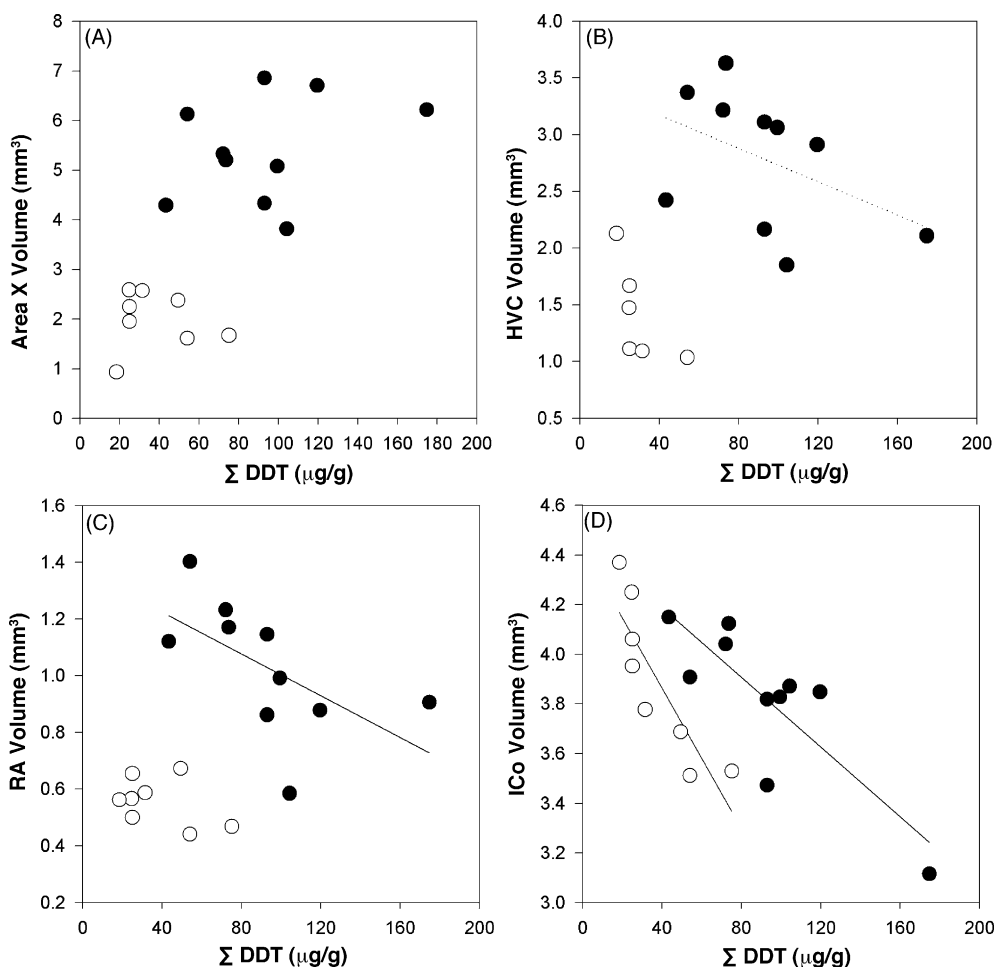


Fig. 3. Scatterplots of log-transformed volumes of (A) area X, (B) HVC, (C) nucleus robustus arcopallialis (RA) and (D) nucleus intercollicularis (ICo) plotted against the sum of all DDT isomers and congeners ( $\Sigma$ DDT in  $\mu\text{g/g}$ ). The open circles indicate the females and the closed circles indicate the males. The solid lines indicate significant correlations. Note the dotted line in (B) indicates a negative correlation that fell short of significance ( $P=0.07$ ).

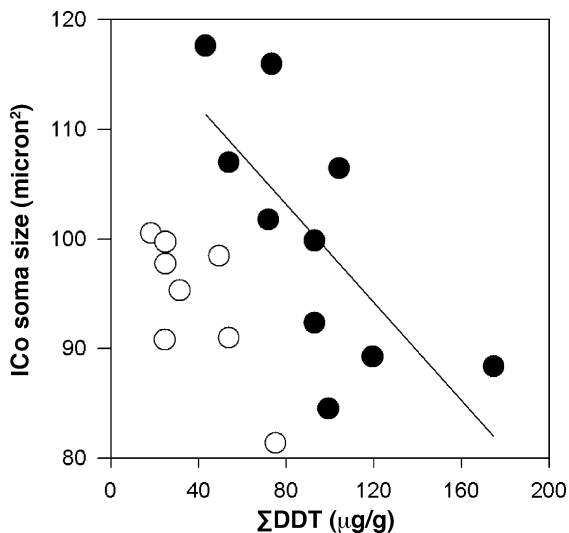


Fig. 4. Scatterplot of soma size of nucleus intercollicularis (ICo) neurons ( $\mu\text{m}^2$ ) plotted against the sum of all DDT isomers and congeners ( $\Sigma$ DDT in  $\mu\text{g/g}$ ). The open circles indicate the females and the closed circles indicate the males. The solid lines indicate significant correlations.

Table 3  
Kendall's  $\tau$  tests of the correlations between each of the brain regions measured and  $p,p'$ -DDE levels

Brain region	Males ( $n=10$ )	Females ( $n=8$ )
Whole brain	-0.45	-0.11
Forebrain	-0.58*	-0.40
Song nuclei		
Area X	0.13	0.04
HVC	-0.49*	-0.59
Nucleus robustus arcopallialis (RA)	-0.49*	-0.11
Septum (S)	-0.03	0.29
Olfactory bulbs (BO)	-0.09	0.32
Nucleus rotundus (Rt)	0.09	-0.18
Nucleus geniculatus lateralis, pars ventralis (GLv)	-0.22	0.05
Nucleus intercollicularis (ICo)	-0.54*	-0.76**
Nucleus mesencephalicus lateralis, pars dorsalis (MLd)	0.13	-0.11

Note that HVC could only be measured in seven of the females.

\*  $P < 0.05$ .

\*\*  $P < 0.01$ .

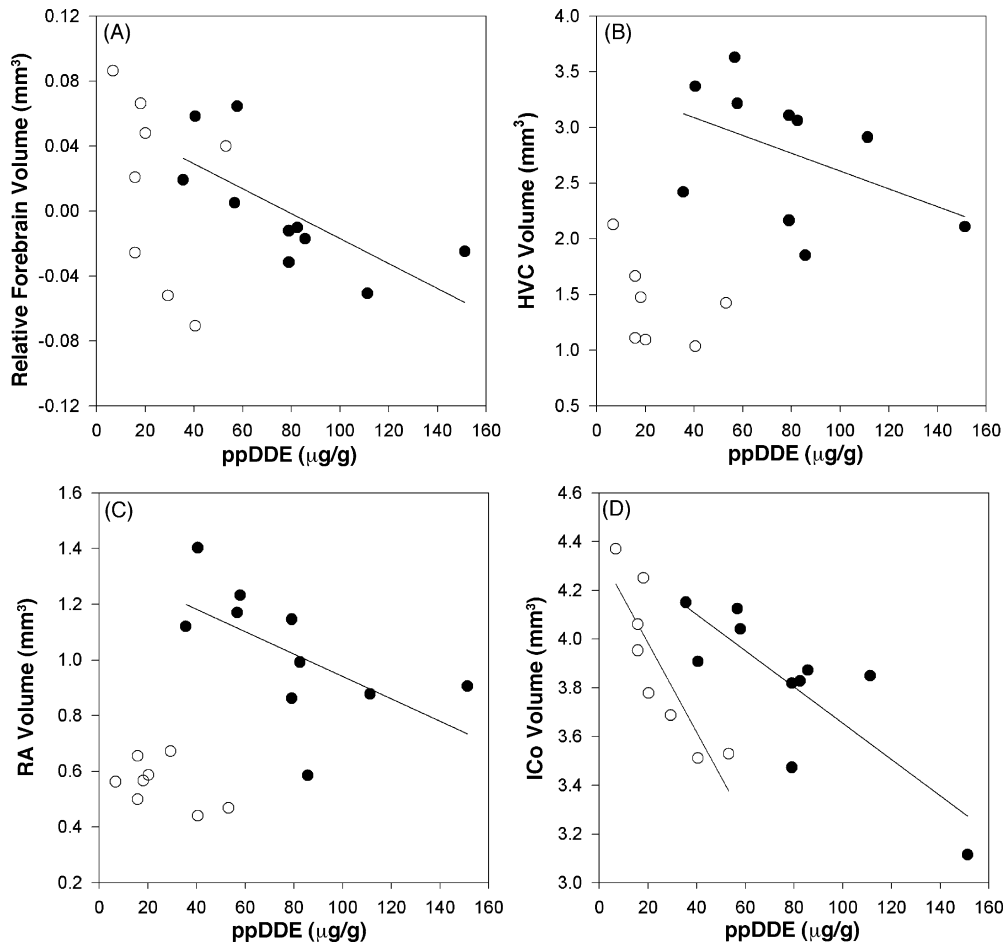


Fig. 5. Scatterplots of log-transformed volumes of (A) relative forebrain, (B) HVC, (C) nucleus robustus arcopallialis (RA) and (D) nucleus intercollicularis (ICo) plotted against *p,p'*-DDE (µg/g). The open circles indicate the females and the closed circles indicate the males. The solid lines indicate significant correlations.

### 3.3. Correlations with other POPs

No significant correlations between brain region volumes and other POP levels were detected (all  $P > 0.06$ ). Thus, nonachlor (both *cis*- and *trans*-), tris(4-chlorophenyl) methanol (TCPM) and polychlorinated biphenyl (PCB) levels were not significantly correlated with the volumes of any of the brain regions measured.

## 4. Discussion

Our results clearly indicate that early exposure to environmental levels of DDT has significant effects on the brains of American Robins. Relative forebrain and absolute song nuclei and ICo volumes were significantly reduced with increasing levels of DDT exposure in males and a similar effect only on ICo was present in females. Although our study was based on a relatively small sample size (total  $n = 18$ ), these effects are startling, particularly when the exposure to DDT was limited to embryonic and early post-hatching development and the birds were sacrificed in their second year. This suggests that whatever effects DDT is having on the brain during development, the effects are long-lasting. Whether these effects are mediated strictly by endocrine disruption or by other mechanisms, such

as an increase in stress hormones or direct neurotoxic effects is, however, uncertain.

Although we detected several significant correlations with DDT levels, we detected no such relationship between DDT levels and the septum. The septum plays a critical role in mediating social and agonistic interactions [36,37], which are impaired by DDT [88], and contains androgen and estrogen receptors [56], but it is composed of multiple nuclei that vary in their neurochemistry [36]. We were unable to delineate nuclei within the septum, but it is possible that DDT does affect the size of nuclei within the septum without causing a detectable effect at the level of total septal volume.

It is also worth noting that we primarily detected significant effects in males. The exposure levels of the males were not only significantly higher than the females, but also broader (males: 43.293–174.671 µg/g; females: 18.431–75.115 µg/g). Because we had no *a priori* knowledge of exposure levels or sexes of the nestlings (they were sexed after fledging), this difference in exposure levels was entirely by chance. Nevertheless, this sex difference in exposure levels could have led to the detection of more significant effects in the males because of a broader range of exposure levels. Alternatively, males may be more susceptible to DDT-induced changes in the brain because of sex differences in hormone sensitivity or because the females ‘dumped’

some of their contaminant load in eggs. We cannot, however, distinguish between these and other alternatives in our current sample.

Lastly, our analysis was constrained by assaying POP levels only during early development and we were unable to assay organs and tissues of the adult birds. Thus, how POP levels experienced *in ovo* correlates with levels in adult birds is uncertain. With the development of techniques to assay POP levels in feathers of adult birds [22,48], it will be possible in future studies to assess POPs and behaviour throughout development without the need for invasive or destructive sampling.

#### 4.1. Effects on brain and forebrain volumes

Although we did not expect to see an effect of DDT exposure on brain and relative forebrain volumes, it was nevertheless present. In males, higher DDT exposure levels were associated with smaller brains and relatively smaller forebrains. Such widespread changes throughout the brain being associated with DDT exposure levels could reflect endocrine disruption, but there are at least two additional reasons: stress and direct neurotoxicity.

DDT exposure can have significant effects on stress hormone levels. DDT can influence the adrenal glands indirectly *via* effects on the thyroid and pituitary glands [10,49], act as a stressor itself [61] and in some species can even act as a corticosterone agonist [43]. High levels of stress hormones impair neuronal survival [39] and are often associated with retarded neurobehavioural development [58]. In fact, developmental stress can result in significant changes in both the brain and behaviour of songbirds [14,58,76,77]. Thus, high stress levels precipitated by DDT exposure during early development could have led to small brain and relative forebrain volumes in the robins.

DDT can also have a number of direct neurotoxic effects. DDT reduces the expression of brain-derived neurotrophic factor (BDNF) [45], disrupts  $\text{Ca}^{2+}$  uptake in neurons [45] and interferes with sodium channels [57,82]. In addition, exposure to DDT during early development results in a decrease in muscarinic acetylcholine receptors and marked differences in spontaneous activity [27–29]. Given that muscarinic cholinergic neurons are found throughout the songbird brain [6], brain development is strongly dependent on BDNF [59] and the modulation of calcium and sodium channels disrupts signal transduction [45,57], these direct neurotoxic could have affected most of the brain and resulted in the observed decrease in brain and relative forebrain volumes.

#### 4.2. Effects on the song control system

The song control system of songbirds is extremely sensitive to hormonal manipulations. Lower levels of testosterone generally result in small song control nuclei and decreases in singing activity and song repertoire [5,40,41,65,86]. Given this sensitivity, we expected that DDT exposure would result in decreases in the volume of the song control nuclei. Our results indicate that increasing levels of DDT, and in particular the degradant *p,p'*-DDE, are correlated with decreases in the size of two song control nuclei:

HVC and RA. Although increases in stress and direct neurotoxicity could have affected HVC and RA in a similar fashion to the forebrain and brain as a whole (see above), an equally plausible explanation for these changes is endocrine disruption. Both HVC and RA contain relatively high concentrations of androgen receptors [32,56] and the volume of both regions increase with testosterone [2,7,40,80]. *p,p'*-DDE is a potent androgen receptor antagonist and inhibits androgen-induced transcriptional activity [51]. Robins exposed to *p,p'*-DDE are therefore analogous to experimental approaches where birds have been castrated and have correspondingly lower HVC and RA volumes [7].

The lack of a significant change in area X volume supports the hypothesis that RA and HVC volumes were affected by endocrine disruption. If the reductions in HVC and RA were primarily due to stress or direct neurotoxicity, we would have expected similar changes in area X as well as all of the control regions. However, no significant correlation was detected between either  $\sum\text{DDT}$  or *p,p'*-DDE levels and the volumes of these regions. Furthermore, area X lacks the androgen and estrogen receptors present in the other two nuclei [56] and does not undergo the same dramatic changes in volume with respect to the hormonal environment [7].

The functional implications of a relatively smaller HVC and RA can be inferred from experimental and comparative studies. In general, smaller HVC and RA volumes are associated with smaller song repertoires and decreased rates of singing [24,33,53,78]. Behavioural observations of some of the robins suggested that singing rates did decrease with *p,p'*-DDE levels [72], but there was an insufficient amount of data to provide a direct comparison with our measurements. However, based on the volumetric changes in HVC and RA with respect to DDT and *p,p'*-DDE levels, we would expect to find correlated changes in singing rate and song repertoire. A recent study on the effects of heavy metal pollution on the song of Great Tits (*Parus major*) found that higher levels of contamination were correlated with decreased song repertoires [38], so it is reasonable to suggest that similar qualitative changes in song repertoire also occur in the robins.

#### 4.3. Effect on the intercollicular nucleus

ICo contains relatively high levels of androgen and estrogen receptors in songbirds and nonsongbirds [56]. In a similar fashion to the song system, the volume of the dorsomedial part of ICo is correlated with testosterone levels [41]. The size of cells is dorsomedial ICo also respond to testosterone; higher levels of T are correlated with larger cells [9,60]. Although we were unable to reliably differentiate between dorsomedial and ventrolateral regions throughout the rostrocaudal extent of ICo in the robins, our study nevertheless found that the volume of the entire ICo and size of cells within the dorsomedial part were negatively correlated with both  $\sum\text{DDT}$  and *p,p'*-DDE levels. As with the song control nuclei, this suggests androgen antagonism of ICo by *p,p'*-DDE. Again, the reduction in ICo and ICo neurons could result from stress and/or direct neurotoxic effects, but if this were the case, there should also have been reductions in the adjacent MLd as well as other control regions.

Several behavioural studies have documented significant impairments and abnormalities in birds exposed to DDT that are at least partially mediated by ICo. One of the most commonly reported behavioural abnormalities is a decrease in courtship behaviours [88]. Although the precise function of ICo remains uncertain, it does play a significant role in courtship. ICo neurons are responsive to visual, auditory and tactile stimuli [8,17,69] and are activated during vocal displays and copulation [15,31,55]. As mentioned previously, lower testosterone levels are correlated with reductions in the size of dorsomedial ICo and neurons [9,41,60] as well as decreases in the frequency of courtship behaviours [1,7,68]. Thus, one potential outcome of a smaller ICo may be impaired courtship and related behaviours, which would support the behavioural abnormalities reported in previous studies [72,88]. This is not necessarily a unidirectional relationship either. For example, reductions or deficits in courtship behaviour resulting from endocrine disruption may have driven a decrease in ICo. Teasing these causal relationships apart is beyond the scope of the present study, but offers several avenues for future research.

#### 4.4. Conclusions

Environmental exposure to DDT early in development clearly results in significant neuroanatomical changes. The mechanism underlying these changes, however, remains highly speculative. Although there is a strong indication that endocrine disruption, and specifically androgen antagonism, has played a prominent role in these changes, we cannot rule out the possibly confounding effects of stress and direct neurotoxic effects. It should also be emphasized that regions other than those measured may also be affected by DDT exposure. Hypothalamic nuclei (*e.g.*, medial preoptic nucleus) and other regions of the song system (*e.g.*, tracheosyringeal portion of the hypoglossal nucleus (nXIIIts)) are not only connected to the song system and ICo [12,64,84,85], but are also androgen-sensitive [65,83] and mediate behaviours that are affected by DDT exposure, such as singing, nesting, courtship and parental care [72,88]. Furthermore, changes in these other regions could indirectly affect the song system by interfering with singing behaviour and/or their input to the song system and thereby affecting the song control nuclei. Regardless of whether these additional regions are affected or not, our results demonstrate environmental exposure to DDT during early development has a significant affect on the songbird brain. DDT is present in most environments [71] and persists for decades after use thus providing some degree of exposure to most humans and wildlife throughout the world. Our results suggest that this exposure needs to be monitored carefully because environmental exposure to DDT will not only lead to reproductive impairments, but could also lead to significant neurological changes in both humans and wildlife.

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