REPRODUCTIVE AND BEHAVIORAL EFFECTS OF TWO BROMINATED FLAME RETARDANTS IN CAPTIVE AMERICAN KESTRELS (*FALCO SPARVERIUS*)

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ABSTRACT

Brominated flame retardants (BFRs) are persistent organic pollutants that have reached a global distribution in the environment. Many of the BFRs are lipophilic and bioaccumulative, and consequently several of them have been found in wildlife tissue with some of the highest levels recorded in several raptor species. The overall aim of this study was to determine the effects of exposure to environmentally relevant levels of the two most prominent BFRs, polybrominated diphenyl ethers (PBDEs: DE-71 mixture) or hexabromocyclododecane (HBCD) on the reproductive success, behaviour and physiology of captive American kestrels. In 2007, males exposed in ovo to DE-71 at three mean egg exposure concentrations of 289 ng/g ww for the low-exposure males, 1131 ng/g ww for the high-exposure males, or background levels of 3 ng/g ww for controls, were paired with unexposed females, and euthanized in the following year for testes extraction. In 2008, kestrel pairs were exposed via diet to technical HBCD (0.32 μg/μl wet weight (ww) daily) or the vehicle only for controls; testis mass and histology was examined in an additional group of males exposed to the same concentrations for three weeks. Pairs with males exposed in ovo to DE-71 demonstrated decreased clutch size, and egg mass, reduced fertility, and delayed timing of egg-laying when compared to controls. Both members of these pairs displayed reduced courtship behavior, including copulation frequency, and males demonstrated reduced parental behavior compared to controls. The testes of males exposed in ovo to DE-71 were enlarged and contained more seminiferous tubules with lumen, though the number of tubules containing final spermatids decreased with increasing in ovo exposure to some PBDE congeners.

Additionally, testosterone levels were reduced during breeding in these males. Pairs exposed to HBCD via diet laid their clutches earlier and their average clutch size of eggs was larger, however no associated increase in reproductive success was noted. Though eggshell thickness was unaffected, HBCD-exposed females produced lighter and smaller eggs. Both members of HBCD-exposed pairs showed reduced courtship behavior and males additionally demonstrated reduced parental care. Males exposed to HBCD had elevated testosterone levels, reduced circulating thyroxin (T_4) and increased body mass at certain time-points throughout the breeding season. Unpaired males had larger testes than controls with more seminiferous tubules containing final spermatids. The results presented herein demonstrate that both of these BFRs affect reproduction in American kestrels. Since exposure levels in the present study were environmentally relevant, wild birds receiving similar exposure may experience comparable effects.

RÉSUMÉ

Les ignifugeants bromés sont des polluants organiques tenaces, répandus globalement dans l'environnement. Lipophiles et bioaccumulatifs, on les retrouve dans les tissus animaux, dont ceux des rapaces qui possèdent parmi les concentrations les plus élevées. Cette étude avait comme objectif global de déterminer les effets sur la reproduction, le comportement et la physiologie de crécerelles d'Amérique (*Falco sparverius*) captives exposées à deux ignifugeants brominés d'importance, soit les polybromodiphényléther (PBDE : mélange DE-71) et l'hexabromocyclodécane (HBCD), à des concentrations représentatives des niveaux environnementaux. En 2007, trois groupes de mâles exposés *in ovo* à des concentrations moyennes (± l'erreur-type) de DE-71 respectives de 288,60 ± 33,35 ng/g mh (faible exposition), de 1130,59 ± 95,34 ng/g mh (forte exposition) et de 3,01 ± 0,46 ng/g mh (contrôle), ont été accouplés avec des femelles non-exposées, et euthanasiés l'année suivante pour en extraire les testicules. En 2008, des couples furent exposés au HBCD dans leur alimentation quotidienne, soit à une concentration de 0.544 µg/µl mh ou à un niveau-contrôle. De plus, la masse et l'histologie testiculaires de mâles non-accouplés exposés pendant trois semaines à ces mêmes concentrations respectives furent analysées. Comparés aux couples-contrôle, les couples avec mâles exposés au DE-71 ont subi une réduction dans la grandeur de leurs couvées et dans la masse et la fertilité de leurs œufs, ainsi qu'un délai dans leur ponte. Chacun des membres de ces couples démontra une diminution des comportements nuptiaux, dont la fréquence de copulation, et les mâles ont fait preuve de soins parentaux réduits. Ces derniers avaient également des testicules hypertrophiés contenant un plus grand nombre de tubules séminifères pourvus de lumen, cependant le nombre de tubules

contenant des spermatides finales décrut en fonction de la croissance du niveau d'exposition à certains congénères PBDE. De leur part, les couples exposés au HBCD ont connu des pontes précoces comportant un plus grand nombre moyen d'œufs que les couples-contrôle, par contre leur succès reproductif global demeura inchangé. Bien que l'épaisseur des coquilles ne fut pas affectée, leurs œufs étaient anormalement petits et légers. Encore une fois, chacun des membres de ces couples démontra une baisse dans les comportements nuptiaux et l'apport de soins parentaux fut réduit chez les mâles. Ces derniers présentèrent également à certains moments des niveaux de testostérone et des masses corporelles supérieurs ainsi que des niveaux de thyroxine (T_4) inférieurs aux mâles-contrôle, et les mâles non-accouplés développèrent de plus gros testicules avec plus grand nombre de tubules séminifères contenant des spermatides finales. L'ensemble de ces résultats démontre que les deux ignifugeants bromés en question, à leurs niveaux environnementaux, agissent effectivement sur la reproduction chez les crécerelles d'Amérique. Il est donc admissible de supposer que les crécerelles à l'état sauvage, voire même autres oiseaux, éprouvent des impacts comparables.

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CONTRIBUTIONS OF COAUTHORS

This thesis is written in manuscript style and follows the format of the journal, Environmental Toxicology and Chemistry. Several people contributed to each of the four manuscripts presented in this thesis that have been or will be submitted for publication. The second chapter, titled *Multi-generational effects of polybrominated diphenylethers exposure: Embryonic exposure of male American kestrels (Falco sparverius) to DE-71 alters reproductive success and behaviors,* is published in Environmental Toxicology and Chemistry, and is co-authored by David Bird, Laird Shutt, Robert Letcher, Ian Ritchie and Kim Fernie. The third chapter, titled *Embryonic exposure to the polybrominated diphenylether mixture, DE-71, affects testes and circulating testosterone concentrations in adult captive American kestrels (Falco sparverius)***,** is co-authored by Sarah Kimmins, David Bird, Robert Letcher, Ian Ritchie, and Kim Fernie and a variation on this text has been accepted for publication in 2011 in Toxicological Sciences. The fourth chapter, titled *Dietary exposure to technical hexabromocyclododecane (HBCD) alters breeding behavior and reproductive success in captive American kestrel (Falco sparverius) pairs,* is co-authored by David Bird, Robert Letcher, Ian Ritchie and Kim Fernie the content of which will submitted in two manuscripts to Environmental Toxicology and Chemistry. The fifth chapter, titled *Diet exposure to technical hexabromocyclododecane (HBCD) affects testes and circulating testosterone and thyroxin levels in captive American kestrels*, and is co-authored by Sarah Kimmins, David Bird, Robert Letcher, Vince Palace, Ian Ritchie, and Kim Fernie and will be submitted to Environmental Toxicology and Chemistry.

All co-authors provided advice and expertise in their respective fields of research to make each chapter a collaborative effort and additionally made the following contributions, in no particular order. Robert Letcher provided the technical mixtures of DE-71 and HBCD and conducted the analysis of chemical concentrations in plasma and egg samples for all of the chapters. Ian Ritchie provided expertise and logistical advice for all data collection and matters pertaining to the study subjects for all of the chapters. Sarah Kimmins provided laboratory facilities and protocols for conducting histology for chapters 3 and 5. Vince Palace provided thyroid hormone analysis for chapter 5. Kim Fernie, Laird Shutt and David Bird provided planning and direction for the project, which encompasses this thesis. All of the above contributed to the editing of chapters specifically co-authored by them, but David Bird and Kim Fernie, as my thesis co-supervisors, contributed extensively to the editing of the entire thesis.

STATEMENT OF ORIGINALITY

This thesis contains four manuscript chapters of which I am the first author, having collected and analyzed the data and held the role of primary writer. This study fulfills the requirement of originality for the degree of *Doctor of Philosophy* in five respects:

1) This study is the first to determine the reproductive effects of embryonic exposure to polybrominated diphenyl ethers (PBDEs) in breeding birds.
2) This study is the first to examine the reproductive effects of hexabromocyclododecane (HBCD) exposure in breeding birds.
3) This study is the first to examine parental care as an endpoint for exposure to any brominated flame retardant in the laboratory.
4) This is the first study to examine avian testicular histology and testosterone levels with respect to any brominated flame retardant.
5) This is the first study to examine testicular histology of any raptorial species.

CHAPTER 1

GENERAL INTRODUCTION

Brominated Flame Retardants in the Environment and Wildlife

Brominated flame retardants (BFRs) have been used since the 1970s in commercial products including high-impact polystyrenes, textiles, foams, upholstery, electronics, insulation and building materials [2]. Seventy-five different BFRs are currently in use and approximately 204,000 tons were produced in 2001 [3]. Today, the three most widely used BFRs are Tetrabromobisphenol-A (TBBPA), which made up 59% of market demand for BFRs in 2001, which remains the most recent estimate to date, followed by polybrominated diphenyl ethers (PBDEs) at 27 % and hexabromocyclododecane (HBCD) at 8%; the remaining 6% is made up of a wide variety of other BFRs [3]. All three of these main BFRs are organic, halogenated compounds that contain varying numbers of bromine atoms. TBBPA is a reactive BFR, and thus is bound to the polymers in final products, however both PBDEs and HBCD are additive, meaning that they are mixed into the polymers but not bound. They are thus prone to slowly dissociate from end-products over their lifespan, and leach into the environment [2, 4]. As a result, PBDEs and HBCD have become the two most prominent pollutants among the BFRs.

PBDEs can have between 1 and 10 bromine atoms, giving rise to 209 possible variations (Fig. 1), and commercial products are mixtures of several congeners. The main mixtures in use, both currently and historically, are: 1) DE-71, which contains tetra-, penta-, and hexa-BDE congeners (Bromkal 70-5DE was the European equivalent), 2) DE-79, which contains tetra- and hexa-BDE congeners, and 3) deca-BDE, which contains mainly BDE-209 (97-98%) but also nona-BDEs [5]. Historically, the DE-71

mixture was the most widely employed until the voluntary global discontinuation of production and use in 2006; today, only the deca-BDE mixture is still employed. Hexabromocyclododecane (HBCD) contains 6 bromine atoms (Fig. 1) and the commercial mixture is made up of three isomers dominated by γ-HBCD (80%), followed by α-HBCD and β-HBCD (reviewed in: [2]). As of 2009, HBCD has been under review for regulation by the United Nations Environment Program's Stockholm Convention (http://chm.pops.int/default.aspx).

The chemical properties of HBCD and PBDEs predispose them to becoming persistent organic pollutants: they are stable with long half-lives and are capable of transport via air and water [6]. Though few production sites have, or do exist [3], the products containing them are distributed worldwide, contributing to their ubiquitous presence in the environment [7, 8]. Thus, their global distribution is likely due to the leaching from these end-products followed by their transport via air and water (e.g. [9, 10]). The PBDEs have been used for a longer period and in greater quantities than HBCD, and consequently are present in higher concentrations (e.g. [11]).

Due to their lipophillic nature, both PBDEs and HBCD bioaccumulate in animal tissue, making them important environmental pollutants with respect to wildlife health. Both BFRs have been detected in animal tissues worldwide, including taxa at low trophic positions such as invertebrates, frogs, fish (reviewed in: [7, 9]) and passerine birds [12]. As a function of their elevated trophic standing, predatory birds show some of the highest recorded levels of BFRs (reviewed in: [7, 9]) in marine [13-16], freshwater [13, 17, 18] and terrestrial food webs [15, 19-21]. Predictably, BFRs are found in wildlife living in highly contaminated urban areas, such as ospreys (*Pandion haliaetus*) in the Delaware

River [22] and common kestrels (*Falco tinnunculus*) in urban China [21]. However, they have also been recorded at lower levels in wildlife residing in remote areas such as - Atlantic kittiwakes (*Rissa tridactyla*) in the Canadian Arctic [11], confirming their transport far from initial sources.

Levels of PBDEs have shown exponential increases in many taxa [9], reflecting the trend seen in abiotic material [24]. The greatest increases in wildlife occurred in the mid-1980s to mid-1990s. While many avian species are now showing decreasing trends in PBDE residue levels [13, 16, 25], levels in certain populations in urban or industrialized areas are stable [18] or still increasing [13]. Studies outlining temporal trends in birds (particularly regarding penta-BDEs) show a doubling time of 10 years in peregrine falcons (*F. peregrinus*) in Greenland between 1986 and 2003 [23]; 5.7 years in herons and cormorants near the Vancouver area between 1979 and 2002 [13]; and 2.6 to 3.1 years in Great Lakes herring gulls (*Larus argentatus*) between 1981 and 2000 [26] (Fig. 2). The PBDE congeners most commonly found in biota are those which are most widely used and most highly bioaccumulative: BDE-47, -99, -100, -154, -153, -183 [27], all of which have been identified in the DE-71 PBDE mixture [5]. Debromination into lower congeners and other metabolites can occur during metabolism [28-30] or in the environment as a result of microbial activity [31] or by photodegredation [32].

HBCD is more of an emerging BFR, and was first detected in wildlife tissue in the 1980s with levels that have slowly risen since. For example, mean concentrations of α-HBCD in herring gull eggs in Norway rose sevenfold from 16 ± 9 ng/g to 108 ± 48 ng/g lipid weight (lw) between 1983 and 2003 [33] and concentrations of ΣHBCD in the eggs of black guillemots in the Baltic Sea rose four-fold from 34 to 140 ng/g lw between

1983 and 2001 [16] (Fig. 3). Though the technical mixture, and hence environmental contamination, of HBCD is dominated by γ-HBCD, α-HBCD is more prevalent in animal tissue. This is likely related to three factors: that it is more easily absorbed [34], is less readily metabolized [35], and bio-isomerisation can occur in vivo, where the β-HBCD and γ-HBCD are converted into the α-HBCD isomer [36].

Reproductive Implications of Exposure to PBDEs and HBCD

Endocrine disrupting potential of PBDEs and HBCD

Endocrine disruptors are defined as chemicals that alter the functioning of the endocrine system by various mechanisms, including binding to hormone receptors and altering the synthesis, transport and/or metabolism of hormones (reviewed in: [37]). Many environmental pollutants, including BFRs [38], show the potential to deregulate steroid hormone axes (sex and thyroid) [37]. To evaluate these potentials, a review of their affinities for hormone binding proteins (Table 1) and their endocrine effects in vivo is useful.

PBDEs are accepted thyroid disruptors [39]. Several congeners can bind competitively to the thyroid transport protein, transthyretin (TTR), causing inhibition [1, 40], and the metabolite, OH-BDE-47 is a strong thyroid receptor agonist [1] (Table 1). Exposure to PBDEs in vivo appears to increase liver elimination of thyroxin (T_4) [41, 42] and circulating levels of T_4 were reduced in exposed rats [41-49], birds [50], and fish [51, 52]. While less is known for HBCD, α- β-, and γ-HBCD are all moderate to strong thyroid receptor agonists [1, 53]. In vivo, circulating T_4 levels were reduced in exposed

laboratory rats (*Rattus norvegicus*) [48, 54] and rainbow trout (*Oncorhynchus mykiss*), which may be related to increased liver metabolism of T_4 [51]. In amphibians, metamorphosis, a process controlled by T_3, was inhibited with exposure to PBDEs in vivo (BDE-47 and DE-71: [55]) and ex vivo (BDE-206: [56]) and was potentiated with HBCD exposure in the same ex vivo tail tip assay [56], reflecting their contrasting in vitro affinity for thyroid receptors [1].

Both PBDEs and HBCD also demonstrate some potential to disrupt sex steroid pathways. Some PBDE congeners and all three isomers of HBCD are mild to moderate antagonists of progesterone receptors [1]. As well, some PBDE metabolites (OH-BDE-47), and α- and γ-HBCD are mild to moderately anti-estrogenic through competitive binding to receptors [1] (Table 1); BDE-100, -47 and the DE-71 PBDE mixture are also somewhat estrogenic [57, 58]. Some evidence for estrogenic effects have been noted in male rats exposed developmentally to 10 μg/kg BDE-99 on gestational days (GD) 1 - 18 by way of an increase in sweet preference, a sexually dimorphic trait normally seen in females and an accepted biomarker for estrogenic effects in the rat model [59]. However, overall, neither HBCD [54] nor PBDEs [38] appear to have strong effects on estrogenic pathways in vivo in rodents.

Some PBDE congeners additionally are androgen receptor agonists and the congener BDE-100 was found to be even more potent than the anti-androgenic drug, flutamide [1] (Table 1). Additionally, some PBDE metabolites (OH-PBDEs) can inhibit aromatase [60], the enzyme that converts testosterone to estrogen in the brain (reviewed in: [61]). Male rats exposed by oral gavage to minimum doses of 60 μg BDE-99 per kg body weight (bw) for 4 – 5 days as weanlings displayed a delay in the onset of puberty

and decreased mass of androgen-dependent organs, both being established biomarkers for anti-androgenic activity in the rat model [59]. The α- and γ-HBCD isomers are also moderate inhibitors of the androgen receptor in vitro [1], but to date in vivo evidence for their activity on this axis is lacking in rats [54].

Effects of PBDEs on reproduction

Hormones have a large impact on a number of physiological processes; thyroid hormones affect most systems and sex steroids are integral in the regulation of reproductive, immune and neurological systems [37, 62]. Through either or both of these processes, hormones may exert impacts upon the reproductive systems of animals. A number of endpoints can be used as indicators of endocrine disruption with regards to reproductive toxicity. These include, but are not limited to, gonadal morphology, circulating hormone levels, levels of neurotransmitters in the brain, and the timing of sexual maturation [63]. Reproductive behavior is also a useful marker because alterations in behavior are often the first noticeable change after chemical exposure, and are the manifestation of numerous physiological processes [62, 64].

The DE-71 PBDE mixture has a combination of estrogenic and anti-androgenic properties that could be particularly detrimental to reproductive behavior and fertility of males. The male reproductive tract has shown sensitivity to PBDE exposure in rats. Males exposed by oral gavage to minimum doses of 60 μg BDE-99/kg bw for 4 – 5 days as weanlings displayed a delay in the onset of puberty [47, 65] and decreased mass of ventral prostate and seminal vesicles [47]. Male rats exposed developmentally to BDE-99 at maternal dose levels of 60 or 300 μg/kg bw on GD 6 demonstrated reduced

epididymal mass relative to body mass at both dose levels, and testis mass at the higher dose level. These changes were coupled with permanent decreased spermatogenesis at both dose levels [66]. Male mice (*Mus musculus*) exposed to 500 mg/kg bw BDE-209 by oral gavage on post-natal days (PND) 21 and 70 had decreased mitochondrial membrane potential in sperm and reduced amplitude of lateral head displacement, though no morphological alterations were seen in testes histology [67]. Additionally, with developmental exposure to 1 mg/kg bw or 10 mg/kg bw on GDs 10 - 18 of BDE-99, male offspring demonstrated decreased circulating estradiol and testosterone as weanlings, which became more pronounced in adulthood [59]. Female rats appear to be less reproductively affected by exposure to PBDEs. However, exposed females also displayed a delay in the onset of puberty [47]. Moreover, a reduction in ovarian primary and secondary primordial follicles was noted at maternal dose levels of 1 mg/kg bw and 10 mg/kg bw on GD 10 - 18 and dams displayed reduced circulating levels of estradiol [59]. Overall, reproductive success and sexual behavior were not affected by exposure to BDE-99 at dose levels up to 300 μg/kg bw on GD 6 in rats, however, some reduction in male sexual motivation was noted [66].

Though reproductive behavior (copulation) and success are not altered in PBDE-exposed laboratory rodents [66], these endpoints have been affected in other animal models. Ranch mink (*Mustela vison*) exposed to 2.5 ppm DE-71, a level that did not affect reproductive success in rats, displayed complete reproductive failure [68]. American kestrels (*F. sparverius*) exposed via diet to DE-71 at two environmentally relevant concentrations of 2.5 μg or 12 μg, administered daily demonstrated altered courtship behavior, including copulation [69]. Reproductive success parameters were

affected at both exposure levels in the kestrels: egg-laying was delayed, fewer pairs laid eggs and eggs were smaller with thinner eggshells [70]. Additionally, fertility of eggs and offspring production was reduced compared to controls [70]. In the fathead minnow (*Pimephales promelas*), spawning was reduced with high exposure to mean concentrations of 3μg BDE-47 daily, with complete cessation occurring after 10 days of exposure. Males additionally demonstrated reduced body condition and a 50% decrease in mature sperm in the testes [71]. These results suggest that wildlife species may be more sensitive to reproductive effects resulting from exposure to PBDEs than laboratory rodents; despite the fact that dose levels are lower in the former, effects appear to be more pronounced.

Effects of HBCD on reproduction

Less is known about the effects of exposure to HBCD on reproduction in animals. In rats exposed to technical HBCD via diet, reproductive output and behavior were not affected even at high exposure levels up to 1363 mg/kg bw daily [54, 72-74]. Similar results have been noted for fish (*Platichthys flesus*) exposed via food and sediment to 8000 μg/g total organic carbon [75]. Adult male rats (F_0) exposed to technical HBCD had decreased epididymides and reduced sperm counts, and exposure at high doses of 1000 mg/kg bw [54] induced sperm lateral head displacement. Conversely, F_1 generation rats receiving developmental exposure at a maternal dose of 100 mg/kg bw daily of technical HBCD, followed by dietary exposure to the same amount during breeding, had increased testicular mass but no differences in gross histopathology of the testes, sperm count or sperm motility [54]. In the same study, follicle-stimulating hormone (FSH) was reduced

in F_0 males at high mean daily doses of 300 and 1000 mg/kg bw and F_1 males exhibited higher circulating levels of 5α-dihydrotestosterone (DHT) and increased testis mass at high dose levels of 300 mg/kg bw daily. However, these results were not consistent across generations, nor were they dose-dependent [54]. In another study [73], developmental exposure to technical HBCD at doses ≥1.3 mg/kg body weight per day resulted in decreased testicular mass without affecting circulating thyroid hormone levels in either sex. The contrasting results of these studies were attributed to the differing rat strains [54]. In females, the number of ovarian primordial follicles was reduced in rats with developmental exposure at high maternal dose levels of 1142 mg/kg bw, and dams demonstrated reduced circulating FSH [54].

The effects of exposure to HBCD in birds have not been thoroughly investigated. In previous research with American kestrels exposed via diet to two environmentally relevant levels of DE-71 of 36 µg or 180 µg per day, some accidental exposure to small amounts of HBCD occurred at mean egg concentrations of 3.27 or 15.67 ng/g wet weight in eggs, respectively [69, 70]. The concentrations of HBCD in this study were associated with decreased eggshell thickness and delayed egg-laying dates in diet-exposed females [70]. Additionally, chicken embryos exposed to 100 or 10,000 ng/g HBCD via injection into the egg's air sac demonstrated decreased hatching success with only 35% and 64% of embryos successfully hatching, respectively, suggesting some embryo toxicity at these high doses [76]. Similar to rodents, however, reproductive output and behavior were not affected in European flounder (*Platichthys flesus*) exposed via food and sediment (8000 µg/g total organic carbon [75]).

Effects PBDEs and HBCD on parental care

No laboratory studies have been conducted on the effects of BFRs on parental care in birds or mammals, or on its endocrine regulation by prolactin. Reductions in brood-related behaviors have resulted in decreased growth and survival of chicks, which can ultimately inhibit the reproductive success if one or both parents are exposed to a toxicant [77]. In wild glaucous gulls (*L. hyperboreus*), circulating concentrations of PBDEs were correlated with decreases in baseline prolactin levels in males [78]. Additionally, plasma levels of other organohalogens have been associated with reduced nest attentiveness (DDT [79] and PCBs [80]) and defense (DDT: [81]) in wild birds. In the laboratory, dietary exposure daily to 10 – 400 μg/kg bisphenol-A, an estrogenic endocrine disruptor, caused female mice to spend less time in the nest, lactating their pups [82] and performing other maternal behaviors [83]. In birds, doves (*Streptopelia ristoria*) exposed to an environmentally relevant mixture of organohalogen pollutants (including PCBs, DDE, etc.) spent less time feeding and brooding young and had altered prolactin levels [84]. These findings suggest that the investigation of such endpoints in relation to BFR exposure is warranted.

The American Kestrel as a Toxicology Model

Birds are more sensitive to toxicant exposure than mammals, as emphasized by the above review, and thus it is important that they undergo toxicity testing to model the potential effects in wildlife [85]. Birds differ from classic mammalian models in many respects. Generally, these include high rates of food consumption and metabolism and naturally and regularly occurring periods of starvation where lipids are metabolized [86].

Fundamental reproductive differences exist, including seasonal breeding, highly ritualized and hormone-controlled behaviors, an oviparous method of reproduction, and the need for external sources of food for the initial provisioning of the young [63, 86]. Additionally, birds have relatively smaller livers and fewer detoxification enzymes, making them less adept at metabolizing and removing toxins from the system [87]. The reproductive physiology of birds is complex, requiring the neuroendocrine integration of properly timed social, environmental, and physiological cues for reproductive success [88, 89]. Several of these processes are reliant on steroid hormone regulation and henceforth, may be susceptible to disruption from chemical such as PBDEs and HBCD.

Raptorial birds, given their high trophic standing, are at particular risk of accumulating some of the highest contaminant residues for which PBDES and HBCD are examples [7, 9]. American kestrels are excellent toxicological subjects for modeling the effects in wild predatory birds [90]: they are small, easy to maintain, and breed successfully in captivity. Additionally, large colonies have been kept for almost four decades at the Avian Science and Conservation Centre (ASCC) of McGill University in Montreal and the Patuxent Wildlife Research Center in Laurel, Maryland by the U.S. Geological Survey [90]. As such, kestrels have been used to investigate the effects of several organic pollutants including DDT/E [91, 92], PCBs [93-96] and PBDEs on various endpoints [50, 69, 70, 97].

The American kestrel is a small migratory North American raptor that breeds seasonally [98], for which the fertile period stretches from mid-March to mid-May [99] (Fig. 4). Kestrels display reversed sexual dimorphism, females being larger, and both sexes engage in similar courtship behaviors [100]. They are socially monogamous and

mate with a new partner each year [98]. Copulations occur at a high frequency and begin before a tight pair-bond is formed, which may be integral in inducing female readiness to breed [101]. A complete clutch is usually four or five eggs, laid at two-day intervals [98, 102]. Females conduct the majority of incubation (80%), though both sexes develop a brood patch [100]. Kestrel hatchlings are semi-altricial and males supply all the food during the first 7 to 10 days while the female remains in the nest to thermoregulate the young; afterwards both sexes provision the brood [101]. In captivity, the courtship period usually lasts from 10 days to 4 weeks, incubation lasts 27-29 days and the nestling rearing phase is 26-32 days; once chicks fledge from the nest, the parents feed them for another 2-3 weeks [102, 103]. American kestrels are currently demonstrating unexplained population declines in the wild at sites with historically consistent occupation [104-106].

In comparison to classic animal models such as laboratory rats, mice, and quail, our knowledge of the physiology of American kestrels with respect to toxicological markers is sparse. However, the value in using the kestrel for toxicological research is unparalleled in that it is the only captive falcon species that can be housed and bred in sufficient quantities [90]. Thus, it is the only avian model available that is closely related to other raptorial species demonstrating the highest levels of toxic residues in the wild, such as the peregrine falcon [107]. The standard gallinaceous avian models, the Japanese (*Coturnix japonica*) and bobwhite quail (*Colinus virginianus*), may not be ideal avian models for raptorial birds, because their natural history and physiology differs in so many fundamental respects. On the whole, birds of prey have longer life spans, carnivorous diets, longer molt cycles and migratory life strategies. With respect to reproduction

specifically, birds of prey, in contrast to one or the other of these quail, display reversed sexual dimorphism, annual breeding, a longer period between hatching and first reproduction, monogamous pair bonding, and semi-altricial young [98, 108-110].

To model natural conditions of wild birds as closely as possible, one- or two-generation reproductive studies in kestrels are subject to a number of constraints in comparison with studies using laboratory rodents or quail. These include the following: 1) they require more space for successful breeding; 2) all individuals must be bred in-house as there are no other readily available sources from which to obtain substantial amounts of new stock; 3) kestrels are highly sensitive to disturbance within their enclosures, limiting the number of times the pair or brood can be removed for sampling in order to minimize cannibalism of eggs and young and heightened stress levels; 4) kestrels breed seasonally once a year under natural photoperiods, and F_1 young require one year to mature, thus 2-generation studies are two years in length; and 5) due to the complete seasonality of this species, gonads and gametogenesis in adults can only be examined during the three months comprising the fertile period.

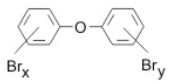
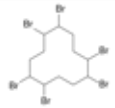
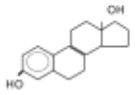
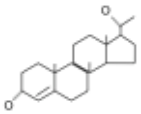
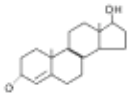
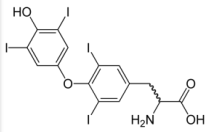
Research Aim and Objectives

Chemicals with sub-acute toxicity that ultimately affect endocrine, physiological and behavioral endpoints, which in turn influence the reproductive success of individuals, are of concern for the stability of wildlife populations [111]. For birds in particular, reproductive physiology is complex, requiring the neuroendocrine integration of properly timed social, environmental, and physiological cues for successful production of young [88, 89]. Chemicals capable of disrupting these functions at any level may

result in reduced breeding success. Both PBDEs and HBCD have demonstrated endocrine disrupting potential and alter a number of reproductive parameters in rodents, fish and birds in the laboratory. Both of these BFRs have shown increasing concentrations in wildlife tissues globally over the last 20 years, and little is known about how they affect birds. Thus it is critical to determine the reproductive effects of exposure in raptorial birds in particular, because they have shown reproductive and population sensitivity to organohalogen contamination in the past [107].

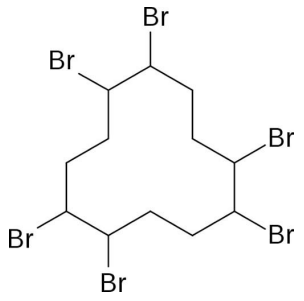
The overall aim of this study was to determine the effects of exposure to environmentally relevant levels of PBDEs and HBCD on reproduction in captive American kestrels. The first specific objective was to evaluate the effects of developmental exposure to DE-71, which was accomplished by examining the reproductive success and behavior of exposed males paired with unexposed females (Ch. 2), as well as testicular physiology and related endocrinology in these same males (Ch 3.). The second specific objective was to determine the effects of dietary exposure to HBCD, accomplished by examining the reproductive success and behavior of exposed male and female pairs (Ch. 4), and again testicular physiology and endocrinology in a second set of males (Ch. 5).

Table 1: Endocrine disrupting potential of PBDEs and HBCD as determined by in vitro profiling by Hamers et al. [1].

	 **PBDEs**		 **HBCD**	
	Action	**Potency**	**Action**	**Potency**
 **Estrogen** (ER-CALUX)	*Agonist*	47: **2** 100: **3**	*Antagonist*	α: **3** β: **2** γ: **3** TM: **2**
	Antagonist	OH-47: **4**		
 **Progesterone** (PR-CALUX)	*Antagonist*	47: **2** OH-47: **3** 99: **2** 100: **3**	*Antagonist*	α: **3** β: **2** γ: **3** TM: **3**
 **Testosterone** (ER-CALUX)	*Antagonist*	47: **3** OH-47: **3** 99: **3** 100: **5**	*Antagonist*	α: **3** β: **2** γ: **3** TM: **2**
 **Thyroid** (T-Screen)	*Agonist*	47: **2** 100: **3**	*Agonist*	α: **4** β: **3** γ: **4** TM: **3**
	Antagonist (TTR-binding)	OH-47: **4**		

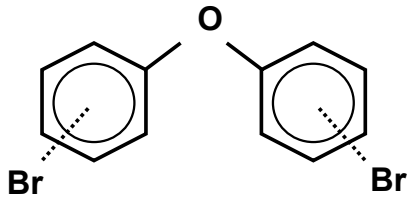
The relative affinities for hormone receptors were determined with the use of CALUX and T-Screen bioassay. For PBDE thyroid antagonism, the potency for the transport protein transthyretin was determined with a TTR-binding bioassay. The potency is ranked on a scale as classified by the authors of the research, where 2 is mild, 3 is moderate, 4 is strong, and 5 is a very strong effect [1]. Chemicals are classified as an antagonist if there is an inhibitory effect, and as an agonist if there is potentiation of the pathway in question as determined by the authors [1]. For the polybrominated diphenyl ethers (PBDEs), the potencies of the top three congeners found in the DE-71 mixture (BDE-47, -99, and -100) as well as the hydroxylated metabolite of BDE-47 (OH-47) are depicted. For hexabromocyclododecane (HBCD), the potencies of all three isomers (α, β, and γ) and that of the technical mixture (TM) are depicted.

Hexabromocyclododecane
(HBCD)



$C_{12}H_{18}Br_6$

Polybrominated diphenyl ethers
(PBDEs)



$C_{12}H_{10-x}Br_x$

$1 < x < 10$

Fig. 1: Chemical structure and formula for hexabromocyclododecane (HBCD) and polybrominated diphenyl ethers (PBDEs).

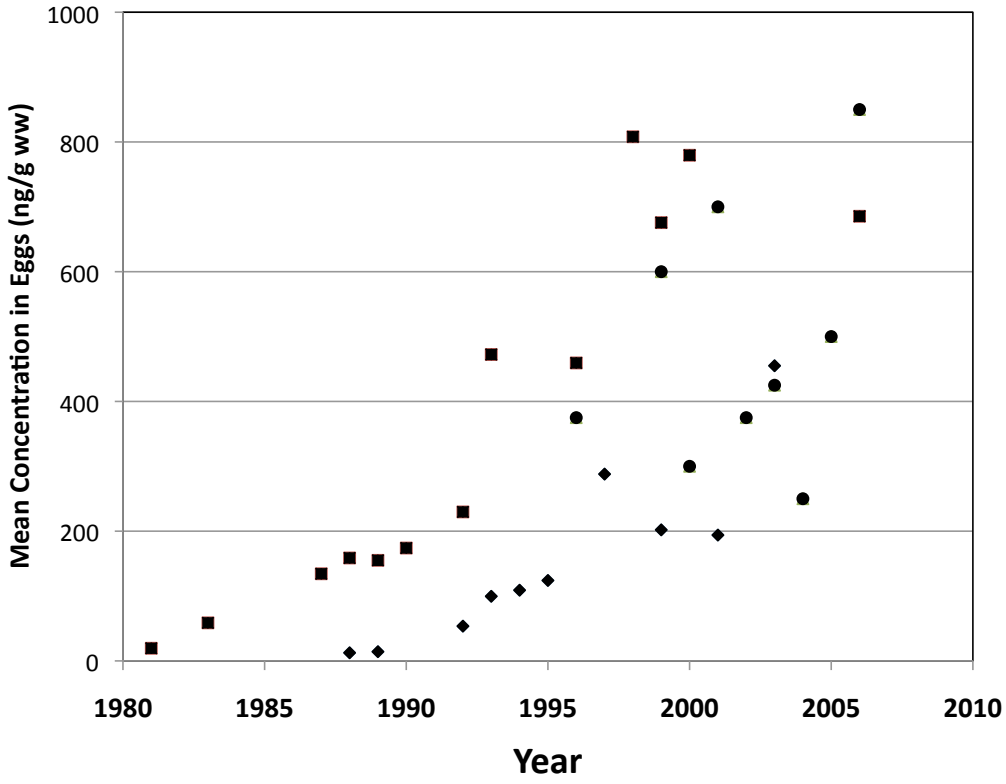


Fig. 2. Temporal trends in the levels of PBDEs in the eggs of predatory birds. Sum concentrations (ng/g wet weight egg) in the eggs of peregrine falcons (*Falco peregrinus*) in the US (●) [20], herring gulls (*Larus argentatus*) from the great lakes (■) [26] and great blue herons (*Ardea herodias*) from Vancouver, Canada (◆) [13] are shown.

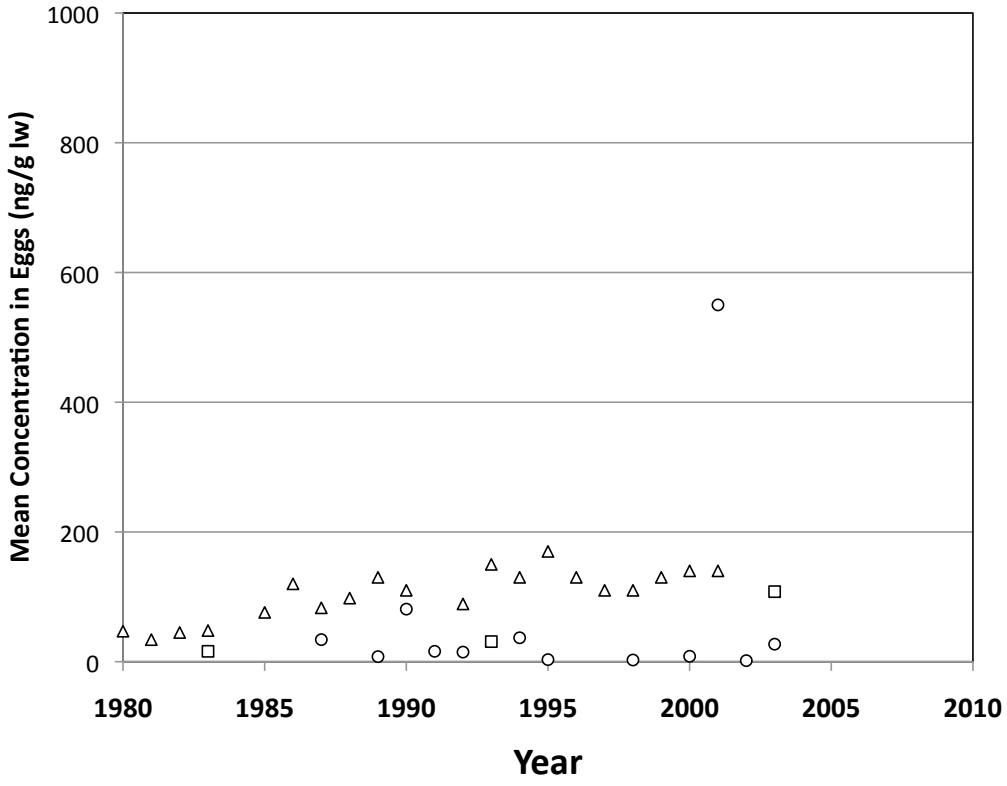


Fig. 3. Temporal trends in the levels of HBCD in the eggs of predatory birds. The sum or α-HBCD concentrations (ng/g lipid weight egg) in the eggs of peregrine falcons (*Falco peregrinus*) from Greenland and Sweden (○) [23, 112, 113], herring gulls (*Larus argentatus*) from Norway (□) [33] and guillemots (*Uria algae*) from the Baltic Sea (△) [16] are shown.

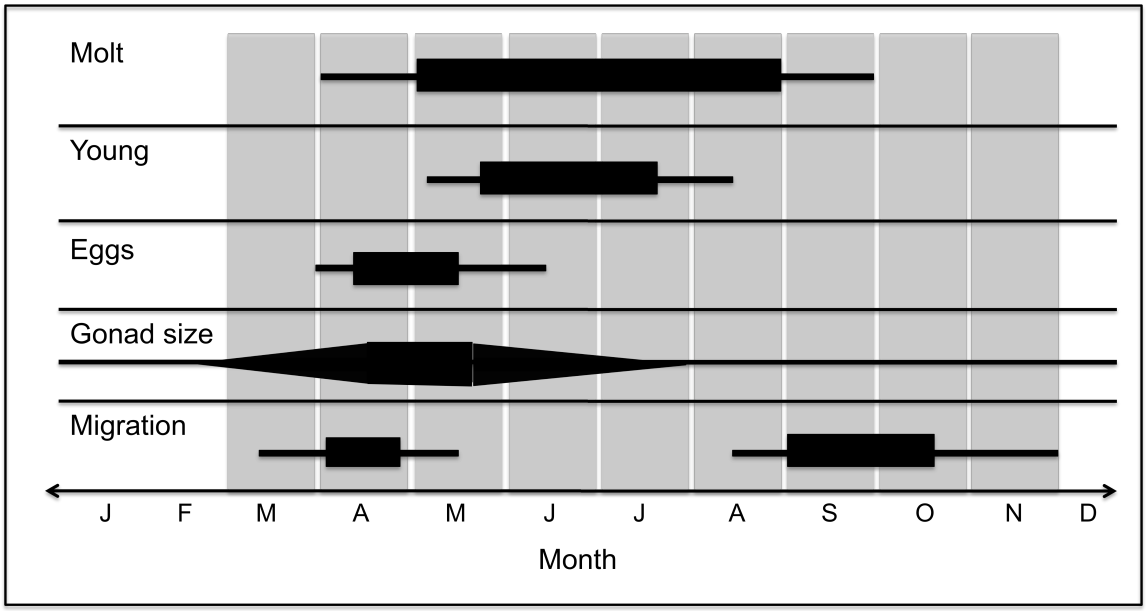


Fig. 4: Annual breeding cycle of the American kestrel (*Falco sparverius*). Migration, egg, young and molt data are modified from [98], depicting the timing of the majority of individuals (■) as well as the extremities (-). Gonad size is extrapolated from that known for a closely related species, *Falco tinnunculus,* in Great Britain [114] where the relative size is depicted by the thickness of the bar.

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CONNECTING STATEMENT 1

Historical research at the Avian Science and Conservation Centre of McGill University in Montreal in 2005 and 2006 examined the dietary effects of environmentally relevant levels of the DE-71 PBDE mixture on reproduction and behavior of American kestrels in comparison to controls. Among the various findings, reproductive success was reduced and courtship behavior was altered. The need for determining whether similar endpoints would be affected in the F_1 generation was identified. Exposure to DE-71 may be particularly important for males, given its combined mild estrogenicity and strong anti-androgenic potential. Therefore, male offspring from the previous diet-exposed study pairs, exposed in ovo to DE-71 by direct maternal transfer to the egg only, were paired with unexposed females to isolate the effect of exposure to the male. In the following chapter, the reproductive success and behavior of these pairs is presented.

CHAPTER 2:

MULTI-GENERATIONAL EFFECTS OF POLYBROMINATED DIPHENYL ETHERS EXPOSURE: EMBRYONIC EXPOSURE OF MALE AMERICAN KESTRELS (*FALCO SPARVERIUS*) TO DE-71 ALTERS REPRODUCTIVE SUCCESS AND BEHAVIORS

Marteinson, SC, Bird, DM, Shutt, JL, Letcher, RJ, Ritchie, I, Fernie, KJ. 2010. Multi-generational effects of polybrominated diphenyl ethers exposure: Embryonic exposure of male American kestrels (*Falco sparverius)* to DE-71 alters reproductive success and behaviors. *Environmental Toxicology and Chemistry.* 29 (8): 1740 - 1747.

Abstract

Polybrominated diphenyl ethers (PBDEs) are additive flame retardants that are environmentally persistent and bioaccumulative compounds of particular concern to species at high trophic levels including predatory birds. The developmental effects of in ovo exposure to male birds at environmentally relevant levels of the PBDE technical mixture, DE-71, on reproductive success and behaviors using captive American kestrels (*Falco sparverius*) were determined. Males were exposed in ovo by direct maternal transfer to DE-71 and unintentionally to low concentrations of hexabromocyclododecane (HBCD) at three mean ± standard error DE-71 concentrations of 288.60 ± 33.35 ng/g wet weight (low-exposure), 1130.59 ± 95.34 ng/g wet weight (high-exposure), or background levels of 3.01 ± 0.46 ng/g wet weight (control). One year following exposure, males were paired with unexposed females. Reproductive success was lower in the high male exposure pairs with 43% failing to lay eggs while all other pairs laid complete clutches. They also laid smaller clutches and produced smaller eggs with reduced fertility, parameters that were negatively correlated with paternal in ovo concentrations of all PBDEs as well as individual congeners and HBCD. Throughout courtship, there were fewer copulations by all in ovo exposed males, fewer mate-calls made by high-exposure males, and decreasing trends in pair-bonding and nest-box behaviors across treatments that continued during brood-rearing. The reductions in clutch size and fertility were associated with the reduced frequencies of male courtship behaviors, and were associated with increasing concentrations of the PBDE congeners BDE-47, -99, -100, -53, -138 and HBCD. The results of the present study confirm effects noted in the F_0 generation and

demonstrate that exposure to DE-71 affects multiple generations of this predatory avian species at environmentally relevant levels of exposure.

Introduction

Polybrominated diphenyl ethers (PBDEs) are added to many commercial plastics and household materials, and have been a widely used class of flame retardants since the 1970s. The penta-BDE technical mixture, DE-71, was used the most extensively until its global voluntary discontinuation of production and use in 2006. The DE-71 mixture is comprised of the congeners BDE-99 (44%), BDE-47 (38%), BDE-100 (13%), and BDE-153 (5.5%) [1], and these congeners are some of the most prominent PBDEs in the environment, wildlife and humans [2]. The PBDE congeners that comprise the penta-BDE mixture are generally stable in the environment, degrade slowly, and are now ubiquitous worldwide, and though the penta-BDE mixture is no longer in use, it continues to enter the environment by leaching out of end products (reviewed in T.A. McDonald [3]).

Polybrominated diphenyl ethers are lipophilic and bioaccumulative and the highest levels have been recorded in several avian top predators including the common kestrel, *Falco tinnunculus,* in which concentrations averaged 12 300 ± 5 540 ng/g lipid weight in muscle [4]. A doubling time of 4.9 to 8.7 years (1981 to 2006) has occurred in eggs of the Great Lakes herring gull, *Larus argentatus,* in which average egg concentrations ranged from 321 to 1191 ng/g wet weight [5] with the most dramatic increases occurring in the 1990s and early 2000s. For the penta-BDE derived congeners some avian species (and in their eggs) are now showing decreasing trends [5,6].

However, as of 2005 to 2006, PBDE levels from some avian populations in closer proximity to urban or industrialized areas may be stabilizing [6], while other species are still demonstrating increasing or non-decreasing temporal trends, including the peregrine falcon (*F. peregrinus*) [7], which is another *Falconidae* species like the American kestrel.

Polybrominated diphenyl ethers and/or their degradation products are now classified as endocrine disruptors, and exposure to PBDEs has numerous reproductive physiological repercussions. In vitro studies have shown that DE-71 [8], some of its individual congeners, and their hydroxylated metabolites [9, 10] have mild estrogenic effects. The DE-71 technical mixture [11] and several of its individual congeners, are also potent anti-androgens [10]. Developmentally exposed male rodents demonstrated a delay in the onset of puberty [11] and a decrease in androgen-dependent reproductive organ weights following developmental exposure to BDE-99 [12] and DE-71 [11]. Male rats exposed developmentally to BDE-99 showed an increase in sweet preference, a sexually dimorphic trait normally seen in females, which may indicate estrogenic effects [13]. Thus, DE-71 has a combination of estrogenic and anti-androgenic properties that may be detrimental for the reproductive behavior and fertility of developmentally exposed males.

Changes in endocrine function can affect reproductive behaviors integral to avian reproductive success. Avian courtship behaviors and reproduction are governed by testosterone, estrogen, and thyroid hormones and PBDEs interfere with all three of these hormones [8-13]. In American kestrels (*F. sparverius*) specifically, decreased circulating thyroxin [14], decreased courtship behaviors [15] and decreased reproductive success

[16] have been noted in kestrels exposed via diet to environmentally relevant concentrations of DE-71. Avian parental care is regulated by prolactin, and while no laboratory studies have been published on the effects of PBDEs on this axis, PBDE levels have been correlated with decreased circulating prolactin in wild male glaucous gulls, *L.arus hyperboreus* [17].

The objective of the present study is to determine the reproductive effects of embryonic developmental (in ovo) exposure to DE-71 on adult male birds. Specifically, reproductive success, courtship and parental behaviors are examined in American kestrels. Their in ovo exposure levels (mean 288.60 ± 33 ng/g and 1130.59 ± 95.34 ng/g wet weight in eggs) are environmentally relevant, mimicking in ovo exposure concentrations reported in wild peregrine falcons [7] and other birds [4, 5]. To the best of the authors' knowledge, the present study is the first to determine the multi-generational effects of exposure of PBDEs, including in ovo exposure in birds, and it is the first to address their effects on parental care in a controlled study.

Methods

This study used captive-bred American kestrels (*Falco sparverius*) with documented histories of all individuals housed at McGill University (Montreal, QC, Canada). Paired birds were placed in breeding chambers with a nest-box and one-way glass window for observation. Birds were subject to natural temperature fluctuations and photoperiod, and fed untreated, day-old frozen-thawed cockerels (*Gallus domesticus*) ad libitum prior to daily behavioral data collection. The treatment and care of the kestrels

was conducted in accordance with the Canadian Council on Animal Care Guidelines [18] and was approved by the Animal Care Committee of McGill University.

In 2007, 21 breeding pairs were grouped according to the males' embryonic exposure to DE-71. One treatment group involved male kestrels exposed embryonically to higher concentrations of DE-71 (n_H = 7), and another group consisted of males exposed embryonically to relatively lower concentrations of DE-71 (n_L = 7) as defined by the exposure categories of their parents [15,16], and labeled herein as high-exposure and low-exposure accordingly. A third group of controls (n_C = 7) was used for comparison. The pairs consisted of unexposed females and one-year-old male kestrels, exposed in ovo either to the control vehicle (safflower oil) or to DE-71 and unintentionally to low concentrations of another brominated flame retardant (BFR), hexabromocyclododecane. These males were the F_1 progeny of the 2006 dietary exposure F_0 study subjects [15]. The parents of treated males had been exposed to one of two environmentally relevant levels of DE-71 found in Great Lakes herring gull eggs [5, 19] and European peregrine falcons [20]. Parental exposure involved injecting DE-71 into their food source (18 μg per individual) at a concentration of 0.658 μg DE-71 per μl of safflower oil for high-exposure parents or 0.140 μg/μl for low-exposure parent birds; controls were exposed only to the vehicle (for details see Fernie et al. [15]). These F_0 birds were exposed as described from three weeks before pairing through incubation (average of 75 d) until the first chick hatched. Thus, the in ovo exposure subjects from this study were never exposed by diet, but only during the 28-d embryonic period via direct maternal transfer to the egg.

The first egg from the same clutch in which study males hatched (sibling egg) was analyzed for PBDE concentrations as a determinant of approximate embryonic exposure. Female European starlings (*Sturnus vulgaris*) exposed to PBDEs via silastic implants laid eggs with increasing concentrations across their 5-egg clutches, and with increasing variation in PBDE concentrations of the latter laid eggs [21], although the sample size was small (n = 3). Thus, the approximate exposure levels of sibling eggs may underestimate the in ovo exposure levels of the present study subjects. Mean ΣPBDE concentrations found in these eggs were 3.01 ± 0.46 ng/g wet weight for controls, 288.60 ± 33.35 ng/g wet weight for low-exposure and 1130.59 ± 95.34 ng/g wet weight for high-exposure males; detailed descriptions of the chemical analysis and concentrations of PBDEs and HBCD in these eggs are described elsewhere [15, 16]. All 14 monitored congeners were detected in the sibling eggs of in ovo exposed males, in the following proportions: BDE-99 (43%), -153 (18%), -100 (15%), -47 (9%), -154 (12%), -85 (1.3%), and the remaining 2.5% consisted of BDE-66, -138, -49, -190, -209, -28, -17, -183 in decreasing order. Hexabromocyclododecane (HBCD) was not expected to be present in the egg samples and was likely an exposure artifact during the dosing of the birds; the concentrations found in the eggs were low, 0.002 ± 0.002 ng/g wet weight for controls, 3.27 ± 0.68 ng/g wet weight in low-exposure eggs and 15.61± 2.63 ng/g wet weight in high-exposure eggs [15,16]. Total-HBCD was not detected (<0.001 ng/g) in the safflower oil vehicle, nor in the technical mixture [16].

Throughout the breeding season, all reproductive parameters were recorded, including the dates of laying, hatching and fledging for each chick. All eggs were weighed when first laid, and length and width measurements were recorded with the use

of digital calipers, from which volume was calculated [22]. All eggs were candled at mid-incubation to determine their fertility by the presence of an embryo and/or blood veins. Adult males were weighed on a weekly basis.

Reproductive measures of the pairs were calculated following Fernie et al. [15] and include: Julian lay date (number of days from pairing to egg laying), clutch size (number of eggs in the first clutch), fertility (the total number of fertile eggs in the clutch) and percent fertility (percent of eggs that were fertile per pair), hatching and fledging success (the total number of hatchlings or fledglings produced per pair), and overall reproductive success (the number of eggs that produced fledglings).

Reproductive courtship behaviors were recorded from the second day after pairing until incubation of the eggs began. Brood-rearing behaviors of all pairs producing young were recorded for 20 days after the first chick hatched. Each pair was observed in random order, twice daily (morning, afternoon) during courtship, and once daily (morning) during brood-rearing to minimize disturbance. Their proximity to each other, the incidences of courtship and brood-rearing behaviors (copulation, nest-inspections, food-transfers, bonding-displays, flight-displays, vocalizations) [23], activities (feeding, perching, flying, preening), and their location within the pen, were recorded.

Statistical analysis

Mean behavioral frequencies were calculated for each individual bird (total number of incidences divided by the number of behavioral samples) for the entire courtship period, the 9 d prior to egg-laying, and during brood-rearing. For pairs that

never laid eggs, the latest lay-date defined their entire courtship period and they were excluded from other analysis. Data were tested for normality and homogeneity of variance, and transformed appropriately when necessary. Differences among treatment groups in reproductive and behavioral measures were analyzed by one-way analysis of variance (ANOVA), followed by pair-wise comparisons of the least square means (LSM). When data were not normal, Kruskal-Wallis tests were used. The number of F_1 pairs successfully raising chicks was extremely small (n_C = 4, n_L = 5, n_H = 3 pairs), thus, sample size was increased by including 8 additional captive control pairs from 2008 in the analyses, where exactly the same method of data collection was used and no statistical differences were found between the two groups. Additionally, the F_1 low- and high-exposed pairs were pooled together into a general exposure category (n = 7) after determining that their brood- rearing behaviors were statistically similar. The differences between controls and males receiving some exposure were analyzed by Mann-Whitney U tests. Spearman's Rank correlation analyses were conducted between F_1 paternal in ovo PBDE concentrations, male behaviors and significant reproductive parameters to determine the possible correlations among in ovo exposure levels, male parental behavior and reproductive success. Significance was considered to be at $p \leq 0.05$.

Results

Courtship behavior

Throughout the present study, the adult male kestrels were similar in body weight ($p \geq 0.240$). However, the in ovo DE-71 exposed pairs performed fewer courtship

behaviors (Table 1), including lower copulation rates (p = 0.002) (Fig. 1) that were strongly and negatively associated with the males' embryonic exposure to concentrations of ΣPBDEs (Fig. 2), individual PBDE congeners and HBCD (Table 2). Compared to the controls, the high-exposure males also made fewer chitter-calls (LSM $P_{C\text{-}H}$ = 0.020) and whine-calls (LSM $P_{C\text{-}H}$ = 0.057) (Table 1), which were negatively associated with the males' in ovo exposure to ΣPBDEs, various individual PBDE congeners, and HBCD (Table 2). During the 9 d prior to laying, a period critical to fertility and egg laying [24], the high-exposure in ovo males continued to copulate less often (X^2 = 5.65, p = 0.059) and their female mates made fewer whine-calls (X^2 = 6.36, p = 0.042) compared to the controls.

The amount of time the males spent in their nest-boxes decreased significantly with increasing embryonic exposure to BDE-99 and -100 ($p < 0.046$) (Table 2), which is reflected by the comparison between controls and high in ovo exposed males (LSM $P_{C\text{-}H}$ = 0.055). Similarly, the number of food-transfers between mates was significantly and negatively associated with the males' in ovo exposure to ΣPBDE levels (p = 0.049) (Table 2) and marginally differed among the groups (X^2 = 5.539, p = 0.063). Conversely, males exposed in ovo to DE-71 performed more flight-displays (Table 1) which were negatively associated with their embryonic exposure to concentrations of BDE-49 (p = 0.026) and positively associated with BDE-209 levels (p = 0.032) (Table 2).

Changes in the courtship behaviors of males were associated with behavioral changes in their mate. Females paired with high in ovo DE-71 exposed males made fewer whine-calls than controls (p = 0.012) (Table 1) and this was associated with fewer

whine-calls by their males (r_s = 0.60, p = 0.005) and fewer food-transfers (r_s = 0.56, p = 0.009). As there were declines in male courtship behaviors, females participated in fewer pair-bonding displays and nest-inspections, and made fewer chitter-calls (Spearman r_s < 0.60, $p < 0.035$).

Courtship behaviors and reproductive success

As with the F_0 generation of parent kestrels [15,16], courtship behavioral changes were consistent with reproductive changes of the in ovo DE-71 exposed kestrels. Although female kestrels were not exposed to PBDEs, when paired with males exposed in ovo to low or high DE-71 concentrations, they laid significantly smaller clutches (p = 0.020) of smaller eggs (weight: $p < 0.001$; volume $p < 0.001$) that were less fertile (p = 0.007) when compared to females paired with control males (Table 1). Furthermore, male in ovo exposure to increasing ΣPBDE concentrations was strongly and negatively correlated with clutch size (r_s = -0.56, p = 0.010) and fertility (p = 0.005) (Table 2, Fig. 3), as were the concentrations of individual PBDE congeners and HBCD ($p \leq 0.047$) (Table 2). Additionally, these reproductive parameters were influenced by the males' courtship behaviors: clutch sizes were larger when males made more nest-inspections (Spearman r_s = 0.53, p = 0.015), copulations (r_s = 0.59, p = 0.007), chitter-calls (r_s = 0.60, p = 0.005), whine-calls (r_s = 0.49, p = 0.028) and food-transfers to their mates (r_s = 0.60, p = 0.005), and the mass of the first egg was weakly and positively associated with copulation frequency throughout courtship (r_s = 0.43, p =0.088) and during the 9 d prior to egg-laying (r_s = 0.45, p =0.069).

Increasing delays in egg-laying were also associated with the increased frequencies of flight-displays by males (r_s = 0.52, p = 0.033). The 7-d delay in the commencement of egg laying by the female kestrels paired with males from the low in ovo exposure group (Table 1) is consistent with the F_0 generation [16], and may be biologically important since birds laying later in the breeding season generally have reduced reproductive success [25].

The extent to which the F_1 males were exposed as embryos to concentrations of BDE-100, -138, 153, and -154 was negatively associated with the number of hatchlings they produced as adult birds ($p \leq 0.044$) (Table 2). Similarly, their embryonic exposure to BDE-138 and -153 was also strongly and negatively associated with the number of fledglings they produced as adults ($p \leq 0.042$) (Table 2). These negative correlations are reflected by the trend in reproductive success with the high in ovo exposure F_1 males producing relatively fewer hatchlings and fledglings than the controls (Table 1).

Brood-rearing behavior

During the 10 days after the first chick hatched, males exposed in ovo to DE-71 continued to perform fewer bonding-displays than controls ($U_{12,9}$ = 44.0, p = 0.034), which was associated with a decrease in the number of hatchlings (r_s = -0.64, p = 0.018) and fledglings (r_s = -0.54, p = 0.055) produced. The number of times that males entered the nest-box or spent time in it were negatively correlated with their in ovo exposure to concentrations of BDE-138 (r_S = -0.682, p = 0.010), BDE-17 (r_S = -0.576, p = 0.039) and BDE-49 (r_S = -0.556, p = 0.048). The more time the male spent in the nest-box, the more hatchlings were produced by the pair (r_s = 0.66, p = 0.014). During the first 20 d after

hatching, kestrel chicks experience rapid and maximal growth [24]; during this time, the treatment males also performed fewer bonding-displays ($U_{12,9}$ = 22.5, p = 0.023) and tended to enter the nest-box less frequently ($U_{12,9}$ = 28.5, p = 0.069). As their in ovo exposure to BDE-209 levels increased, the males spent more time flying (r_S = 0.684, p = 0.010).

Discussion

The present study shows that similar to the dietary exposure of American kestrels to DE-71 (the F_0 generation) [15], embryonic exposure of F_1 male American kestrels to environmentally relevant concentrations of PBDEs in the DE-71 mixture alters reproductive behaviors and reduces reproductive success. These results are especially surprising given that only the male bird of the pair was exposed. Specifically, like their parents [15], the F_1 generation of in ovo exposed males showed decreased rates of copulation, a decrease in the number of fertile eggs, the number of pairs laying, and in egg size [the present study, 16]. In the present F_1 generation, the pairs with high in ovo DE-71 exposure males also had smaller clutches and as in ovo exposure to specific PBDE congeners increased, fewer nestlings and fledglings were produced. These results for the F_0 and F_1 generation of DE-71 kestrels parallel the multi-generational findings involving kestrels exposed to PCBs [26]. Furthermore, the results of the present study and that of Fernie et al. [16] confirm the association between PBDE body burdens and decreased brood size in wild peregrine falcons [7], the negative correlation between reproductive productivity of free-ranging ospreys and only in ovo ΣPBDE concentrations

[27], and are consistent with the reduction in pipping and hatching success of American kestrels exposed as embryos to DE-71 [28].

As previously demonstrated [15,16], PBDE exposure concentrations in the present study are similar to levels recorded in wild peregrine falcons [20] and herring gulls in the Great Lakes between 2004 and 2006; in herring gulls, mean egg concentrations of Σ_{39}PBDEs ranged from 321 to 1191 ng/g wet weight from seven locations [5]. In addition, several other closely related species of predatory birds currently show higher PBDE concentrations, including common kestrels where levels averaged 12 300 ± 5540 ng/g lipid weight in muscle [4], and peregrine falcons where concentrations in eggs ranged from 680 to 39,000 ng/g lipid weight [7,20]. In the present study, the specific PBDE congeners (BDE-47, -49, -85, -99, -100, -138, -153, -183) primarily associated with reproductive behaviors and reproductive success, especially those making up 88.5 % of the in ovo concentrations (BDE-47, -99, -100, -153), are the congeners found in the highest proportions in wildlife and have the greatest bioactivity in animals (BDE-47, -99, -100, -154, -153) (e.g., [19]), suggesting that these reproductive effects may also occur in wild birds exposed to similar or higher PBDE concentrations.

In the present study, the alterations in courtship behaviors of the pairs in which unexposed females were paired with males exposed in ovo to DE-7, are consistent with the courtship behavioral changes experienced by the F_0 generation of kestrels exposed by diet to DE-71. The establishment of the nest-site was delayed, evidenced by fewer nest-site inspections occurring as the in ovo exposure of the F_1 males to BDE-99 and -100 increased. Similarly, the F_0 males spent less time in the nest-box overall [15]. There was also an overall decrease in pair-bond quality in both generations; the high in ovo

exposure F_1 males performed fewer pair-bonding behaviors that are important for kestrels [24], including vocalizations, nest-inspections, food-transfers and copulations. Furthermore, the frequencies of these pair-bonding behaviors by the F_1 pairs were negatively and strongly correlated with the egg concentrations of one or more of the PBDE congeners. A reduction in these courtship behaviors, the correlation of these behavioral changes with clutch and egg size, and that 43% of females paired with high in ovo exposure males did not lay eggs, adds evidence to what Fernie et al. [15] suggested for the F_0 generation of kestrels: that a reduction in pair-bonding behavior, in relation to PBDE exposure, plays an important role in reducing reproductive success and occurs in multiple generations.

There is a clear link between the reproductive behavioral changes and the changes in reproductive success of the kestrels exposed in ovo to the DE-71 mixture. This highlights the importance and specificity of changes in male behavior from PBDE exposure in determining reproductive success, as well as suggesting that behavioral, physiological, endocrine, and embryonic mechanisms likely explain the associations between reproductive changes and PBDE exposure [7, 16, 27, 28]. Female kestrels were chemically unexposed, but those paired with high in ovo DE-71 exposed males were less likely to lay eggs, and produced smaller clutches with smaller eggs, suggesting that behavioral and physiological changes in the males resulting from their embryonic exposure to DE-71 influenced female reproductive physiology. Female reproductive readiness in seasonally breeding birds depends on several factors. Ultimately, photoperiod and the presence of a mate and nest-site are important, which were controlled for in this experiment. However, females also require appropriate behavioral

signals from the male to induce egg laying. In kestrels [24] and other birds [29, 30], these signals include vocalizations and nest-inspections, and most importantly, copulation. In the present study, the clutch size of the kestrels decreased with reductions in nest-inspections, copulations, mate-calls, and food-transfers, as well as with increasing concentrations of the major PBDE congeners to which the F_1 males were exposed as embryos. Furthermore, many of the same specific PBDE congeners, BDE-47, -99, -100, -138, -153, -85, and HBCD, were negatively associated with both reproductive and behavioral parameters, specifically clutch size, copulation rates, and mate-calls during courtship.

A female's investment in egg size is also influenced by mate quality, where females paired with higher-ranking males [31], or males with more prominent sexual traits [32], produce larger eggs that are consistently associated with increased hatching success in birds (reviewed in T.D. Williams [33]). This suggests that the decrease in male courtship behavior observed in the F_1 in ovo DE-71 exposed pairs, may be affecting female reproductive physiology by decreased sexual stimulation as well as by a decrease in the male's attractiveness and suitability as a mate. Ultimately, this may affect the reproductive success of the kestrel pairs, thereby partially explaining the reduced number and size of the eggs laid by chemically unexposed females paired with males exposed in ovo to DE-71.

The DE-71 F_1 pairs also experienced reduced fertility of their eggs, consistent with the reduced fertility of their parent F_0 kestrels. Like the F_0 generation, this reduction in fertility is consistent with the reduced copulation frequencies throughout the courtship period, and particularly during the 9 d preceding egg laying when fertility of the eggs is

determined in kestrels. The reduction in the fertility of the in ovo exposed kestrels may also be a function of the reduced quality of sperm of the same males (Marteinson et al, Chapter 3, this volume). Once again, there are consistent negative correlations between the concentrations of the same individual PBDE congeners, specifically BDE-47, -99, -100, -138, -153, and HBCD, and the copulation activity and fertility of these F_1 birds. Together these results suggest that the fertility of birds exposed in ovo to environmentally relevant PBDE concentrations may be affected through one or multiple mechanisms: behavioral, physiological, and/or exposure to PBDEs at environmentally relevant concentrations.

Changes in avian behavior can be highly indicative of underlying physiological alterations in relation to chemical exposure. Male courtship behavior in birds is directly regulated by testosterone produced in the testes and is dramatically reduced by exposure to synthetic estrogens such as genistein [34] or anti-androgens such as flutamide [35]. Decreased plasma testosterone levels have accompanied BDE-99 exposure in lab rats [13] and since several DE-71 congeners have shown mild estrogenic or potent anti-androgenic capabilities in vitro [8-10], and their hydroxylated metabolites can inhibit aromatase in vitro [36], it is plausible that male embryonic exposure to DE-71 disrupts the functioning of sex hormones in adult kestrels, and testosterone levels were reduced (Marteinson et al, Chapter 3, this volume). An alternative or additional explanation involves thyroid hormone disruption, a well-established effect of exposure to DE-71, BDE-47 and -99 (reviewed in C.E. Talsness [37]), including in kestrels [14]. In seasonally breeding birds, changes in the length of the photoperiod in conjunction with changes in thyroid hormones promote testes growth and steroidogenesis in adults

(reviewed in Wagner et al. [38]). During development, thyroid hormones affect testes development irreversibly in mammals and likely birds as well [39]. Since embryonic exposure to DE-71 decreased circulating triiodothyronine and thyroxin in the F_1 kestrels when they were nestlings (Fernie et al., Environment Canada, unpublished data), and some PBDEs have shown irreversible effects in brain development, testes functioning and subsequent behaviors in laboratory rodents following exposure during critical periods of brain development (reviewed in C.E. Talsness [37]), it is conceivable that this also occurred during the embryonic development of the F_1 male kestrels with the final observable outcome involving a decrease in testosterone-mediated courtship behaviors as adults. However, exposed males from the present study did not have detectible differences in thyroid hormone compared to controls (Marteinson, et al, Chapter 3, this volume).

In the present study, the number of hatchlings and fledglings produced by the F_1 pairs was negatively associated with the in ovo exposure of the F_1 parental males to increasing concentrations of major PBDE congeners, BDE-100, -153, -154, as well as BDE-138, as well as with these males spending less time in their nest-boxes and performing fewer pair-bonding behaviors immediately after the chicks hatched. In addition, there was a decreasing trend in these reproductive parameters across the treatment groups. These results suggest that the males' in ovo exposure to PBDEs is affecting their ability to successfully raise nestlings. Circulating prolactin concentrations, the hormone controlling incubation and brood-rearing behaviors, is inversely related to PBDE concentrations [17] and was associated with reductions in nest-site attentiveness in wild glaucous gulls [40]. In kestrels, the first 10 d of the brood-rearing period is

critical to nestling survival and the male kestrel delivers most of the food to the nest [24]. During this period, the F_1 in ovo exposed males performed fewer bonding-displays and fewer nest-inspections while the females entered the nest-box more often and fed more frequently outside of the nest-box. The reductions in these male behaviors were associated with a decrease in the number of young, and furthermore, the nestlings of these F_1 in ovo exposed pairs grew more slowly (Fernie et al., Environment Canada, unpublished data). Together, the results suggest that the males had a decreased affinity for the female and the brood and were insufficient at provisioning them with food -- consistent with the negative associations of fewer F_2 hatchlings and fledglings associated with increased embryonic PBDE exposure of the F_1 males.

From the results of the present study, it is apparent that several PBDE congeners of major or minor concentrations consistently affect reproductive behaviors and reproductive success, and different PBDE congeners influence reproductive behaviors at different stages of the breeding season. Decreases in nest-related behaviors were negatively associated with BDE-99 and -100 concentrations during courtship, but with concentrations of BDE-17 and -138 during brood rearing. Nest-related behaviors during courtship are related to sex hormones that can be affected by BDE-99 and -100 [9,10,12,13], while they are regulated by prolactin during brood rearing. The PBDE congeners present in the highest concentrations (and proportions), BDE-99 (43%), -153 (17%), -100 (15%), and -154 (12%), were associated with many changes in reproductive behaviors and reproductive parameters. However, congeners found in small concentrations (e.g., BDE-17, -85, -138, -183, -209) were also consistently associated

with these reproductive parameters, suggesting that minor concentrations of individual PBDE congeners are important too.

The accidental exposure to HBCD also raises the question as to the possibility of synergistic or additive effects of this BFR with the PBDE congeners. Hexabromocyclododecane was negatively correlated with parameters of particular importance to the reproductive success of birds, specifically clutch size, fertility, and copulation behavior. In all three of these cases, other PBDE congeners (e.g., BDE-47, -49, -85, -99, -100, -138, -153, and/or -183) were also associated with these parameters. Because HBCD is present in such small quantities and is correlated with these PBDE congeners and ΣPBDEs (Fernie and Letcher, Environment Canada, unpublished data), it is difficult to determine if indeed HBCD and/or the other PBDE congeners, especially those having minor concentrations, influence reproductive parameters by acting individually, synergistically, or additively, thereby warranting further research.

The findings from the present study clearly demonstrate that in ovo exposure of male kestrels to DE-71 has adverse effects on reproduction and behavior in American kestrels, and changes in these parameters are consistent across generations. Since these exposure levels are environmentally relevant, there is potential for the reproductive behaviors and reproductive success of wild birds to be altered by exposure to PBDEs at similar or higher concentrations, corroborating the findings that in ovo PBDE concentrations with decreased brood sizes in wild peregrine falcons [7] and ospreys [27]. In addition, wild birds are exposed to other ecological, environmental and anthropogenic stressors, including other organohalogens and chemicals that may act additively or

synergistically with PBDEs. These considerations are particularly important for species demonstrating unexplained population declines in the wild.

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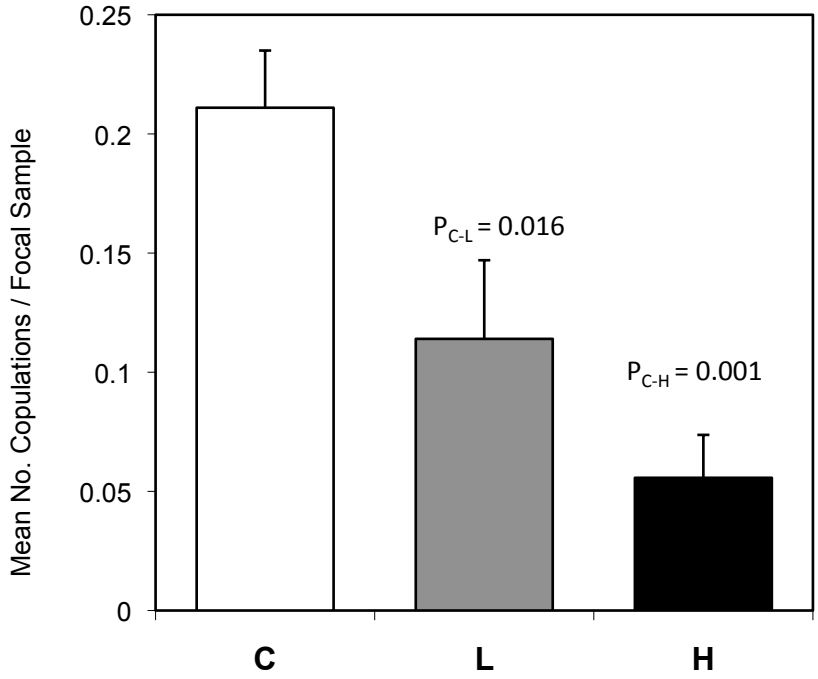

Fig.1. Copulation frequency over the whole courtship period for control (C), low-exposure (L) and high male in ovo DE-71 exposure (H) pairs. Differences between the groups were determined by one-way analysis of variance. Statistical comparisons between control and low-exposure (L vs C) and control and high-exposure (H vs C) males are made.

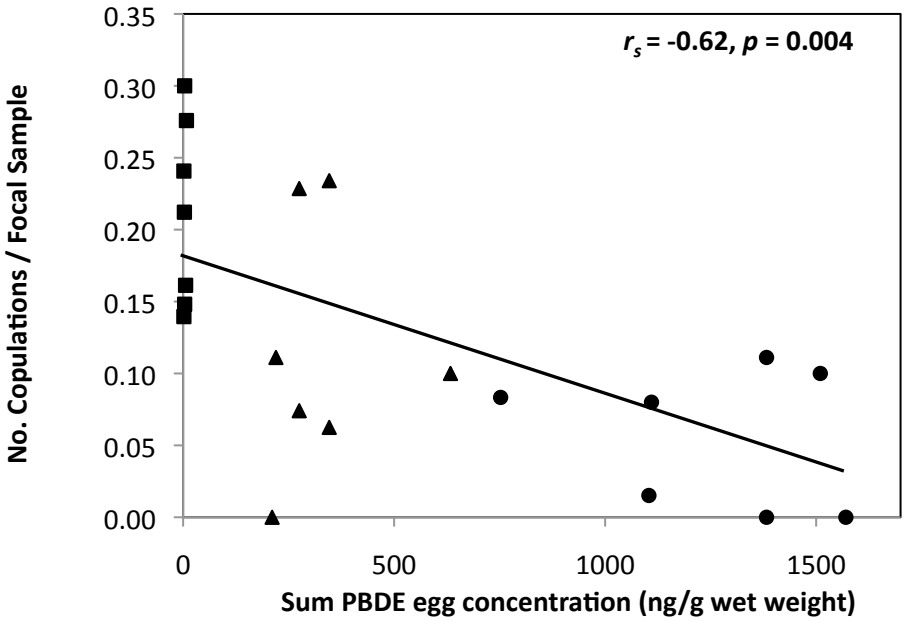

Fig. 2. Copulation frequency in relation to the sum of polybrominated diphenyl ethers (ΣPBDE) in ovo exposure concentrations of the parental F_1 male kestrels (Spearman Rank Correlation). Control (■); low-exposure (▲); high-exposure (●).

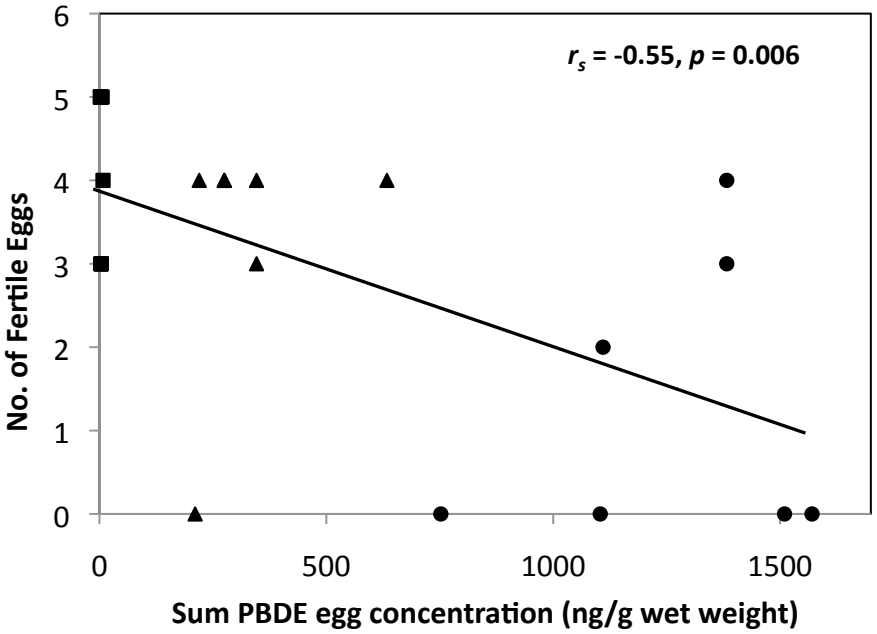

Fig. 3. Number of fertile eggs produced per pair in relation to the sum of polybrominated diphenyl ether (ΣPBDE) in ovo exposure concentrations of the F_1 male kestrels (Spearman Rank Correlation). Control (■); low-exposure (▲); high-exposure (●).

Table 1: Reproductive parameters of American kestrels exposed in ovo to control vehicle (safflower oil) or environmentally relevant levels of DE-71 (low and high) and unintentionally to hexabromocyclododecane (HBCD), (one-way ANOVA).

	F	*p*	Control pairs (*n* = 7)		Low-exposure pairs (*n* = 7)		High-exposure pairs (*n* = 7)	
Reproductive Variable	*df* = 2, 18		Mean	SE	Mean	SE	Mean	SE
% pairs not laying	NA	NA	0	NA	0	NA	43	NA
Julian lay date (d)	1.21	0.327	14.00	1.56	21.29	2.88	18.00	6.34
Clutch size (# eggs laid)	4.69	0.024	5.67	0.21	5.29	0.18	3.00*	1.07
Egg Variable								
Egg weight	30.87	<0.001	16.62	0.08	16.38*	0.09	14.57**	0.11
Egg volume	29.93	<0.001	35.95	0.15	35.72*	0.15	34.53**	0.21
Reproductive Success								
Total # fertile eggs	6.87	0.007	4.17	0.40	3.30	0.57	1.29*	0.64
Total # hatchlings	2.36	0.124	2.50	0.85	2.86	0.55	1.00	0.58
Total # fledglings	1.69	0.205	2.17	0.79	2.57	0.57	1.00	0.58
Overall hatching success	1.66	0.219	0.53	0.17	0.75	0.14	0.33	0.18
Overall reproductive success	0.93	0.414	0.57	0.19	0.76	0.14	0.43	0.20
Fertility (%) [a]	1.59	0.204	72.78	4.82	61.90	10.76	43.33	17.32
Courtship Behavior								
Copulation	9.34	0.002	0.21	0.02	0.11*	0.03	0.06**	0.04
Male chitter-call	3.90	0.039	4.57	1.19	3.88*	0.84	1.90*	1.18
Female whine-call	$X^2 = 8.877$	0.012	0.26	0.12	0.63	0.28	0.05	0.05
Male flight-display	$X^2 = 5.815$	0.055	0.90	0.22	2.15	0.79	2.36	0.74

[a] % fertility: $n_C = 6$, $n_L = 7$, $n_H = 4$. Courtship behavior frequencies are from the whole courtship period. Where X^2 value is reported, data were not normal and were analyzed by Kruskal- Wallis tests.* $p \leq 0.05$, ** $p \leq 0.01$ for differences between exposed groups and controls determined by post hoc Least Square Means tests. *df*: degrees of freedom. NA: not applicable. SE: standard error.

Table 2: Significant correlations among egg concentrations of polybrominated diphenyl ether (PBDE) congeners and hexabromocyclododecane (HBCD) from sibling eggs of in ovo exposed male American kestrels, their reproductive parameters and their behavior frequencies during the whole courtship period (n = 21, Spearman Rank correlation).

		Reproductive Parameters								Courtship Behavior							
		Clutch Size		# Fertile Eggs		# Hatchlings		# Fledglings		Copulation		Nest-inspection		Chitter-call		Whine-call	
Congener	% of ∑PBDE	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
BDE-99	43.26	-0.72	0.001	-0.69	0.002	NS	NS	NS	NS	-0.59	0.008	-0.50	0.029	-0.50	0.030	-0.48	0.038
BDE-153	17.46	-0.67	0.002	-0.76	<0.001	-0.54	0.020	-0.48	0.042	-0.64	0.004	NS	NS	-0.48	0.036	NS	NS
BDE-100	14.93	-0.68	0.002	-0.69	0.002	-0.48	0.044	NS	NS	-0.61	0.005	-0.46	0.047	NS	NS	-0.47	0.045
BDE-154	11.94	-0.67	0.002	-0.71	0.001	-0.40	0.035	NS	NS	-0.59	0.008	NS	NS	-0.46	0.049	NS	NS
BDE-47	8.60	-0.61	0.008	-0.64	0.004	NS	NS	NS	NS	-0.55	0.014	NS	NS	NS	NS	NS	NS
BDE-85	1.32	-0.58	0.011	-0.64	0.004	NS	NS	NS	NS	-0.60	0.006	NS	NS	NS	NS	NS	NS
BDE-17	0.08	NS	NS	NS	NS	NS	NS	NS	NS	NS	-	NS	NS	NS	NS	NS	NS
BDE-49	0.43	-0.47	0.047	-0.48	0.043	NS	NS.	NS	NS	-0.52	0.021	NS	NS	-0.53	0.021	NS	NS
BDE-138	0.045	-0.62	0.006	-0.74	0.001	-0.58	0.012	-0.49	0.012	NS	NS	NS	NS	NS	NS	NS	NS
BDE-183	0.01	NS	NS	-0.51	0.032	NS	NS	NS	NS	-0.62	0.005	NS	NS	NS	NS	NS	NS
∑PBDE		-0.55	0.006	-0.55	0.006	NS	NS	NS	NS	-0.62	0.004	NS	NS	-0.41	0.034	-0.40	0.036
HBCD		-0.67	0.002	-0.67	0.003	-0.47	0.049	NS	NS	-0.62	0.004	NS	NS	NS	NS	NS	NS

BDE: brominated diphenyl ether followed by the congener number. ∑PBDE: sum concentration of all PBDE congeners. r_s: Speaman's rank correlation coefficient, and its corresponding significance level (p). Only significant ($p \leq 0.05$) correlations are displayed; BDE-190, 28, -66, -209 were detected in eggs, but not correlated with any parameters. NS: not significant.

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CONNECTING STATEMENT 2

The previous chapter outlines the impacts of in ovo exposure to polybrominated diphenyl ethers (DE-71 mixture), research that followed investigations on the effects of dietary exposure to the parents of the same study subjects. In the next chapter, to further investigate the effects of in ovo PBDE exposure to male American kestrels, circulating testosterone and thyroid hormones as well as testicular physiology were assessed in the same individuals. During the same reproductive cycle, as described in the previous section, blood samples were taken at strategic time points and analyzed for hormone concentrations. Body mass and sperm numbers were also assessed. One year following this reproductive study, the same males were sacrificed for analysis of testicular physiology.

CHAPTER 3

EMBRYONIC EXPOSURE TO THE POLYBROMINATED DIPHENYLETHER MIXTURE, DE-71, AFFECTS TESTES AND CIRCULATING TESTOSTERONE CONCENTRATIONS IN ADULT CAPTIVE AMERICAN KESTRELS (*FALCO SPARVERIUS*)

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Abstract

Polybrominated diphenyl ethers (PBDEs) are additive flame retardants that are environmentally persistent and bioaccumulative compounds of particular concern to species at high trophic levels including predatory birds. The developmental effects of in ovo exposure to environmentally relevant levels of the PBDE technical mixture, DE-71 on male reproductive physiology in captive American kestrels (*Falco sparverius*) was determined. Males were exposed in ovo by direct maternal transfer to DE-71 at three (mean ± standard error DE-71) concentrations of 289 ng/g wet weight (low-exposure), 1131 ng/g wet weight (high-exposure), or background levels of 3 ng/g wet weight (control). Unintentionally, exposure to very low concentrations of hexabromocyclododecane (HBCD) also occurred. At one year of age, males were paired with unexposed females for breeding and one year later, they were sacrificed for testes extraction. While breeding, high-exposure males demonstrated a trend towards reduced circulating testosterone levels at the time of egg-laying by their partner ($p = 0.056$) when compared to controls. No differences in circulating free T_4 or T_3 were detected at any time. Sperm numbers were elevated on the perivitelline layer of the first egg of both high- and low-exposure males compared to controls ($p \leq 0.028$). High-exposure males had a higher gonadosomatic index ($p = 0.046$) and heavier testes than controls on the right ($p=0.034$) with a similar trend on the left ($p=0.055$). The increased testis mass was associated with increasing in ovo ΣPBDE exposure concentrations as well as those of BDE-100, -47, -85, -183. In the testes, high-exposure males also had more seminiferous tubules containing lumen than did controls ($p = 0.030$). The proportion of tubules containing final spermatids was negatively associated with the in ovo exposure

concentrations of BDE-47, -66, -85, -17, -49,and -28. Concentrations of HBCD were not associated with any parameters measured. The results of the present study demonstrate that embryonic exposure to technical DE-71 affects the reproductive tract in adult male kestrels at environmentally relevant concentrations.

Introduction

Polybrominated diphenyl ethers are lipophillic and bioaccumulative brominated flame retardants that have reached global distribution in the environment and wildlife [1]. The highest levels have been recorded in several avian top predators, including the common kestrel, *Falco tinnunculus,* in which concentrations averaged 12 300 ± 5 540 ng/g lipid weight in muscle [2]. A doubling time of 4.9 to 8.7 years (1981 to 2006) has occurred in eggs of the Great Lakes herring gull, *Larus argentatus,* in which average egg concentrations ranged from 321 to 1191 ng/g wet weight [3] with the most dramatic increases occurring in the 1990s and early 2000s. For the penta-BDE derived congeners some avian species are now showing decreasing trends in eggs [4, 5]. However, as of 2005 to 2006, PBDE levels from some avian populations in closer proximity to urban or industrialized areas may be stabilizing [4], or are still demonstrating increasing temporal trends, as seen in the peregrine falcon (*F. peregrinus*) [6].

Polybrominated diphenyl ethers and/or their degradation products are now classified as endocrine disruptors. The DE-71 mixture has a combination of estrogenic and anti-androgenic properties [7-9] as well as thyroid disrupting capabilities [7, 10, 11], which may be particularly detrimental to the reproductive tract in males. Male rats developmentally exposed to DE-71 demonstrated a delay in the onset of puberty [12, 13]

and a decrease in androgen-dependent reproductive organ weights (testes and epididymus) following developmental exposure to BDE-99 [14] which are biomarkers for anti-androgenic effects in rats [12-14]. Additionally, male rats exposed developmentally to BDE-99 showed an increase in sweet preference, a sexually dimorphic trait normally seen in females, and an accepted biomarker for estrogenizing effects in rats [15]. Circulating estradiol, testosterone [15] and T_4 [13, 16-23] were reduced in male rats exposed developmentally to BDE-99.

American kestrels exposed to the DE-71 technical mixture have demonstrated altered courtship frequencies both with diet [24] and developmental exposure [25] and reduced reproductive success in both cases [24, 25], indicating that they are reproductively sensitive to PBDE exposure. Additionally, circulating T_4 was reduced in young kestrels with developmental exposure via egg-injection [26] and this hormone is critical in the regulation of testis growth during seasonal recrudescence [27]. The objective of the present study is to examine the effects of in ovo exposure to DE-71 at environmentally relevant levels on the male reproductive tract and associated endocrinology in American kestrels. Specifically, sperm numbers and testis mass and histology were assessed as well as circulating thyroid hormone and testosterone levels. To the best of the authors' knowledge, the present study is the first to determine the effects of exposure to PBDEs on the reproductive tract in male birds.

Methods

The study used captive-bred American kestrels (*F. sparverius*) with documented histories, housed at the Avian Science and Conservation Centre at McGill University,

and took place in 2007 and 2008. Birds were subjected to natural temperature fluctuations and photoperiod, and fed untreated day-old frozen-thawed cockerels (*Gallus domesticus*) ad libitum. The treatment and care of the kestrels was conducted in accordance with the Canadian Council on Animal Care Guidelines [28] and was approved by the Animal Care Committee of McGill University (#5007).

The study subjects

The in ovo exposed males used in the present study were the F_1 progeny of the 2006 dietary exposure F_0 study subjects [24] and were all one year of age. The parents of treated males had been exposed to one of two environmentally relevant levels of DE-71 found in the eggs of Great Lakes herring gulls [3, 29] and European peregrine falcons [30]. Parental exposure involved injecting DE-71 into their food source (18 µg per individual) at a concentration of 0.658 µg DE-71 per µl or 0.140 µg/µl of safflower oil; controls were exposed only to the vehicle (for details see [24]). These F_0 birds were exposed as described from three weeks before pairing through incubation (average of 75 d) until the first chick hatched. Thus, the in ovo exposure subjects from this study were never exposed by diet, but only during the 28-d embryonic period via direct maternal transfer to the egg. One treatment group involved male kestrels exposed embryonically to higher concentrations of DE-71 (n_H = 7), and another group consisted of males exposed embryonically to relatively lower concentrations of DE-71 (n_L = 7), as defined by the exposure categories of their parents [24, 31], and labeled herein as high-exposure and low-exposure accordingly. A third group of controls (n_C = 7) was used for comparison. Mean ΣPBDE concentrations found in sibling eggs of these individuals

were 1131 ± 95 ng/g wet weight for high-exposure and 289± 33 ng/g wet weight for low-exposure males. Background levels of 3 ± 0.46 ng/g wet weight were determined in the eggs of controls. Hexabromocyclododecane (HBCD) was not expected to be present in the egg samples and was likely an exposure artifact during the dosing of the birds; the concentrations found in the eggs were low, 16 ± 3 ng/g wet weight in high-exposure eggs, 3.3 ± 0.7 ng/g wet weight in low-exposure eggs and 0.002 ± 0.002 ng/g wet weight for controls [24, 31]. Detailed descriptions of the chemical analysis and concentrations of PBDEs and HBCD in these eggs are described elsewhere [24, 25, 31].

In 2007, at one year of age, the 21 in ovo exposed males in the 3 exposure groups described above were paired with unexposed females and allowed to complete one reproductive cycle (Fig. 1). They are the same individuals for which reproductive success and behavior have been previously described for this same breeding season (Marteinson et al, Chapter 2, this volume; [25]). One year later in the spring of 2008, males were euthanized at two years of age for testes evaluation during the fertile period though males were in unpaired status at this time.

Measurement of circulating hormone levels

One ml of blood was taken at each sampling time point with a heparinized 27.5-gauge needle by jugular venipuncture. This frequency and volume of blood withdrawal does not significantly affect reproductive output or hematocrit in kestrels [32]. Body mass was recorded prior to withdrawal of each blood sample and samples were drawn at

the same time of day (8:30 – 10:30 just prior to feeding) to avoid the effects of diurnal variation in hormone concentrations.

Plasma from the blood samples taken while males were breeding were analyzed for testosterone concentrations at 3 biological reference points: pairing, the week before the pair laid their first egg and the week that the first egg was laid (Fig. 1). Circulating testosterone (T) concentrations in plasma were determined by enzyme immunoassay (EIA), conducted at Environment Canada's National Wildlife Research Centre in Ottawa. A salivary testosterone enzyme immunoassay kit (Salimetrics, State College PA, USA) was used because of its higher sensitivity compared with radioimmunoassay (RIA) kits or serum EIA kits, and thus less plasma was needed. The plasma samples were thawed and 15 µl was diluted by 10 times with 135 µl of the assay diluent. The absorbance of this solution was determined using a Molecular Devices plate reader (SpectraMax 190: s/n NN02060). Plates with 96 wells were coated with rabbit anti-testosterone antibodies. A calibration curve was prepared with 6 concentration levels of T standards in duplicates with low and high levels of T in a saliva-like matrix, for quality assurance. Duplicates of each sample were conduced from which a mean was calculated and used for analysis. The determined T levels were within the acceptable range and inter- and intra-assay variability was below 10% in all cases. The kestrel standards, created using control plasma (not charcoal stripped) were parallel with the salivary standards of the kit with a coefficient of variation of $R^2 = 0.998$. For additional quality control, 12 samples were concurrently analyzed for total T by radioimmunoassay kits (Siemens, Coat-A-Count©, CON6 lot 22), with well-correlated results ($R^2 = 0.97$), thus validating the results obtained from the salivary EIA.

Plasma was also analyzed for the thyroid hormones T_3 and T_4 at pairing and 5 other time points throughout the breeding season (Fig. 1). The levels of circulating free T_4 and T_3 in plasma duplicates were determined with the use of radioimmunoassay (RIA) kits (Siemens Medical Solutions Diagnostics) at Environment Canada's National Wildlife Research Centre in Ottawa. Plasma samples in duplicates were slowly thawed: 25µl for T_4 and 50 µl for T_3. The T_4 levels were analyzed that same day, and the T_3 levels were analyzed the next day; all samples from a given individual were analyzed together to reduce variation. The radioactivity was determined with a Canberra-Packard gamma counter E-5002 (serial number 423345) and was counted during 1 minute. Results were calculated using linear regression of log-logit representation of the respective calibration curve, prepared with 6 levels, for both T_3 and T_4. The percents of variation for most samples were below 10% with only three samples being above 20% variation. All samples fell within the range of the calibration curve. Commercial controls at various concentrations were also analyzed for quality assurance.

Measurement of sperm numbers during breeding

Sperm nuclei become trapped in the perivitelline layer (PVL) of the egg yolk, which reflect insemination sperm counts [33-35]. The first egg was removed on the day of laying and frozen until analysis to preserve spermatozoa. Eggs were thawed and the PVL was stretched on a microscope slide and stained with a DNA-binding fluorochrome (Hoechst 33342, Merck). Spermatozoa were irregularly dispersed on the PVL, thus total numbers were estimated by counting all sperm nuclei under a Nikon fluorescence microscope at 200x magnification. In certain areas of high density, quadrate

multiplication was conducted when necessary [34, 36]. An additional 8 controls from 2008 found not statistically different from the present controls, were also included to increase the sample size (n_C = 15) and to expand the range of what is normal, since this analysis has never before been conducted on kestrels. All low-exposure pairs laid eggs (n_L = 7), but only 4 high-exposure pairs laid eggs [25], all of which were successfully evaluated.

Evaluation of epididymal sperm numbers, testes mass and histology

These same individuals were sacrificed one year later on May 5th and 6th, 2008 to coincide with the onset of spermatogenesis [37] and thus seasonal testicular development in American kestrels. Both testes were extracted within 30 min of death and weighed with an analytical balance. Body mass was taken just prior to death and the gonadosomatic index was calculated as the total testes mass × 100 / body − testes mass [38].

The left and right epididymides were immediately removed and placed in 3.5 cubic centimeters of phosphate buffered saline solution (PBS) at body temperature (40°C). These organs were then thoroughly minced and incubated at body temperature for 30 minutes to allow sperm to swim out. One drop was placed on an improved Neubauer hemacytometer; sperm were counted in all squares because their numbers were low and only swimming sperm could be identified because the samples were contaminated with debris and red blood cells. Four replicates were conducted per individual, from which a mean was calculated.

The larger left testis was selected for histological analysis. Testes were fixed in Bouin's solution, embedded in paraffin; and 6 μm sections were stained with hematoxylin and eosin. The number of seminiferous tubules containing final spermatids (Fig. 2) was counted on two testis cross-sections from different levels in the testis per individual under 670x magnification, from which a mean was calculated. The number of tubules containing lumen (Fig. 2) were counted for one cross-section per individual from a digital photograph, using the automated counting tool in Adobe Photoshop 11. The total number of tubules was also counted from which a proportion of tubules containing lumen or final spermatids were calculated.

Statistical analysis

For all parameters measured, the high-exposure (n_H = 7), low-exposure (n_L = 7) and control (n_C = 7) males were compared using one-way analyses of variance (ANOVA) with Least Square Difference (LSD) as a post hoc test. For hormone concentrations, the ANOVAs were conducted both with and without the covariates of body mass. For proportions, data were arcsine-transformed. Mean hormone concentrations (T, T_3, T_4) and body mass were also compared for differences in the pattern of change over time using a repeated measures (RM) ANOVA. Pearson's correlation analyses were conducted between the physiological parameters measured and the in ovo exposure concentrations of PBDE congeners as determined in sibling eggs. Statistical analysis was conducted using SPSS 17.0, all data were tested for normality and homogeneity of variance and significance was considered to be $p \leq 0.05$.

Results

Circulating hormone concentrations and body mass

While breeding, T levels in high-exposure males were lower than those of controls at the time the first egg was laid (ANOVA $F_{2,18} = 2.10$, LSD $p = 0.056$) (Fig. 3) but did not differ over time, nor at testes extraction. While males were breeding, there was no difference in circulating T_3 or T_4 at any point, nor did the pattern over time differ among groups. Testosterone and thyroid levels were not associated with the in ovo exposure concentrations of PBDEs. There was no difference in body mass between DE-71 exposed males and controls at any time.

Sperm counts

The number of sperm trapped in the PVL of the first egg produced while males were breeding differed between the three groups ($F_{2,23} = 4.96$, $p = 0.021$). Both the low- (LSD $p = 0.022$) and high-exposure males (LSD $p = 0.028$) had higher PVL sperm counts than controls only when the set of pooled controls was used (Fig 4). At the time of testes extraction one year later while males were in unpaired condition, there was no difference in the concentration of motile sperm in the epididymides between DE-71-exposed and control males.

Testes mass and histology

Two years after their embryonic exposure to DE-71, the high-exposure males had a heavier right testes than did controls ($F_{2,18}$ = 2.69, LSD $P_{C\text{-}H}$ = 0.034) with a similar trend for the left testis ($F_{2,18}$ = 2.16, LSD $P_{C\text{-}H}$ = 0.055) (Fig. 5), and overall, a higher gonadosomatic index than controls ($F_{2,18}$ = 2.34, LSD $P_{C\text{-}H}$ = 0.046). The ratio of left to right testis mass did not differ between the groups. Testis mass was positively associated with *in ovo* exposure concentrations of ΣPBDEs and the individual congeners, BDE-100, -47, -85, and -183 ($p \leq 0.046$) (Table 1). High-exposure males had more seminiferous tubules containing lumen than did controls ($F_{2,18}$ = 3.02, LSD $P_{C\text{-}H}$ = 0.030) (Fig. 6) which was also positively associated with testis mass (r_p = 0.79, $p < 0.001$) and low-exposure males had more tubules with lumen in proportion to the total number of tubules ($F_{2,17}$ = 3.74, LSD $P_{C\text{-}L}$ = 0.016). Testis mass was also positively associated with the total number of tubules (r_p = 0.69, p = 0.001) and the number of tubules with final spermatids (r_p = 0.59, p = 0.005). The mean percent of tubules containing lumen increased with exposure from 50% for controls to 69% and 65% for low- and high-exposure males respectively. Conversely, the mean percent of tubules containing final spermatids was decreased to 43% in high-exposure males compared 53% in controls and 59% in low-exposure males (Fig. 7). The proportion of tubules containing final spermatids decreased with increasing *in ovo* exposure to BDE-47 (Fig. 8), -85, -49, and -28 ($p \leq 0.049$) (Table 1).

Discussion

The present study demonstrates that embryonic exposure to high environmentally relevant levels of DE-71 had long-term effects on the reproductive physiology of adult male American kestrels. High in ovo exposed males demonstrated reduced testosterone levels at egg laying, however, unlike young kestrels receiving egg injections of DE-71 [26], there was no evidence of hypothyroidism. At the onset of spermatogenesis, high exposure males had heavier testes with more seminiferous tubules containing lumen than controls. However, there was no evidence for any associated increase in spermatogenesis. High-exposure males had proportionally fewer tubules with final spermatids as in ovo exposure concentrations increased for several major and minor PBDE congeners and epididymal sperm counts were similar to those of controls. Perivitelline sperm counts were highly elevated in both low- and high-exposure males, which may at first appear contradictory. However, they may actually be demonstrating an increase in ejaculate concentration which can result from reduced copulation frequency [39, 40], an outcome that was strongly noted for both the low- and high-exposure males used in this study [25]. Additionally, higher PVL sperm numbers are usually related to more fertilized eggs [41]; however, though the high-exposure males had elevated levels of sperm reaching the ovum, they demonstrated reduced egg fertility [25], suggesting that sperm may actually have decreased fertilization potential. These results are consistent with those for laboratory rodents exposed developmentally to BDE-99 that demonstrated permanent reductions in circulating testosterone [15] and spermatogenesis although testis mass was reduced [14].

Androgens play a critical role in the reproductive behavior of male birds [42], such that when their function is suppressed experimentally, reductions in courtship behavior are seen [43, 44]. Though there was no statistical correlation (Marteinson et al., unpublished data), it is likely that the reduced plasma testosterone (this study) and impaired courtship behavior frequencies [25] are biologically related in these males as illustrated by the congeners that appear to be causative. Testis mass was positively associated with increasing in ovo exposure concentrations of ΣPBDE and those of BDE-100, -47, -85, and -183. The proportion of tubules containing final spermatids decreased with increasing in ovo exposure to BDE-47, -85, -49, and -28. In the same males, as reported elsewhere, clutch size, the number of fertile eggs and copulation frequency were also associated with BDE-100, -47, -85, and -183 as well as other congeners including BDE-99, -153, -154, and -138 [25]. Because males were paired with unexposed females, the alterations in male behavior were implicated in the decreased clutch size and fertility in the same high-exposure individuals [25]. As demonstrated in the present research, the alterations in male reproductive physiology may have also been a factor in the reduced reproductive success.

Testicular size is a sensitive endpoint with chemical exposure and is useful as an indicator of function and reproductive state of the testis [45]. Most commonly, testicular mass is reduced in mammals and birds exposed to endocrine active chemicals which is usually indicative of reduced spermatogenesis [45], as seen in laboratory rodents exposed to PBDEs [14]. However, increased testis mass can also occur with chemical exposure as a result of one or two mechanisms: increased cell proliferation during development [46] or increased fluid in the testes [45].

With respect to the first possibility, it is well documented in mammalian models that triiodothyronine (T_3) inhibits somatic cell proliferation [46]. Henceforth, hypothyroidism (subnormal levels of T_4) during development causes increased final numbers of Leydig [47] and Sertoli cells which are followed by heavier testes and greater sperm production in adult mammals [48]. This mechanism has been recently confirmed in fish [49] and birds [50], and has been implicated in increased testicular mass in rodents exposed to PCBs [51, 52], environmental pollutants with similar chemistry to PBDEs. Several PBDE congeners and their metabolites have the capacity to disrupt the thyroid axis [7, 10] and hypothyroidism has been recorded in numerous in vivo studies [13, 16-23, 53, 54] including in young American kestrels exposed by egg injection to environmentally relevant levels of PBDEs [26]. Surprisingly, there was no evidence for any alterations in thyroid function in the present study subjects at the time points examined. Additionally, there was no evidence of increased sperm production which usually accompanies hypothyroid enlarged testes [48]: epididymal sperm counts were not higher in high-exposure males and the proportion of tubules containing final spermatids was decreased compared to controls.

The second possible mechanism for increased testicular mass involves a disturbance of the fluid balance in the male reproductive tract that can ultimately result in fluid retention in the testis which accounts for the increased mass [45]. In mammals, this may occur as a result of overproduction of fluid by Sertoli cells within the testis, or by a backflow of fluid into the testes by the impaired functioning of the efferent tubules either from a blockage or by reduced fluid resorption (reviewed in: [55]). All three of these possibilities usually result in the dilation of the seminiferous tubule lumen as a

recognizable outcome [55]. In the present research, it did not appear as though the tubules were dilated, however the fact that more seminiferous tubules contained a lumen at all in the high-exposure males when compared to controls may explain the greater testes mass of these males.

Conclusion

The present study demonstrates that embryonic exposure to technical DE-71 at high environmentally relevant levels has long-term effects on multiple aspects of the male reproductive tract in adults. Due to the experimental design of the present research, and because the testicular histology of kestrels has never previously been examined, it could not be determined whether the enlarged testes in the kestrels were related to thyroid disruption or fluid imbalance in the testes. However, there is no evidence for any alterations in thyroid function in these birds, nor any evidence for increased spermatogenesis compared to controls. The reduced testosterone levels during courtship as well as the reduction in the proportion of tubules containing final spermatids in high-exposure males may be related to the reduced courtship behavior and reproductive success in the same individuals [25]. Because exposure concentrations are environmentally relevant and are similar to those recorded in the closely related peregrine falcon [6, 56] and other species [3, 4], wild male birds may be subject to similar alterations in the reproductive tract.

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Table 1: Significant correlations among egg concentrations of polybrominated diphenyl ether (PBDE) congeners from sibling eggs of in ovo exposed male American kestrels and testicular mass and percent of seminiferous tubules containing final spermatids (f.s.) (n = 21, Pearson's correlation). NS: not significant.

	% of	Left Testis (g)		Right Testis (g)		% Tubules with f.s.	
Congener	ΣPBDE	r_s	p	r_s	p	r_s	p
BDE-100	14.93	NS	NS	0.463	0.046	NS	NS
BDE-47	8.6	0.49	0.034	0.583	0.017	-0.51	0.003
BDE-85	1.32	0.53	0.019	0.53	0.019	-0.46	0.049
BDE-49	0.43	NS	NS	NS	NS	-0.64	0.003
BDE-28	0.09	NS	NS	NS	NS	-0.64	0.003
BDE-183	0.01	0.62	0.004	0.60	0.004	NS	NS
ΣPBDE		0.44	0.046	0.50	0.021	NS	NS

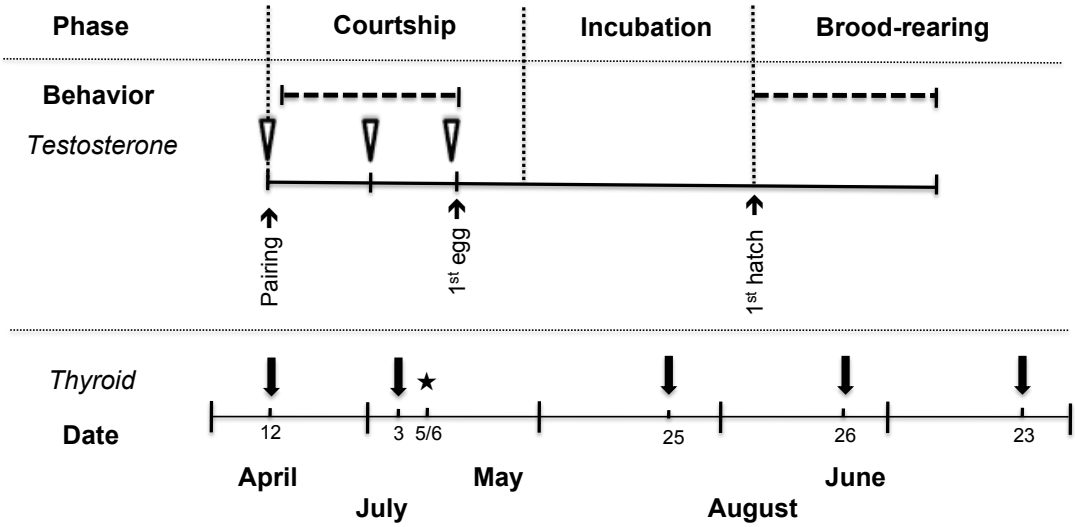

Fig. 1: Timeline for data collection during each reproductive phase of American kestrels. A blood sample was taken for testosterone analysis three times during courtship at the biological reference points of pairing, the week before laying and the week the first egg was laid (▽). A blood sample was taken for thyroid hormone analysis at pairing and once per month four times thereafter (⬇). Behavioral data were collected during the courtship and brood-rearing periods the timing of which is marked by the dashed lines. Body mass was recorded at the same time as all blood samples. The reproductive phases line up in approximation with the timeline of dates but vary between the pairs. In the year following breeding, the same males were euthanized for testis extraction (★).

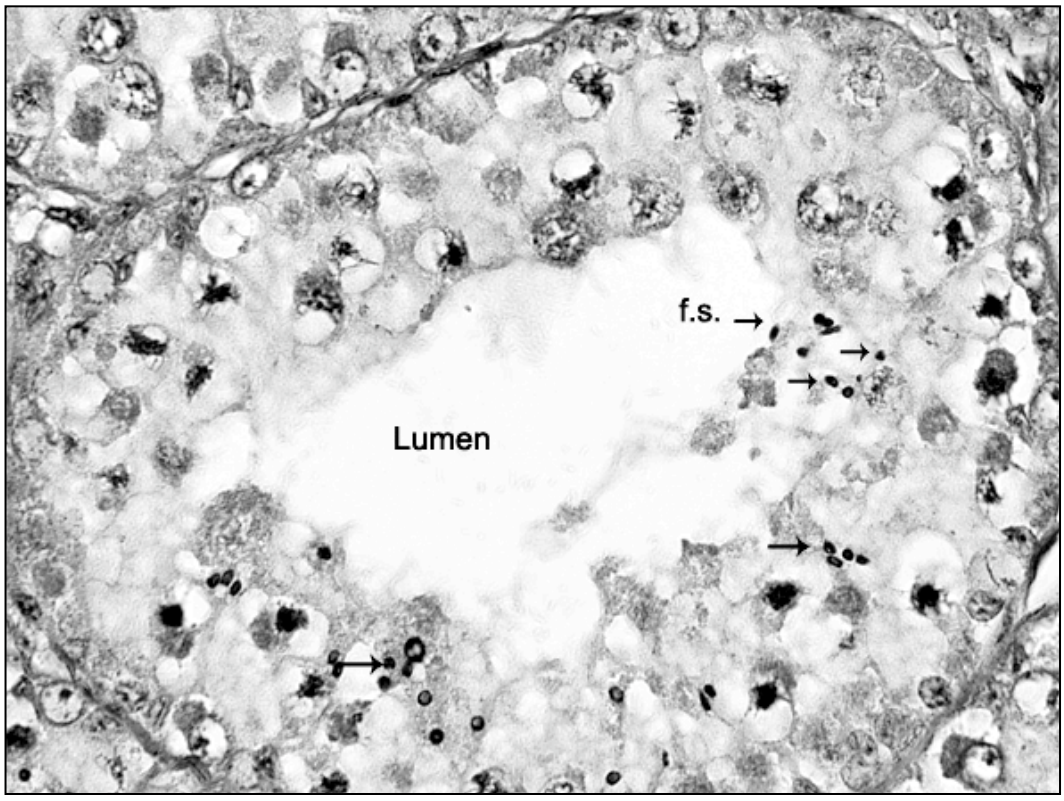

Fig. 2: Cross-section of a seminiferous tubule from the left testis of an unpaired American kestrel at the onset of spermatogenesis. The lumen is visible as are final spermatids (f.s. ➔) in the epithelium.

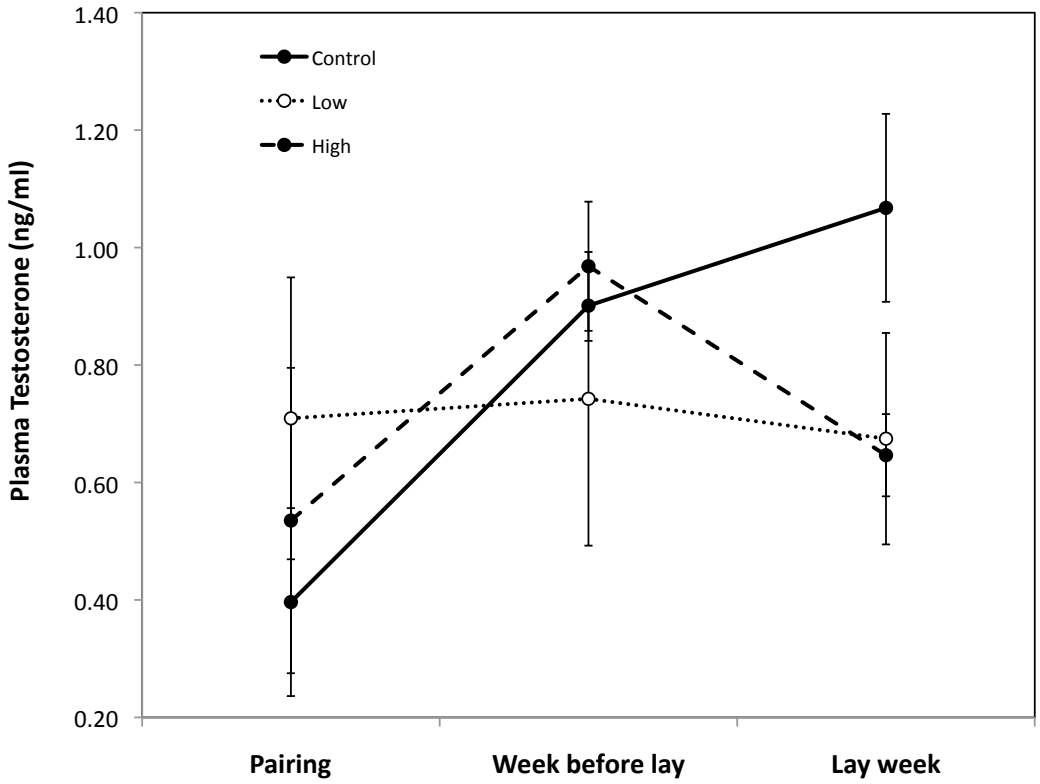

Fig. 3: Plasma testosterone concentrations for breeding male American kestrels exposed in ovo to higher or lower levels of DE-71 and controls. Means for the biological reference points of pairing, the week before egg-laying and the week the first egg was laid are displayed (1-way ANOVA $p = 0.056$). The difference in pattern over time was not different among the groups (RM ANOVA).

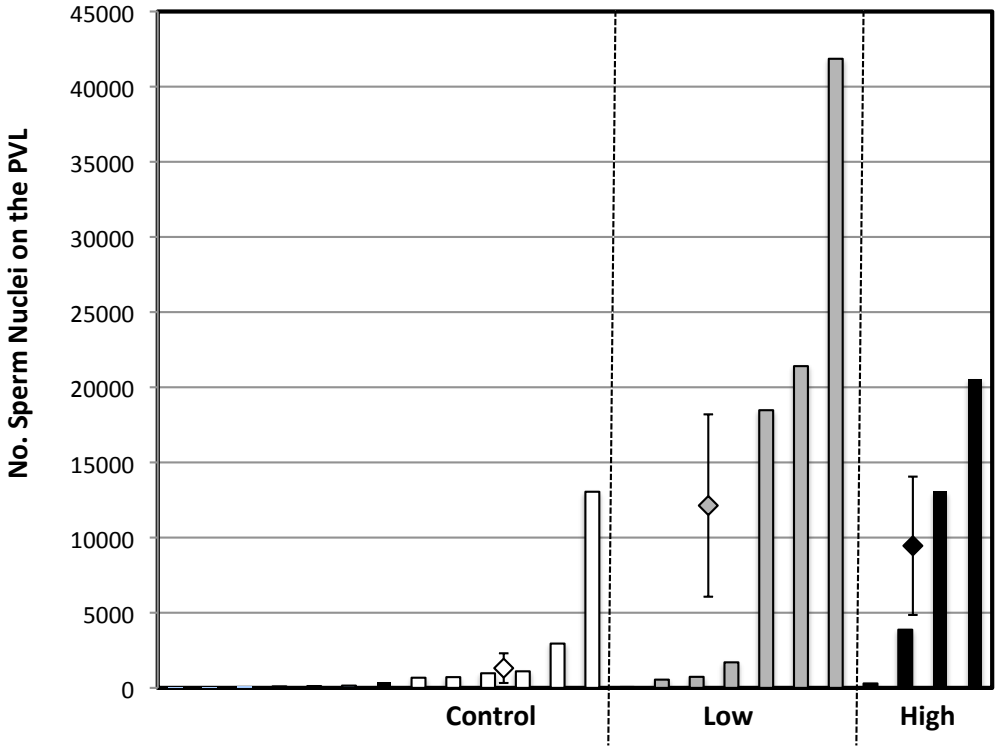

Fig. 4: Counts for all sperm nuclei in the perivitelline layer of the first egg in each clutch of male American kestrels exposed in ovo to DE-71 (n_C = 15, n_L = 7, n_H = 4). Each column represents the count from one individual; means for controls (C), low- (L) and high-exposure (H) are also depicted (◆). The eggs of exposed male American kestrels had more sperm in the perivitelline layer than did controls (ANOVA $F_{2,23}$ = 4.96, p = 0.021).

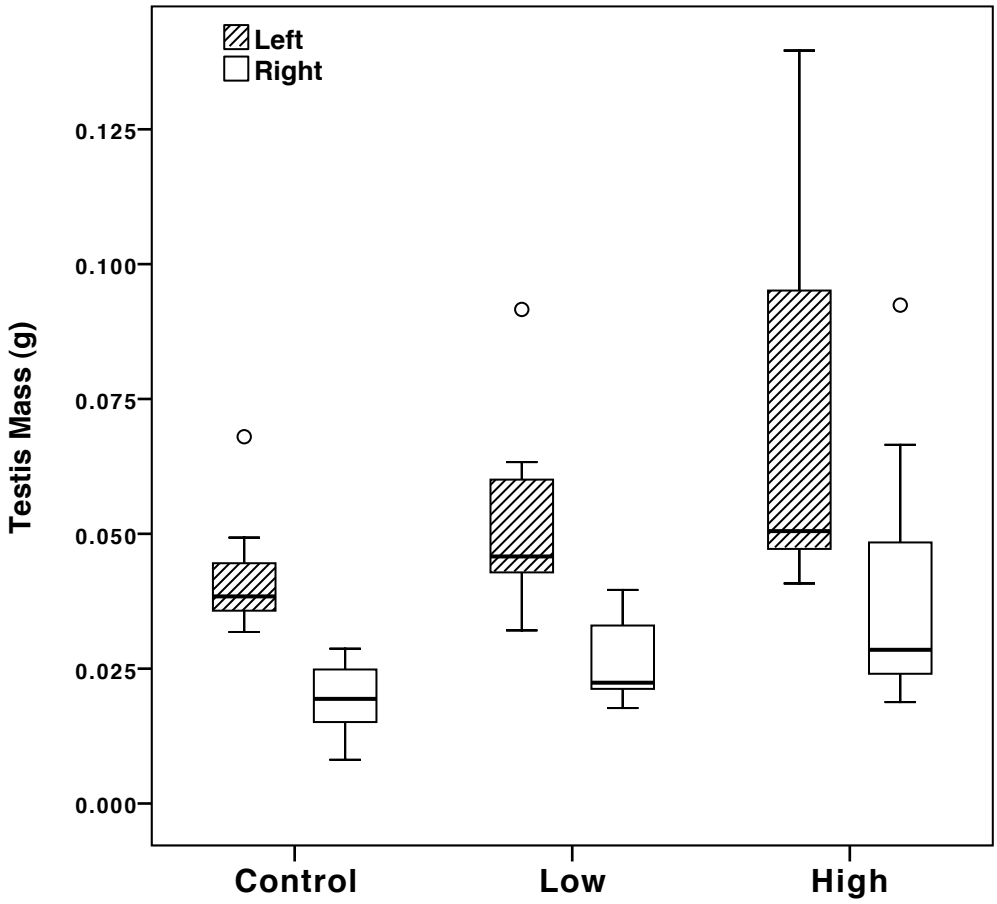

Fig. 5: Left and right testis mass for control, low and high in ovo DE-71 exposed male American kestrels. Males were in unpaired condition, but testes were collected in May during the fertile period. The left testis of DE-71 high-exposure males was heavier than those of controls on the left (LSD $p = 0.055$) and right (LSD $p = 0.034$).

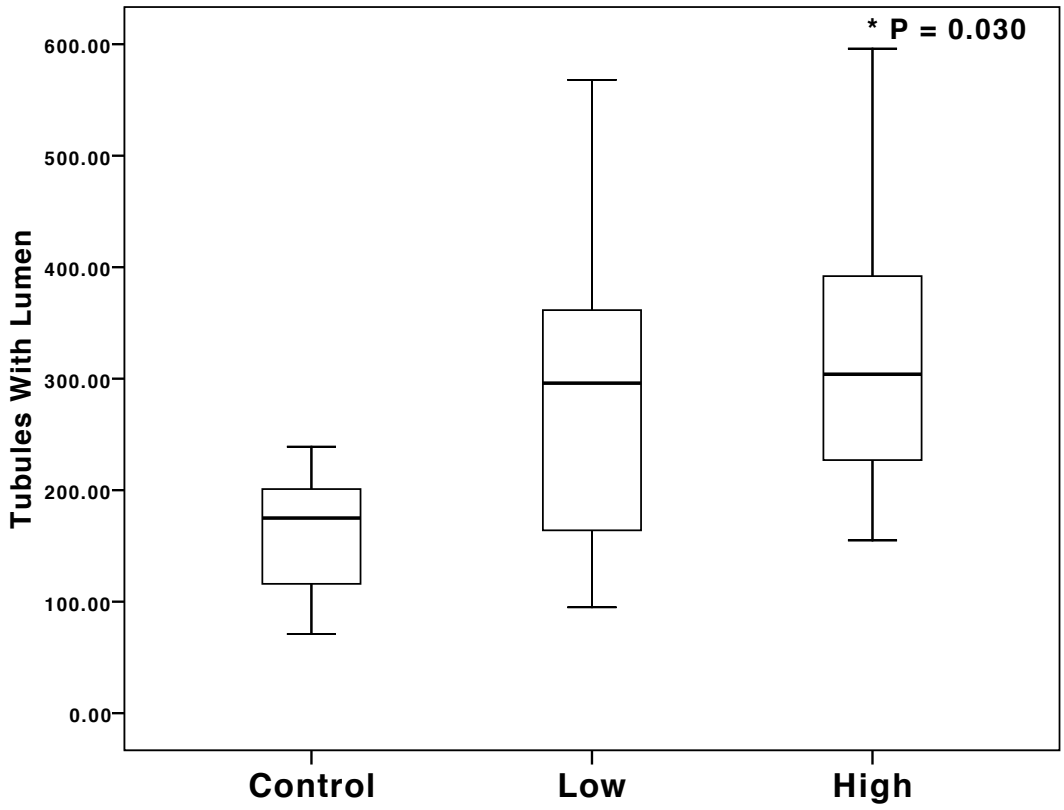

Fig. 6: Seminiferous tubules with lumen for control, low and high in ovo DE-71 exposed American kestrels. Males were in unpaired condition, but testes were collected in May during the fertile period. High-exposure males had more tubules with lumen than did controls (ANOVA $F_{2,18} = 3.02$, LSD $P_{C\text{-}H} = 0.030$).

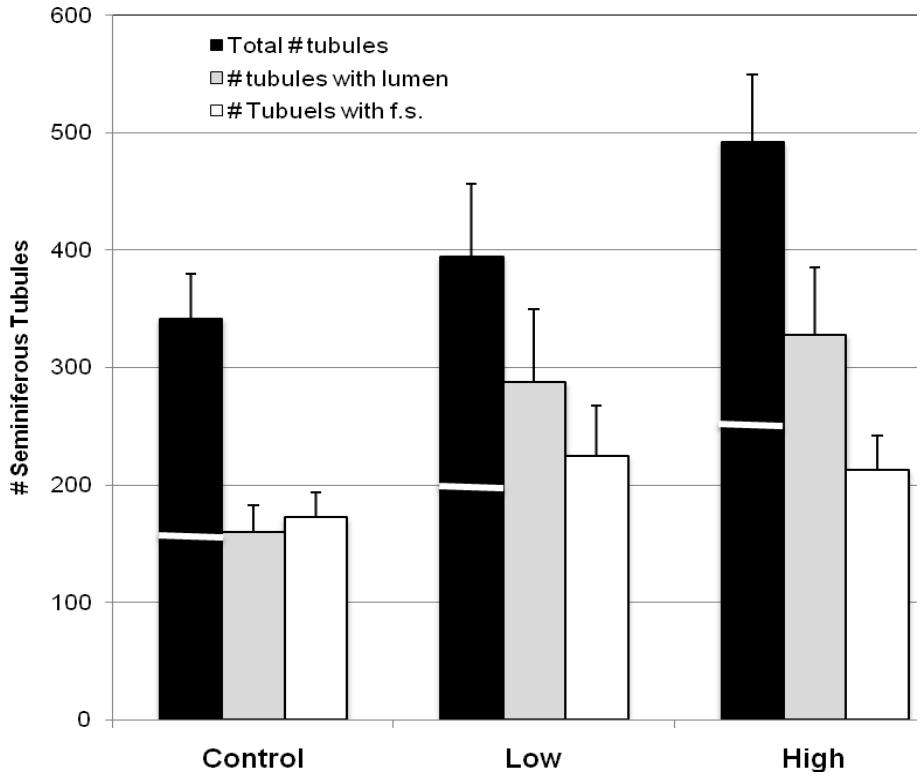

Fig. 7. The number of seminiferous tubules in cross-sections of the left testis to illustrate the proportion of tubules containing lumen or final spermatids (f.s.) in unpaired male control, low and high in ovo DE-71 exposure American kestrels. The 50% mark in the total number of tubules in the cross section is marked with a white line. Both low and high exposure males had grater than 50% of the tubules containing lumen. Only high exposure males had less than half of tubules containing f.s..

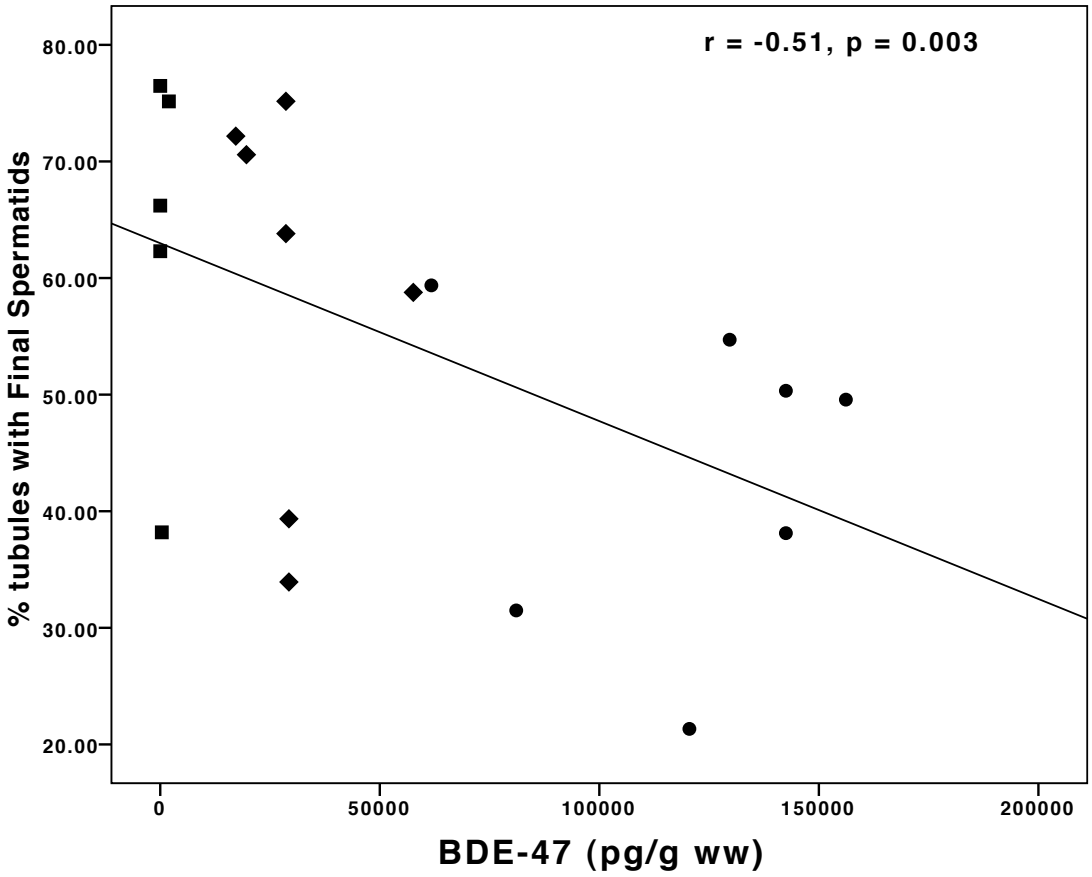

Fig. 8: The percent of seminiferous tubules with final spermatids (f.s.) in relation to the BDE-47 *in ovo* exposure concentrations for the F_1 control (■), low-exposure (◆) and high-exposure (●) male American kestrels (p = 0.003). Seminiferous tubules were counted in histological sections of the left testis, collected from unpaired males in the fertile period.

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CONNECTING STATEMENT 3

The previous two chapters outline the impacts of in ovo exposure to polybrominated diphenyl ethers (DE-71 mixture), research that followed previous investigations on the effects of dietary exposure to the parents of the same study subjects. This research confirmed the multigenerational outcomes of exposure to DE-71 and has provided substantial insight into the reproductive effects of PBDEs and potential relationships with HBCD. However, these findings highlighted the need to investigate similar endpoints in other, more current BFRs such as hexabromocyclododecane (HBCD). Additionally, it was imperative that the effects of HBCD alone be examined to identify reproductive outcomes that may be attributed to individual effects of this BFR, versus synergistic or additive effects with PBDEs. The following chapter presents the effects of diet exposure to environmentally relevant levels of technical HBCD on reproductive success and behavior in breeding American kestrel pairs.

CHAPTER 4

DIETARY EXPOSURE TO TECHNICAL HEXABROMOCYCLODODECANE (HBCD) ALTERS BREEDING BEHAVIOR AND REPRODUCTIVE SUCCESS IN CAPTIVE AMERICAN KESTREL (*FALCO SPARVERIUS*) PAIRS

Abstract

Hexabromocyclododecane (HBCD) is a high production volume brominated flame retardant in current use that has been detected in the environment and wildlife at increasing concentrations. Though some of the highest recorded levels have been found in the tissues of birds of prey, the impact of HBCD exposure on reproduction in birds is unknown. The objectives of this study were to determine the effects of dietary exposure to environmentally relevant levels of HBCD on reproductive success and behavior in captive American kestrels. Twenty kestrel pairs were exposed daily to 0.32 μg/μl wet weight per day of technical HBCD mixture from 4 weeks prior to pairing until chicks hatched. Ten pairs of controls, receiving the safflower oil vehicle only, were used for comparison. HBCD-exposed pairs initiated egg-laying earlier than controls by an average of 5 days ($p = 0.009$) and had larger clutch sizes by an average of one egg ($p = 0.008$). However, there was no associated increase in the number of fertile eggs, nor in the number of young produced (hatchlings or fledglings), and fewer pairs initiated a second clutch compared to controls. Additionally, females exposed to HBCD laid eggs that were 5.5 % smaller than those of controls ($p = 0.001$). Some important courtship behaviors were reduced in both sexes, including vocalizations and nest-inspections ($p < 0.050$). Males also displayed reduced frequencies of key parental behaviors, including entering the nest-box, food-retrievals, and pair-bonding displays ($p < 0.040$). This study demonstrates that HBCD exposure influences courtship behavior and reproductive success in birds.

Introduction

Hexabromocyclododecane (HBCD) is a high production volume brominated flame retardant (BFR) used in commercial products such as polystyrene foams and textiles, and is one of the three major BFRs in current use today (www.bsef.com). HBCD has been used since the 1980s, and despite its short half-life, is a ubiquitous persistent organic pollutant that is currently under review by the United Nations Environment Program's Stockholm Convention as of 2009. The technical mixture of HBCD consists of three isomers (α-HBCD, β-HBCD, and γ-HBCD). The mixture is dominated by γ-HBCD (80%,), which is found in the highest concentrations in abiotic environmental compartments. However it is the α-HBCD isomer that has been shown to accumulate in animal tissues (reviewed in: [1]).

HBCD is lipophilic and bioaccumulative and has been detected in humans and wildlife globally (reviewed in [2]). Though current levels in wildlife are lower than other BFRs such as the polybrominated diphenyl ethers (PBDEs), HBCD concentrations are increasing in biota. For example, mean concentrations of α-HBCD in herring gull (*Larus argentatus*) eggs in Norway rose from 16 ± 9 ng/g lipid weight (lw) in 1983 to 108 ± 48 ng/g lw in 2003 [3] and concentrations of ΣHBCD in the eggs of guillemots (*Uria algae*) in the Baltic Sea rose from 34 to 140 ng/g lw between 1983 and 2001 [4]. Some of the highest HBCD levels have been recorded in raptorial birds, including the maximal ΣHBCD concentration ever recorded (39 000 ng/g lw) in a peregrine falcon (*Falco peregrinus*) egg [5].

The reproductive effects of HBCD exposure in animals are still not well understood. HBCD can bind competitively in vitro to estrogen, androgen, progesterone, and thyroid receptors, resulting in inhibition of these steroidal pathways [6]. Additionally, liver biosynthesis of cholesterol was down-regulated and metabolism of estrogen was up-regulated in rats exposed to technical HBCD, which could result in deregulation of steroid hormone processes with prolonged exposure [7]. Furthermore, HBCD has demonstrated neurological toxicity and can inhibit the re-uptake of dopamine in the neurons of the brain [8]. Despite these properties no significant evidence for in vivo sex steroid disruption has been shown in fish [9] and mammalian in vivo models [10]. Specifically, reproductive output and behavior were not affected even at high exposure levels in rats (maximum 1363 mg/kg daily [10-13]), and similar results have been noted for fish (*Platichthys flesus*) exposed via food and sediment (8000µg/g total organic carbon [9]).

However, exposure to HBCD can alter thyroid homeostasis as evidenced by reductions in circulating thyroxine (T_4) and increases in thyroid gland weight in rats [10, 13] and reduced circulating T_4 and increased follicular cell height in fish [14]. Thyroxine is critically important in controlling reproductive cycles in seasonally breeding species (reviewed in: [15]), and dopamine is an important neurotransmitter in the expression of reproductive behaviors of birds [16]. Thus, birds exposed to HBCD may be differentially sensitive to reproductive alterations than rats. Few studies have examined the effects of HBCD exposure in birds. Chicken embryos exposed to 100 or 10,000 ng/g egg weight of technical HBCD via injection into the egg's air sac demonstrated decreased hatching success, where only 35% and 64% of embryos

successfully hatched, respectively [17]. As well, in previous multi-generational research with American kestrels (*F. sparverius*) exposed to the DE-71 polybrominated diphenyl ether (PBDE) technical mixture, some accidental exposure to HBCD occurred at concentrations that were 100 times lower than the intended ΣPBDE exposure [18-20]. These HBCD exposure concentrations were associated with some reproductive endpoints, including eggshell thickness and earlier laying dates in the F_0 diet-exposed females [18], as well as depressed copulatory behavior, reduced fertility, and clutch size in their developmentally exposed F_1 offspring [20]. The design of these kestrel-PBDE studies did not provide a means to determine whether HBCD was directly associated with some of these reproductive effects or whether it may have acted synergistically or additively with the PBDE congeners and further investigation is warranted.

The overall aim of the present study is to determine the effects of dietary exposure to environmentally relevant levels of HBCD on the reproductive success and behavior of birds, using captive American kestrels exposed during breeding and seasonal gonadal development (recrudescence). The same endpoints and methods used in the previous PBDE-kestrel research [19, 20] will be examined for comparison, as well as to isolate the potential effects of exposure to technical HBCD alone. The levels of HBCD exposure in the present study are environmentally relevant, and represent a ten-fold increase from the highest category in the previous research [18]. This is the first study to publish the reproductive and behavioral effects of HBCD exposure in breeding birds.

Methods

The present study used captive-bred American kestrels with documented histories of all individuals housed at McGill University. Paired birds were placed in breeding chambers (1.0 m x 2.4 m x 2.4 m) with a nest-box and one-way glass window for observation (0.1 m x 0.3 m x 0.006 m). Birds were subjected to natural temperature fluctuations and photoperiod, and fed day-old frozen-thawed cockerels (*Gallus domesticus*) ad libitum prior to daily behavioral data collection. The treatment and care of the kestrels was conducted in accordance with the Canadian Council on Animal Care Guidelines [21] and was approved by the Animal Care Committee of McGill University (#5548).

In April 2008, 31 kestrel pairs consisting of previously unexposed males and females were randomly assigned to one of two groups: control (n_C = 11) or HBCD-exposed (n_{HBCD} = 20). Pairs were randomly assigned to either group and the age and experience of the individuals were split evenly between them. Exposed pairs were subjected to 54.4 µg of technical HBCD formulation purchased from Wellington Laboratories (Guelph Ontario), which was dissolved in safflower oil (0.544 µg/µl or 800ppm) and injected into the brains of cockerels daily; control pairs were exposed to the vehicle only (following [19]). Exposure began 4 weeks prior to pair formation, and continued throughout the courtship period. During this time the birds were undergoing physiological reproductive development (recrudescence) for seasonal breeding, which is a sensitive window for exposure to endocrine disruptors [22]. The exposure then continued through incubation until two days before chicks hatched, providing a mean

exposure period of 75 days. HBCD exposure concentrations were environmentally relevant and reflected the levels recorded in the eggs of wild peregrine falcons [23].

The first egg in each clutch was analyzed for α, β, γ HBCD concentrations at the National Wildlife Research Centre (Letcher lab). Eggs were stored frozen (-20°C) and thawed to room temperature for analysis. Approximately 0.1 – 1 g of tissue homogenate were thoroughly mixed with diatomaceous earth and spiked with 100 ul of the internal standard solution (Mα, β, γ-HBCD). The HBCD isomers were extracted by accelerated solvent extraction (ASE: Dionex Company) using dichloromethane/hexane. One ml of the extract was used to determine the lipid concentration (%). Samples were then cleaned up with sulfuric acid silica and the α, β, γ HBCD isomers were detected using high performance liquid chromatography (Waters Alliance 2695) with an electrospray source (HPLC-ESI-MS/MS). For quality assurance, a blank sample, undergoing the whole procedure but without the tissue was included, and an in house egg reference material (double crested cormorant, [24]) were analyzed with each batch. A five-point linear calibration curve was performed daily, from which HBCD isomer concentrations were calculated using QuanLynx 4.0. The recovery of the internal standard was verified by an external standard recovery method. The eggs of exposed pairs were dominated by α-HBCD (90.9% of ΣHBCD), but β-HBCD (7.7%) and γ-HBCD (1.4%) were also detected; background levels of all three isomers were detected at similar levels in control eggs, the means of which are reported in Table 1 (Letcher and Fernie, Environment Canada, unpublished data).

Data collection

Throughout the breeding season, all reproductive parameters were recorded, including dates of laying, hatching and fledging for each chick. All individual eggs were weighed when first laid, and length and width measurements were recorded with the use of digital calipers, from which volume was calculated [25]. At mid-incubation, all eggs were candled to determine their fertility by the presence of an embryo. Females were weighed at pairing, twice during incubation and twice during brood rearing. Male body mass is recorded elsewhere (Marteinson et al. Ch 5, this volume). Eggshell thickness was determined at five points around the equator of the first egg in each clutch, from which a mean was calculated [26].

Reproductive measures of the pairs were calculated following Fernie *et al.* [18] and include: Julian lay date, the lay lag (number of days from pairing to egg laying), clutch size (number of eggs in the first clutch of a pair), fertility (the total number of fertile eggs in the clutch) and percent fertility (percent of eggs laid that were fertile per pair), hatching and fledging success (the total number of hatchlings or fledglings produced per pair), and overall reproductive success (the number of eggs that produced fledglings).

Following Marteinson et al. [20], reproductive courtship behaviors were recorded from the second day after pairing until the first egg was laid. Brood-rearing behaviors of all pairs producing young were recorded for 20 days after the first chick hatched. Each pair was observed in random order, twice daily (morning, afternoon) during courtship, and once daily (morning) during brood-rearing to minimize disturbance. Their proximity to each other, the incidences of courtship and brood-rearing behaviors (copulation, nest-

inspections, food-transfers, bonding-displays, flight-displays, vocalizations) [27], activities (feeding, perching, flying, preening), and their location within the pen were recorded (Table 2).

Statistical analysis

Reproductive measures were analyzed for statistically significant differences between the two treatment groups using analysis of variance (ANOVA), with the Julian lay date as a covariate. Results are reported only for significant treatment effects. Nineteen additional control pairs from 2005-2006 (found to be statistically similar to the 2008 controls with a t-test) were included to increase the sample size. The difference in egg volume between the groups was analyzed by nested one-way ANOVA with the clutch laid by each female as the nested variable. Female body mass was analyzed by t-tests between the two groups, and by a repeated measures ANOVA for differences in body mass patterns over time.

Following Marteinson et al. [20], mean behavioral frequencies were calculated for each individual bird (total number of incidences divided by the number of behavioral samples). Means were calculated for the entire courtship period, as well as the 5 days after pairing, and the 5 days prior to egg-laying. For the brood-rearing period, behavioral means for each pair were calculated for the entire period as well as the first and second 10-day periods. Additional control pairs from 2007 (statistically similar to the 2008 controls) were included to balance the statistical designs: 7 for the courtship period, and 4 for the brood-rearing period. Control pairs from 2007 were used in this behavioral component because of the similarities in observation methods between these two studies

but which differed from the methods used in the 2005 and 2006 DE-71 kestrel behavioral study [19].

For the courtship period, only pairs that laid eggs were included in the analysis (n_C = 17, n_{HBCD} = 18 pairs), and for the brood-rearing period, only pairs that produced young that successfully fledged were included (n_C = 12, n_{HBCD} = 12 pairs). Data were tested for normality and homogeneity of variance, and log- or square root-transformed when necessary.

For behaviors that were normally distributed for both sexes, 2-factor (treatment and sex) ANCOVAs were used to determine significant differences between the mean behavior frequencies, with the interaction term (treatment × sex) used to determine if males and females were differentially affected by HBCD exposure. When the behavioral data were normally distributed for one sex only, the data were then analyzed using General Linear Model (GLM) procedures equivalent to a one-way, one-factor ANCOVA. When data were not normal, Mann-Whitney U non-parametric tests were used to identify statistically significant differences between the two treatment groups. For all GLM behavioral procedures, the covariate of Julian lay date for the courtship period, and the covariates of Julian hatch date and the number of chicks per pair for the brood-rearing period were included. The Julian dates account for both the timing of breeding, which can be related to behavior, as well as the varying amount of exposure received by the pair. The covariate of Julian lay date was not used for the 5 days after pairing since there was no variation in exposure at this time. Correlation analyses were conducted between reproductive parameters, some behavior frequencies of both sexes, and concentrations of HBCD (α, β, γ and their sum) measured in the first egg as an estimate of exposure to the

pair. Pearsons's correlation analysis was used when data were normally distributed, otherwise Spearman's Rank correlation analyses were used. For all statistical analyses, significance was considered to be at $p \leq 0.05$.

Results

Reproductive success

HBCD-exposed pairs initiated their clutches earlier than controls by an average of 5 days ($p = 0.009$), and had larger clutch sizes with a median of 6 eggs compared to 5 eggs in control clutches ($p = 0.008$) (Table 3). This increased clutch size was associated with the earlier lay dates (Spearman: $r_s = -0.39$, $p = 0.036$) as well as the concentrations of α-HBCD ($r_s = 0.36$, $p = 0.054$) in the first egg. However, there was no corresponding increase in fertility or reproductive success associated with these larger clutch sizes, (Table 3) nor was there any difference in the sex ratio of chicks produced. Although HBCD-exposed pairs initiated egg-laying earlier than controls, they seemed less likely than controls to lay a second clutch of eggs, i.e. only one HBCD-exposed pair (5%) initiated a second clutch compared with 27.3 % of control pairs. Body mass did not differ between HBCD-exposed and control females.

The first egg in each clutch was smaller for HBCD-exposed females both in weight ($p < 0.001$) and volume ($p < 0.001$) (Table 3) by approximately 5.5%, the size of which was associated with in ovo β-HBCD ($r_p = -0.37$, $p = 0.046$) and ΣHBCD concentrations ($r_p = -0.40$, $p = 0.046$). Eggshell thickness was similar between HBCD-exposed and control groups (Table 3).

Courtship behavior

Courtship vocalizations were affected by HBCD exposure. Males made fewer chitter-calls (GLM: $F_{1,32} = 4.31$, $p = 0.046$) and marginally fewer whine-calls ($F_{1,32} = 3.96$, $p = 0.055$) in the 5 days after pairing. During the whole courtship period, both males and females made fewer chitter-calls than respective controls (2-factor ANCOVA: $F_{4,65} = 3.31$, treatment main effect $p = 0.050$) and in the 5 days before egg-laying, this trend continued for males ($F_{1,29} = 4.31$, $p = 0.047$) (Fig. 1). Additionally, HBCD-exposed females performed fewer bonding-displays than controls (GLM: $F_{1,32} = 6.92$, $p = 0.013$) in the initial 5 days after pairing. While the vocalization frequencies were not correlated between members of a pair, the number of times a male vocalized was positively associated with the frequency of bonding-display performances by their mates over the whole courtship period (Pearson: chitter-call: $r_P = 0.56$, $p = 0.003$; whine-call: $r_P = 0.50$, $p = 0.008$). The number of chitter-calls performed by the male was related to the mass of the first egg produced by their mate ($r_P = 0.46$, $p = 0.019$). There was no difference in copulation frequency between control and HBCD-exposed pairs at any time point.

During the overall courtship period, exposure to HBCD altered the activity budgets: both males and females ($n_C = 17$, $n_{HBCD} = 18$ pairs) demonstrated decreases in the amount of time spent feeding (2-factor ANCOVA: $F_{4,65} = 4.49$, treatment main effect $p = 0.038$) and in preening ($F_{4,65} = 5.54$, treatment main effect: $p = 0.022$). As a result, both sexes were more inactive when compared to controls (corrected $F_{4,65} = 14.08$, $p < 0.001$; treatment main effect $p < 0.001$) (Fig. 2).

Brood-rearing behavior

Males exposed to HBCD demonstrated a reduced affinity towards their broods compared to control males. During the first 10 days after the chicks hatched (n_C = 12, n_{HBCD} = 12 pairs), HBCD-exposed males entered the nest-box less often than controls and HBCD-exposed females compensated by entering the nest-box more often (treatment × sex p = 0.004) in a directly inverse relationship when compared to controls (Fig. 2). During this time period, HBCD-exposed males also spent less time engaged in pair-bonding behaviors ($U_{12,12}$ = 40.5, p = 0.036).

During the second 10 days after the chicks hatched (n_C = 12, n_{HBCD} = 12 pairs), when emphasis shifts to activities outside the nest-box, HBCD-exposed males performed fewer bonding-displays and their female mates conversely performed more displays compared to control pairs (2-factor ANOVA: corrected p = 0.027) (Fig. 3). HBCD-exposed females also solicited food-transfers more often (Mann-Whitney: $U_{12,12}$ = 40.0, p = 0.031), indicating that females may have still been compensating for reductions in male behaviors. That HBCD-exposed males performed fewer bonding-displays towards the end of the brood-rearing period (Fig. 3) may also be related to the reduced initiation of a second clutch.

Males exposed to HBCD also demonstrated a reduced motivation for provisioning the young compared to controls. When all 20 days of brood-rearing were analyzed together, HBCD-exposed males retrieved food half as much as control males, while HBCD-exposed females compensated with slight increases when compared to controls (2-factor ANOVA, treatment × sex p = 0.025) (Fig. 4). This reduced food

retrieval behavior in males was associated with the concentrations of α-HBCD (Pearson: r_p = -0.49, p = 0.040) in the first egg laid by their mate. HBCD-exposed males also spent less time flying than did control males ($U_{12,12}$ = 35.5, p = 0.032).

Discussion

The results from the present study demonstrate that HBCD, like other organohalogen pollutants including PBDEs and PCBs [18, 19, 28], can alter the reproductive success and behavior of American kestrels. Furthermore, the mean sum concentrations of HBCD determined in the eggs of the HBCD-exposed pairs (163.5 ± 75.1 ng/g wet weight) are comparable to those recorded recently in the eggs of peregrine falcons [23]. Captive kestrel pairs exposed to technical HBCD via their diet during reproductive recrudescence initiated egg laying earlier and had larger clutches than controls. However, reproductive success was not subsequently augmented: pairs did not demonstrate any increase in fertility or additional young, nor an increased propensity for initiating a second clutch. Additionally, HBCD-exposed females produced smaller eggs compared to controls. Both members of the pair performed fewer important courtship behaviors and males additionally displayed reductions in brood-rearing behaviors when compared to controls.

The present study is critical to understanding the possible effects of the unintentional HBCD contamination in previous research where the DE-71 PBDE mixture was the targeted substance under examination [18-20]. In these studies, concentrations of HBCD were statistically correlated with decreased eggshell thickness and the delayed laying dates in diet-exposed females [18]. Depressed copulatory behavior, reduced

fertility and smaller clutch sizes in the developmentally exposed male offspring were also correlated with HBCD in ovo exposure concentrations [20]. With only HBCD exposure in the present study, the opposite was found for the timing of breeding and number of eggs produced, while copulation frequency and egg fertility were not affected. Therefore, the exposure to much lower HBCD concentrations may not have had a direct effect on these endpoints in the DE-71 exposure study. However, for some markers, results from the two studies parallel one another, including the reduction of other courtship behaviors, decreased egg size and the depressed brood-rearing behavior of males. Thus, although the accidental HBCD exposure in the previous research was 10 times lower than the exposure levels used in the present study, synergistic or additive effects with DE-71 cannot be ruled out entirely.

Timing of breeding and reproductive success

Though breeding early can often be advantageous [29], beginning too soon can also lead to reduced reproductive output in wild birds [30]. The onset of breeding in seasonal birds is highly evolved for aligning reproduction with ideal environmental conditions. Brood-rearing is timed with peaks in prey availability and shifts in this synchrony can be accompanied by food shortages that stress both the parent's body condition and the growth of the nestlings [31]. Climatic conditions also play an important role in reproductive success. Exposure to inclement weather, for example, is associated with poorer nest success and decreased chick growth in birds, including kestrels [32]. This can become a critical issue if pairs breed too early in the spring when colder and wetter weather prevail. The timing of reproduction in seasonally breeding birds is

intrinsically regulated by photoperiodic stimulation of the hypothalamo-pituitary-gonad axis in combination with thyroid hormones (reviewed in: [33]), suggesting that one of these axes may have been affected by exposure to HBCD. In the same HBCD-exposed study males, testosterone was increased and thyroxine was decreased compared to controls, confirming this possibility (Marteinson et al. Chapter 5, this volume).

Females exposed to technical HBCD in the present study demonstrated a reduction in egg mass and volume when compared to controls, similar to females exposed via diet to DE-71 [18]. In birds (reviewed in: [34]), including wild American kestrels [35], smaller eggs are associated with reduced hatching success and smaller nestling size [36], which can result in reduced survival [37]. A female's investment in her eggs is an important factor determining the reproductive success of the pair, and the smaller eggs of the birds exposed to HBCD or PBDEs may represent a detrimental effect of exposure to BFRs, particularly if they become even smaller as exposure levels rise. Several factors can decrease egg size, including reduced body condition of the female (reviewed in: [38]), reduced social cues from the male partner [39, 40], and alterations in endocrine regulation [41-43]. HBCD-exposed females did not demonstrate weight loss at any time point during the study and had access to an unlimited food supply, suggesting alternative factors.

Courtship behavior

The reductions in courtship behavior observed in both members of the pairs exposed to HBCD in the present study suggests a reduction in the quality of the pair bond as a result of that exposure, a phenomenon also seen in pairs exposed to DE-71 [19].

Social cues are critically important for successful reproduction in birds. The behavior of the male in particular greatly influences sexual readiness and associated behavioral responses in their female mates [44, 45]. Even egg size can be influenced by the quality of the male partner [39, 40]. Indeed, when only males are exposed in ovo to DE-71, their unexposed female partners also displayed an associated reduction in their courtship behaviors, laid smaller eggs, and fewer females initiated clutches at all [20]. In the present study, reductions in courtship behavior were noted more strongly in males, though less dramatically so, and reductions in chitter-calls made by HBCD-exposed males were associated with decreased display behavior and reduced egg mass in their female partners. However, the size of eggs and number of whine-calls produced by females were also correlated with the HBCD concentrations detected in their first eggs, suggesting that females were affected by HBCD exposure independently as well.

Sexual behaviors are controlled by androgens in males and courtship, nest-building and sexual receptivity are regulated by estrogen and progesterone in females [46-48]. Exposure to androgen-inhibiting agents [49] or agents (e.g., drugs) that block dopamine re-uptake in the brain [16, 50] have been shown to cause declines in sexual motivation in males. Although little is known for female birds, exposure to anti-estrogenic chemicals reduced sexual behaviors in female rats (RU 58668:[51]). Since HBCD has demonstrated the ability to inhibit both the receptors of sex hormones [6] and the re-uptake of dopamine by neurons in vitro [8], as well as enhancing the hepatic metabolism of estrogen in vivo in rats [7], some impact on any or all of these axes in the kestrels may have occurred.

Brood-rearing behavior

The present study adds to the growing evidence that exposure to organic pollutants in birds can decrease brood-related behaviors such as incubation [52, 53], nest defense [54, 55], nest-attentiveness [52, 56] and provisioning [56, 57], including in wild falcons [54]. Little data exist on the effects specifically for brominated flame retardants (BFRs); however, in wild glaucous gulls (*L. hyperboreus*), serum prolactin levels were negatively associated with PBDE body burdens in males [58], and American kestrel males exposed in ovo to DE-71 performed fewer parental behaviors when paired with unexposed females [20]. In the present study, HBCD-exposed males demonstrated reduced motivation for provisioning the young during the first 10 days after the chicks hatched when compared to controls. During this critical period, the male is the main provider of food, which allows the female to remain in the nest-box to thermoregulate the chicks [59]. Reductions in provisioning the young at this time could be detrimental to the success of the brood, particularly in the wild where birds do not have exposure to unlimited food sources and are at risk for predation of nestlings.

The inverse relationship between the number of times males and females entered the nest-box suggests that females were compensating for the male's lack of attentiveness to the brood. Such responses are not uncommon in avian species where both sexes provide food for the young [60, 61]. However, in the wild, females may be unable to compensate effectively or completely because food resources are limited, and chick growth and survival can be compromised as a result [57]. Indeed, in the present study, despite the female's compensatory behavior, the nestlings of HBCD-exposed pairs grew more slowly (Fernie et al., Environment Canada, unpublished data), possibly due to

inadequate male provisioning in the first 10 days. However, these effects were not critical enough to reduce the fledging success of the young at the exposure levels used in this study.

Parental care in mammals and birds is regulated by the hormone, prolactin (reviewed in: [62-64]). Females may be less susceptible than males to disruptions in parental behavior and in general, they maintain higher concentrations of prolactin than males [58, 65]. Since female kestrels normally incubate for 80% of the time [66], they receive more positive feedback by tactile stimulation from the brood patch by contact with the eggs, which perpetuates incubation [65, 67] and promotes parental behaviors thereafter [68]. Testosterone can inhibit pituitary prolactin secretion in birds (reviewed in: [69]). Thus, the elevated levels of testosterone in HBCD-exposed males in the present study, which were associated with the reduction in parental care (as reported elsewhere: Marteinson et al., Ch. 4, this volume), may have contributed to the contrasting effects noted for males and females.

Conclusion

The present study demonstrates that exposure to HBCD alone can affect reproduction in American kestrels at levels that are environmentally relevant. Though HBCD-exposed pairs produced more eggs than controls, which usually improves reproductive success [29], there was no subsequent increase in either fertility or production of young. Several endpoints were affected in ways that may be related to this fact, and that could prove to be detrimental for birds living in the wild. First, breeding may be misaligned with prime environmental conditions and peaks in food availability

for the optimal production of young. Second, HBCD-exposed females produced smaller eggs, which can decrease their reproductive fitness. Third, courtship behaviors were reduced in both sexes, and these are critical in establishing the pair-bond and inducing physiological readiness to breed. Fourth, males demonstrated a reduced propensity for provisioning the young during the critical period of brood-rearing, which can affect chick growth rates and their potential survival. Since the levels of HBCD in the present study are similar to those recorded recently in peregrine falcons [23], similar effects may also be occurring in free-living birds.

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Table 1. Mean hexabromocyclododecane (HBCD) isomer concentrations (ng/g wet weight) determined in the first egg of American kestrel pairs exposed to technical HBCD via diet (Letcher and Fernie, Environment Canada, unpublished data), and the background levels detected in the first egg of control pairs.

	ΣHBCD		α-HBCD		β-HBCD		γ-HBCD	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control eggs	0.3	0.4	0.2	0.1	0.2	0.1	0.6	0.5
HBCD-exposed eggs	163.5	75.1	13.9	5.6	2.6	3.8	179.9	80.6

Table 2: Reproductive behaviors observed during focal samples for male and female captive American kestrels throughout the courtship and brood-rearing periods (following [59]).

Behavior	Description
Bonding-display	Involves bowing forward with head tucked in and running or hopping along perch. Directed towards mate or young.
Copulation	Including all copulatory attempts where males mount the female.
Nest-inspection	Enters nest-box, or perches in the opening.
Chitter-call	Trilling call used in pair-bonding, and brood-rearing.
Whine-call	Food and affiliation-related call that sounds like a nestling begging.
Bring-food-to-nest	When adult brings food into the nest box.

Table 3: Reproductive parameters of captive American kestrels exposed via diet to control vehicle (safflower oil) or environmentally relevant levels of hexabromocyclododecane (HBCD) (one-way ANOVA).

				Control pairs		HBCD-exposed pairs	
Reproductive Variable	*F-value*	*df*	*P*	Mean	SE	Mean	SE
Days to egg laying	7.43	2	0.009	18.19	1.63	12.79	1.07
Reproductive Success							
Clutch size (# eggs laid)	7.75	2	0.008	5.00	0.21	5.63	0.14
Total # fertile eggs	0.14	2	0.780	3.50	0.27	3.68	0.38
Total # hatchlings	0.79	2	0.381	2.63	0.24	3.16	0.44
Total # fledglings	1.34	2	0.254	2.44	0.34	3.11	0.43
Overall hatching success	0.42	2	0.519	0.65	0.08	0.74	0.09
Overall reproductive success	0.072	2	0.401	0.72	0.08	0.83	0.86
Fertility (%)	0.08	2	0.780	0.81	0.07	0.80	0.07
Egg Variable							
Mass (g)	13.42	39	<0.001	16.26	0.12	15.37	0.09
Volume (cm^3)	13.57	39	<0.001	16.59	0.10	15.54	0.11
Shell thickness	1.09	27	0.306	0.179	0.00	0.183	0.003

For the number of days to egg laying and for the reproductive success parameters i.e. clutch size to fertility, $n_C = 29$, $n_{HBCD} = 20$ which includes pooled control pairs from 2005 - 2008. For egg mass and volume, $n_C = 17$, $n_{HBCD} = 20$ and for eggs, $n_C = 89$, $n_{HBCD} = 106$ which includes pooled control pairs form 2007-2008. For eggshell thickness $n_C = 10$, $n_{HBCD} = 19$ from 2008 pairs only.

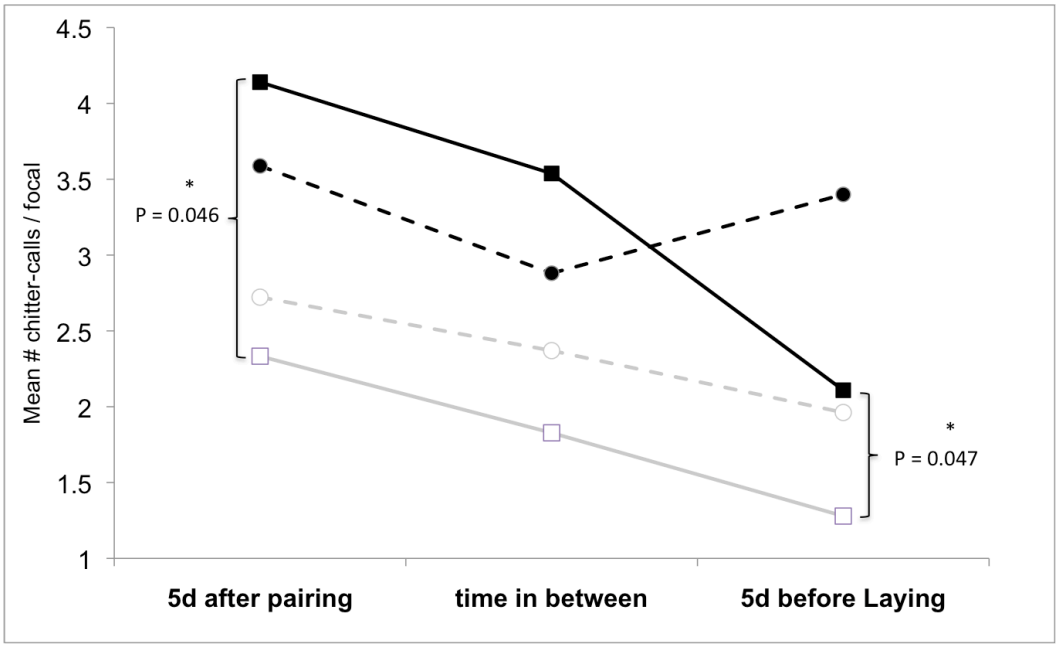

Fig. 1. Mean chitter-call frequencies per focal sample during the first 5 days and last 5 days of the courtship period for HBCD-exposed males (□) and female (○) as well as control male (■) and female (●) American kestrels. Each time point was analyzed separately for each sex using an ANOVA.

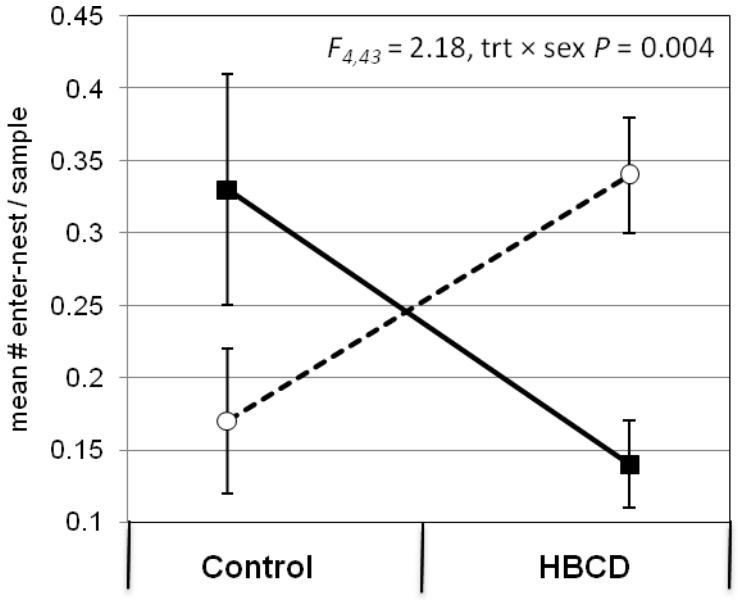

Fig. 2: Frequency of entering the nest in the first 10 days after the nestlings hatched for males (■) and females (○) in HBCD-exposed and control American kestrel pairs (2 factor ANOVA: $n_C = 12$, $n_{HBCD} = 12$).

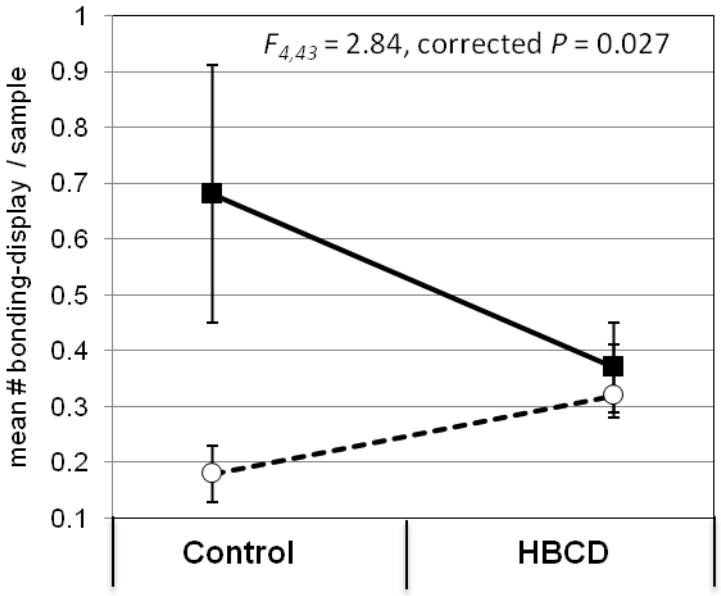

Fig. 3: Frequency of bonding-displays in the second 10 days after the nestlings hatched for males (■) and females (○) in HBCD-exposed and control American kestrel pairs (2 factor ANOVA: $n_C = 12$, $n_{HBCD} = 12$).

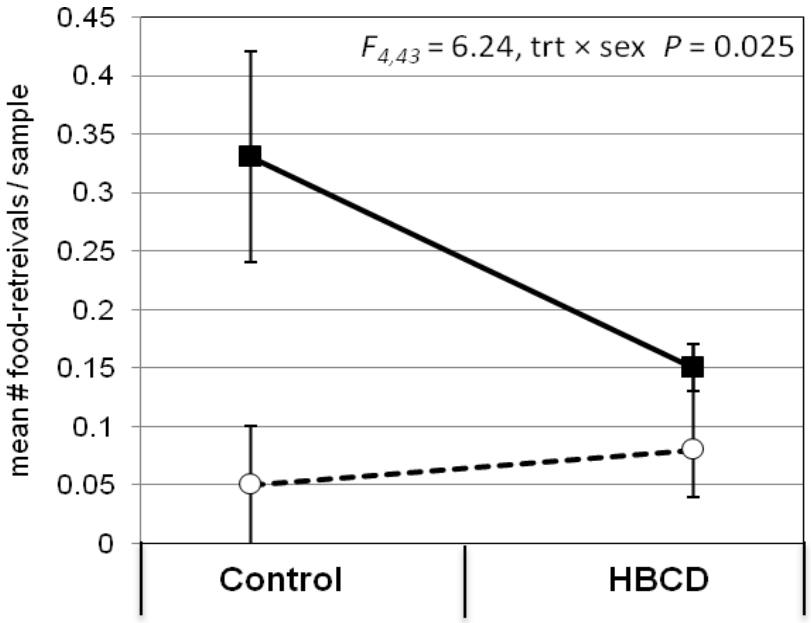

Fig. 4: Frequency of food retrievals in the entire 20 days after the nestlings hatched for males (■) and females (○) in HBCD-exposed and control American kestrel pairs (2 factor ANOVA: $n_C = 12$, $n_{HBCD} = 12$).

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CONNECTING STATEMENT 4

The previous section outlined the impact of diet exposure to HBCD on reproductive success and behavior of American kestrel pairs and demonstrated that their reproduction had been altered. To further investigate the effects of dietary exposure to HBCD on males, circulating testosterone and thyroid hormones as well as testicular physiology were examined in the same study individuals for whom behavior and reproductive success had been determined in the previous chapter. During the same reproductive cycle, blood samples were taken at strategic time points and analyzed for hormone concentrations, body mass and sperm numbers were also assessed and are reported in the next chapter. Additionally a second subset of males in unpaired status were sacrificed for testicular histology by the same methods used in the DE-71 in ovo experiment that were presented in Chapter 3.

CHAPTER 5

DIET EXPOSURE TO TECHNICAL HEXABROMOCYCLODODECANE (HBCD) AFFECTS TESTES AND CIRCULATING TESTOSTERONE AND THYROXINE LEVELS IN CAPTIVE AMERICAN KESTRELS

Abstract

Hexabromocyclododecane (HBCD) is a high production volume brominated flame retardant that is used in products such as polystyrene foams. HBCD has been detected in the environment and in wildlife tissues globally, with some of the highest levels recorded in predatory birds. This study examined the effects of environmentally relevant levels of HBCD exposure on the reproductive physiology of unpaired male American kestrels as well as ones paired with similarly HBCD-exposed females. Two groups of captive males were exposed daily to 0.32 μg/μl wet weight of technical HBCD injected into their food during testicular recrudescence. The first set of unpaired males (n_c = 12, n_{HBCD} = 10) was euthanized three weeks later for testes and sperm analysis. Males from the second group (n_c = 10, n_{HBCD} = 20) were exposed to HBCD, beginning 4 weeks prior to pairing with females receiving the same exposure, until 2 days before hatching of young. For the unpaired males, those exposed to HBCD versus controls had heavier testes ($p \leq 0.017$). The left testis had more seminiferous tubules containing elongated spermatids (p = 0.052) than did controls, and there was an associated increasing trend in plasma testosterone concentrations (p = 0.056). The testosterone levels of the HBCD-exposed breeding males increased during the courtship period to culminate in higher levels than controls by the time the first egg was laid (p = 0.010), and both free and total T_4 were reduced throughout ($p < 0.05$). The number of sperm reaching the perivitelline layer of the first egg did not differ between HBCD-exposed and control pairs. This study shows that HBCD exposure at environmentally relevant levels can alter reproductive physiology in male American kestrels.

Introduction

Hexabromocyclododecane (HBCD) is a high production volume brominated flame retardant (BFR) used in commercial products such as polystyrene foams and textiles, and is one of the major three BFRs in current usage [1]. HBCD is lipophillic and bioaccumulative and has been detected in human and wildlife tissues globally; levels are especially high in species at the top of the food chain such as predatory birds (reviewed in [2]). The technical mixture of HBCD consists of three isomers (α-HBCD, β-HBCD, γ-HBCD) dominated by γ-HBCD (80%). Consequently, γ-HBCD is found to accumulate in the highest concentrations in the abiotic environment. However, α-HBCD predominates in animal tissue (reviewed in: [3]) and increases are being recorded in the eggs of wild predatory birds [4, 5]. The α- and γ-HBCD isomers have been shown in vitro to act as antagonists for androgens, estrogen and progesterone by binding to their receptors [6, 7] and all three isomers are thyroid receptor agonists [7]. Thus, HBCD has the potential to act as a reproductive endocrine disruptor and exposure may result in some alterations related to gonadal physiology in vivo, since both sex steroids and thyroid hormones are important regulators of the reproductive cycle.

The physiological effects of HBCD exposure are still not well understood, including those affecting the male reproductive tract. Adult male rats (F_0) exposed to technical HBCD at high doses (15,000 ppm) had decreased epididymides, reduced sperm counts, and lateral head displacement in sperm [8]. Conversely, F_1-generation rats developmentally exposed to HBCD via maternal transfer followed by dietary exposure during breeding demonstrated increased testicular mass, but no differences in sperm number or motility. Also, no alterations in testicular histology were noted in comparison

with controls [8]. Levels of thyroid hormone (T_4) and follicle-stimulating hormone (FSH) were reduced in F_0 male rats at high doses of HBCD (15,000 ppm and 1500 ppm, respectively). F_1 males exposed to high dose levels of HBCD (1500 ppm) demonstrated higher levels of 5-α-dihydrotestosterone (DHT) which accompanied increased testis mass compared to controls [8]. However, in another study on rats, developmental exposure to technical HBCD resulted in decreased testicular mass without affecting circulating thyroid hormone levels in either sex [9]. The contrasting results of these studies were attributed to the differing rat strains used [8]. Because effects were only seen at high doses, Ema et al. [8] postulated that HBCD did not have a major endocrine disrupting effect on sex steroid axes in rats. Few data exist for other vertebrates, though no changes were seen in behavior or gonad weight and histology in fish (*Platichthys flesus*) exposed to HBCD via food and sediment (8000μg/g total organic carbon) [10].

Birds are often more sensitive to chemical exposure than mammals. In contrast to findings in rats [8] and fish [10] exposed to HBCD, American kestrel (*Falco sparverius*) pairs exposed daily to technical HBCD (800 ppm) displayed a reduction in some pair-bonding behaviors in both sexes, decreased parental behavior in males, and smaller eggs laid by females in comparison to controls (Marteinson *et al.* Ch. 4, this volume). The timing of breeding was also affected in these kestrels, where pairs exposed to HBCD via their diet initiated egg-laying earlier and produced more eggs than controls but with no augmentation in overall reproductive success (Marteinson et al Ch. 4, this volume).

Since predatory birds have shown some of the highest recorded levels of HBCD in the wild [2] and appear to have greater reproductive sensitivity to HBCD exposure than other animal models [8, 10], it is critical to determine the specific effects of HBCD

on reproduction in these species. In the present study, testes mass and histology, circulating testosterone and thyroxine concentrations, and the number of sperm reaching the ovum were examined in captive male American kestrels exposed to technical HBCD via their diet. Effects relating to the female reproductive tract are reported elsewhere (Marteinson et al., Ch. 4, this volume). The present study is the first to report on these endpoints in any avian species and is the first to examine testicular histology in relation to HBCD exposure in birds.

Methods

The present study used captive-bred American kestrels with documented histories, housed at the Avian Science and Conservation Centre of McGill University. They were subjected to natural temperature fluctuations and photoperiod, and fed day-old frozen-thawed cockerels (*Gallus domesticus*) ad libitum. Exposure procedures followed those of previous studies on PBDEs [11]. All exposed birds were subjected to 54.4 μg of technical HBCD formulation purchased from Wellington Laboratories (Guelph Ontario), which was dissolved in safflower oil (0.32 μg/μl or 800ppm) and injected into the brains of cockerels daily; control pairs were exposed to the vehicle only (following [11]). Exposure concentrations of HBCD were environmentally relevant and based on levels recorded in the eggs of wild peregrine falcons (*F. peregrinus*) and herring gulls (*L. argentatus*) [12, 13]. The treatment and care of the kestrels was conducted in accordance with the Canadian Council on Animal Care Guidelines [14] and was approved by the Animal Care Committee of McGill University (#5548).

Tissue was analyzed for α-, β-, and γ-HBCD concentrations at the National Wildlife Research Centre (Letcher lab). Eggs and plasma were stored frozen (-20°C, and -80°C respectively) and thawed to room temperature for analysis. Approximately 0.1 – 1 g of tissue homogenate were thoroughly mixed with diatomaceous earth and spiked with 100 µl of the internal standard solution (Mα, β, γ-HBCD). The HBCD isomers were extracted by accelerated solvent extraction (ASE: Dionex Company) using dichloromethane/hexane. One ml of the extract was used to determine the lipid concentration (%). Samples were then cleaned up with sulfuric acid silica and the α-, β-, and γ-HBCD isomers were detected using high performance liquid chromatography (Waters Alliance 2695) with an electrospray source (HPLC-ESI-MS/MS). For quality assurance, a blank sample, undergoing the whole procedure but without the tissue was included, and an in house egg reference material was analyzed with each batch. A five-point linear calibration curve was performed daily from which HBCD isomer concentrations were calculated using QuanLynx 4.0. The recovery of the internal standard was verified by an external standard recovery method. The detection limits for α-, β- and γ-HBCD, were 0.61, 0.61 and 0.97 ng/g ww, and the replicate mean recoveries were 88 ± 5%, 97 ± 5% and 100 ± 5 %, respectively.

The study subjects

Unpaired males - At the end of April, following three weeks of exposure and a 3-week depuration period, 10 HBCD-exposed and 12 control males aged 1 to 9 years old were blood-sampled and euthanized for testes extraction. These males were euthanized during the fertile period, but were not housed in the presence of females, and are thus

labeled as unpaired males hereafter. The HBCD exposure level in these males was determined from a plasma sample at the time of euthanasia the means of which are presented in Table 1.

Breeding Males - Following 4 weeks of pre-pairing exposure, 20 HBCD-exposed and 11 control males labeled hereafter as breeding males, were paired with identically exposed, unrelated females. Pairs were randomly assigned to either group and males aged 1 to 9 years old were thus split evenly between the exposed and control groups. These breeding pairs were placed in separate chambers (1.0 m x 2.4 m x 2.4 m) and allowed to complete one reproductive cycle. The HBCD-exposure to breeding pairs continued exactly as described above throughout the courtship and incubation periods until two days before chicks hatched, for an average of 75 days of exposure. For a timeline of sample collection, see Fig. 1. These breeding males were not sacrificed because by the time young have fledged, testes regression is well underway [15] and spermatogenesis cannot be assessed [16]. All three isomers of HBCD were detected in the eggs of the exposed breeding pairs, and background levels were detected in controls the means of which are presented in Table 1 (Letcher and Fernie, Environment Canada, unpublished data).

Evaluation of testes mass and histology

Testes were extracted from unpaired males only (n_C = 12, n_{HBCD} = 10) on April 14th and 15th to coincide with the onset of spermatogenesis [17] and thus, seasonal testicular development in American kestrels. Due to restrictions on availability of birds, all males in the control group were one year of age and males in the HBCD-exposed

group ranged from 4 to 9 years of age with an average of 6 years. Both testes were extracted within 30 min of death and weighed with an analytical balance. The larger left testis was used for histological analysis and was fixed in Bouin's solution, embedded in paraffin; and sliced into 6 μm sections that were stained with hematoxylin and eosin. The number of seminiferous tubules containing final (elongated) spermatids was counted on two testis cross-sections per individual under 670x magnification from which a mean was calculated. The total number of tubules and the number of tubules with lumen were counted for one cross-section per individual from a digital photograph using the automated counting tool in Adobe Photoshop 11. An exemplary seminiferous tubule demonstrating these characteristics is shown in Fig. 2.

Measurement of body mass and circulating hormone levels

For breeding males (n_C = 11, n_{HBCD} = 20), blood samples were taken weekly during the courtship period at the same time of day (8:30 -10:30 am prior to feeding) to avoid the effects of diurnal variation in hormone levels. A blood sample was withdrawn from unpaired males (n_C = 12, n_{HBCD} = 10) immediately prior to euthanasia and testes extraction. For each sample, one ml of blood was taken with a heparinized 27.5-gauge needle by jugular venipuncture. This frequency and volume of blood withdrawal does not significantly affect reproductive output or hematocrit [18]. Body mass was recorded prior to withdrawal of each blood sample. For breeding males, body mass was also monitored throughout the reproductive cycle (Fig. 1).

Circulating testosterone (T) concentrations in plasma were determined by enzyme immunoassay (EIA), conducted at Environment Canada's National Wildlife Research

Centre in Ottawa. A salivary testosterone enzyme immunoassay kit (Salimetrics, State College PA, USA) was used because of its higher sensitivity compared with radioimmunoassay (RIA) or serum EIA kits, and thus less plasma was needed. The plasma samples were thawed and 15 µl was diluted by 10 times with 135 µl of the assay diluent. The absorbance of this solution was determined using a Molecular Devices plate reader (SpectraMax 190: s/n NN02060). Plates with 96 wells were coated with rabbit anti-testosterone antibodies. A calibration curve was prepared with 6 concentration levels of T standards in duplicates with low and high levels of T in a saliva-like matrix, for quality assurance. Duplicates of each sample were conduced from which a mean was calculated and used for analysis. The determined T levels were within the acceptable range and inter- and intra-assay variability was below 10 percent in all cases. The kestrel standards, created using control plasma (not charcoal stripped) were parallel with the salivary standards of the kit with a coefficent of variation of $R^2 = 0.998$. For additional quality control, 12 samples were concurrently analyzed for total T by radioimmunoassay kits (Siemens, Coat-A-Count©, CON6 lot 22), with well-correlated results ($R^2 = 0.97$) thus validating the results obtained from the salivary EIA. The levels of circulating total T_4 and free T_4 were analyzed for breeding males only, using radioimmunoassay kits (MP Biomedical, USA), at Environment Canada, Winnipeg, Manitoba. The $R^2 = 0.998$ for both FT_4 and TT_4 and he sensitivity of the assay was 0.76 µg/ dl.

Measurement of sperm numbers during breeding

Sperm nuclei become trapped in the perivitelline layer (PVL) of the yolk and thus reflect insemination sperm counts [19]. The first egg was removed on the day of laying

for all breeding pairs and frozen until analysis to preserve spermatozoa. Eggs were thawed and the PVL was stretched on a microscope slide and stained with a DNA-binding fluorochrome (Hoechst 33342, Merck). Spermatozoa were irregularly dispersed on the PVL, thus total numbers were estimated by counting all sperm nuclei under a Nikon fluorescence microscope at 200X magnification. In certain areas of high density, quadrate multiplication was conducted when necessary [20, 21]. Eight control and 14 HBCD-exposed eggs were evaluated successfully. An additional 7 controls from 2007 found not statistically different from the present controls were also included to increase the sample size to $n_C = 15$ and $n_{HBCD} = 14$.

Measurement of reproductive behavior and success

The reproductive behavior and success of the breeding males are reported in detail elsewhere ([22], Marteinson et al. Chapter 4, this volume) but are used here to determine if there were any associations with male physiology. Briefly, throughout the courtship period, all reproductive parameters were recorded including the laying date, number of eggs and young, and egg mass and volume. Reproductive courtship behaviors were recorded from the second day after pairing until the first egg was laid. Brood-rearing behaviors of all pairs producing young were recorded for 20 days after the first chick hatched (Fig.1). The frequency of important behaviors, including the number of copulations, nest-inspections, bonding-displays, vocalizations and provisioning of the young, were recorded [22, 23].

Statistical analysis

For breeding males, mean plasma testosterone concentrations (T) as well as free and total T_4 were calculated for three biological reference points: pairing (taken just before males and females were placed together), the week before the pair laid their first egg and the week that the first egg was laid (Fig. 1). Mean hormone concentrations were compared between HBCD-exposed and control males for each of these points using t-tests, as well as with a General Linear Model (GLM) with body mass as a covariate for each time point. Repeated measures (RM) ANOVAs were used to determine differences in the pattern over time between the two groups for T levels and body mass. Sperm counts from the perivitelline layer from the same breeding males were analyzed statistically by t-tests between control and HBCD-exposed males. Pearson's correlation analyses were conducted between these physiological parameters and behavior frequencies. Data were tested for homogeneity of variances using a Levene's test, and when the variances differed, a t-test for unequal variances was used.

For the unpaired males, differences in T levels, testis mass and histological counts between the HBCD-exposed and control males were analyzed by t-tests as well as by GLM procedures with the body mass and age of the males as covariates. When examining chemicals that can affect both testicular physiology and body mass in exposed individuals, measuring absolute testis mass is recommended for mammals because it is usually conserved even if changes in body mass occur [24]. However, in adult kestrels (one year and older), testicular productivity has been associated with body mass and not age [17], and therefore, both analyses are presented here. The gonadosomatic index was calculated as the total testes mass × 100 / body − testes mass [25], and was compared

between HBCD-exposed and control birds using a t-test. Pearson's correlation analyses were conducted separately for each group between these physiological parameters. No correlation analyses could be conducted between testicular physiology and the parameters measured during breeding because different individuals were used in the two experiments. Statistical analysis was conducted using SPSS 17.0. All data were tested for normality and homogeneity of variance and significance was considered to be $p \leq 0.05$.

Results

Unpaired males: Testosterone, testis mass and histology

The left testis of HBCD-exposed unpaired males was significantly larger than that of controls by an average of 41%. This was in evidence both for absolute mass ($t_{(19)} = -3.50$, $p = 0.002$) (Fig. 3), as well as when controlling for body mass and age (ANCOVA $F_{3,18} = 6.56$, corrected $p = 0.003$). The less prominent right testis was also 26% larger in HBCD-exposed unpaired males compared to controls (ANCOVA $F_{2,19} = 5.07$, corrected $p = 0.017$) (Fig. 3). Body mass and age had no effect on left testis mass, however, it was related to the mass of the right testis (body mass main effect $p = 0.011$). The ratio of left to right testis mass did not differ between the exposed and control unpaired males nor did the gonadosomatic index.

In the unpaired males, higher mean circulating T concentrations were associated with the larger mass of the left testis (Spearman $r_s = 0.43$, $p = 0.056$). HBCD-exposed unpaired males had marginally greater circulating T concentrations (0.79 ± 0.18 ng T /

ml) than those of respective controls in April (0.34 ± 0.17 ng/ml) ($t_{(19)}$ = -1.81, p = 0.086).

Unpaired males exposed to HBCD had more mean tubules with final spermatids than did controls ($t_{(19)}$ = -2.07, p = 0.052) (Fig. 4) which increased with increasing total mass of the left testis (r_p = 0.46, p = 0.016) (Fig. 5) and circulating testosterone levels (r_p = 0.44, p = 0.054). Age and body mass had no effect on histological parameters. No major differences in the gross histopathology of the testes were noted between HBCD-exposed and control unpaired males.

Breeding males: Body mass, testosterone and sperm numbers

By the end of the courtship period, HBCD-exposed breeding males had gained more weight than controls to reach a peak at day 10 of incubation where there were 7% larger than controls on average ($t_{(27)}$ = -2.68, p = 0.013). During brood-rearing, the HBCD-exposed breeding males subsequently lost weight to return to control levels by the time their offspring were 21 days of age (Fig. 6). However, the pattern of body mass change over time did not differ between HBCD-exposed and control males (RM ANOVA).

The T levels of HBCD-exposed breeding males increased throughout the courtship period to become significantly higher than those of controls by the time the first egg was laid ($t_{(28)}$ = -2.02, p = 0.054) in a pattern that differed from those of controls (RM ANOVA time×treatment $F_{2,25}$ = 5.87, p = 0.010) (Fig. 7). Body mass, as a covariate did not have a significant effect on T levels. Testosterone concentrations at egg-laying were marginally and negatively associated with the number of times breeding males

entered the nest-box during the brood-rearing period (r_s = -0.46, p = 0.055) but were not related to any courtship behavior frequencies.

Both the total and free T_4 concentrations in plasma of HBCD-exposed males were maintained at a lower level than those of controls. For total T_4, levels were significantly lower at pairing ($F_{2,11}$ = 3.38, treatment main effect p = 0.043) and at egg-laying ($F_{2,7}$ = 7.27, treatment main effect p = 0.007) (Fig. 8). At egg-laying the variances were unequal (Levene's F = 6.04, p = 0.022) however, HBCD-exposed males still had significantly lower levels than controls with this considered (t = 2.73, p = 0.015). Body mass had no effect on total T_4 levels. Free T_4 levels also tended to be lower in HBCD-exposed males compared to controls at pairing ($F_{2,7}$ = 17.92, treatment main effect p = 0.007) (Fig. 9) at which time body mass was a factor (p = 0.035), however, variances were unequal between the groups (Levene's F = 14.08, p = 0.003), and when taken into consideration, the groups did not differ significantly.

HBCD-exposed breeding males showed lower mean perivitelline sperm counts, with an average of 597.14 ± 167 compared to 1353.87 ± 859 for controls, though these were highly variable and did not differ statistically.

Discussion

This study demonstrated that exposure to environmentally relevant levels of technical HBCD in the diet altered the reproductive physiology of male American kestrels. When compared to controls, males exposed to HBCD had greater testis mass at the onset of spermatogenesis, with an associated increase in the number of seminiferous tubules containing final (elongated) spermatids. These effects were noted after just three

weeks of exposure during testicular recrudescence. In a separate set of breeding males, plasma testosterone levels and body mass were elevated in HBCD-exposed males compared to controls as the courtship period progressed and circulating levels of T_4 were reduced throughout.

The testicular and endocrine alterations in the kestrels are in line with results noted for laboratory rodents. In rats exposed developmentally to HBCD, testes size and plasma DHT concentrations were increased, but no alterations in gross histopathology of the testis were noted at a maternal dosing level of 11.4 mg/kg/day for 90 days [8]. Circulating T_4 levels were also reduced in these rats [8]. Exposure levels in the current study, at 54.4 μg daily, are considerably lower than the those that elicited effects in rodents [8], suggesting that kestrels may be more sensitive to HBCD exposure with respect to these male reproductive tract endpoints.

Alterations in testicular physiology may present consequences for reproductive success in free-living birds experiencing similar exposure levels of HBCD. Although testes mass and number of spermatid-containing tubules were elevated in the unpaired HBCD-exposed males in the present study, there was no evidence for enhanced reproductive success in their breeding counterparts. In these breeding males there was no increase in the number of sperm reaching the ovum, nor was there any increase in the fertility of the eggs or production of young compared to controls (Marteinson et al. Ch 4, this volume). This suggests that there is a lack of benefit from increased testicular mass and potential productivity.

Higher than normal levels of circulating testosterone can have direct consequences on breeding capacity in males and may represent an outcome detrimental to reproductive success in itself. For wild birds with limited food supplies, elevated testosterone levels have been associated with decreased body mass and fat reserves [26], increased circulating stress corticosteroids [27], decreased immune response, and greater susceptibility to parasitism [28]. Furthermore, elevated levels of testosterone have been associated with decreases in incubation [29] and brood-rearing behaviors [29-31] in males, which can ultimately result in decreased reproductive output [31]. Indeed, the same breeding males from the present study had reduced parental behaviors that were related to their increased testosterone levels.

Testicular size is a sensitive endpoint with chemical exposure and is useful as an indicator of function and reproductive state of the testis [24]. Most commonly, testicular mass is reduced in mammals and birds exposed to endocrine active chemicals which is usually indicative of reduced spermatogenesis [24]. Examples of such chemicals include drugs that are anti-androgenic, such as flutamide or vinclozolin [32-34], or anti-estrogenic such as tamoxifen [35]. As well, reductions in testis mass have occurred with exposure to several xenobiotic contaminants, including DDT [36], 4-octylphenol (OP) [37], butyl benzyl pthalate (BBP) [37], 3-methyl4-nitrophenol [38], and bisphenol-A [39].

Increased testis mass can also occur with chemical exposure as a result of one of two mechanisms: increased fluid in the testes [24] or increased cell proliferation during development [40]. Disturbance of the fluid balance in the male reproductive tract can ultimately result in fluid retention in the testis which accounts for the increased mass

[24]. In mammals, this may occur as a result of overproduction of fluid by Sertoli cells within the testis, or by a backflow of fluid into the testes by the impaired functioning of the efferent tubules either from a blockage or by reduced fluid resorption (reviewed in: [41]). All three of these possibilities usually result in the dilation of the seminiferous tubule lumen as a recognizable outcome [41]. Sex steroids are key regulators of the fluid balance of the mammalian and bird testis [42, 43], and chemicals that disrupt these pathways can result in increased fluid in the testes. In these instances, the increased testis size is usually accompanied by negative impacts on testicular function as evidenced in the histology by reduced seminiferous epithelium and degeneration of tubules [41, 42, 44]. In this study, the tubule lumen did not appear enlarged, and none of the negative impacts described above were apparent in the testis sections of the unpaired HBCD-exposed males. However, further investigation is warranted, particularly with regard to more prolonged exposure, higher exposure concentrations, and exposure during full spermatogenesis.

The second possibility for enlarged testes following chemical exposure involves a deregulation of the thyroid axis. It is well documented in mammalian models that triiodothyronine (T_3) inhibits somatic cell proliferation in the testes during development [40]. The cell-types sensitive to such control are the Sertoli cells which support sperm production and are directly related to testicular mass [40], and the Leydig cells which produce testosterone and are directly related to its circulating levels [45-47]. Henceforth, hypothyroidism (subnormal levels of T_4) during development causes increased final numbers of Leydig [48] and Sertoli cells, which are followed by heavier testes and greater sperm production in adult mammals [49] which has been confirmed more

recently in fish [50] and birds [51]. Elevated levels of circulating testosterone do not usually accompany developmental hypothyroidism in rats [52, 53] but can occur in birds [51, 54], sometimes even more so after photostimulation [55].

Similar to rodents [8] and fish [56], the breeding male kestrels exposed to HBCD demonstrated reduced T_4, which may be related to their ability to bind to thyroid receptors [7] and/or to the increased liver metabolism of T_4, which has been demonstrated in fish [57]. This suggests that a thyroid-disrupting mechanism is a likely explanation for the enlarged testes in the HBCD-exposed unpaired kestrels. Additionally, exposure to another class of organohalogen pollutants, the polychlorinated biphenyls (PCBs), has been shown to increase testicular mass by this mechanism in developmentally exposed rats [58, 59]. The PCB mixtures used in the cited studies, Aroclor 1242, 1254, have similar multimodal endocrine disrupting potential as HBCD in vitro [60-63], confirming that these types of chemicals can have this effect on the testis.

Though the kestrels in the present study were not exposed during embryonic or post-natal development, testicular recrudescence for seasonal breeding involves testicular growth by somatic cell proliferation in mammals [64-67] and birds [68]. In birds, this testicular growth is analogous to puberty in juveniles [69], where in kestrels, testes rapidly grow 12 times larger [15]. This phase represents a form of development that may be sensitive to alterations in thyroid control and has been identified as a sensitive time-period for examining the effects of endocrine disrupting chemicals in fish [70, 71]. Very little is known about how hypothyroidism affects specifically seasonal testicular development in any species. However in birds, T_4 can suppress this testicular growth by inhibiting cell proliferation at this time [72]. As such, mild experimentally induced

hypothyroidism during seasonal testicular recrudescence in the spotted munia (*Estrilda amandava*) caused larger testis volume earlier in the breeding season [73], confirming that this stage can be sensitive to thyroid disruption.

Conclusion

Due to the experimental design of this study, and because the testicular histology of kestrels has never previously been examined, it could not be determined whether the enlarged testes in the kestrels were related to a fluid imbalance or thyroid disruption. However, circulating levels of T_4 were reduced in breeding HBCD-exposed males, confirming previous in vivo research in other animal models [8, 56, 74, 75], and suggesting that hypothyroidism may explain the increased testicular mass. Additionally, the number of seminiferous tubules containing final spermatids was higher in HBCD-exposed kestrels and the tubule lumens did not appear to be dilated in the testicular sections examined making a disruption in fluid balance appear less convincing.

Despite the increased testis mass and number of spermatid-containing tubules, no subsequent increase in fertility or production of young occurred in the similarly exposed breeding males. Furthermore, elevated levels of testosterone in males may have negative implications for wild birds [26-29]. These include the inhibition of parental care [29-31], which was associated with increased testosterone levels in the subjects of the present study. Exposure levels in the present study are environmentally relevant and modeled after those recorded recently in wild birds, including the closely related peregrine falcon (*F. peregrinus*) [12]. Therefore, free-living birds experiencing comparable exposure levels of HBCD may be susceptible to similar physiological responses in the wild.

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Table 1. Hexabromocyclododecane concentrations (ng/g wet weight)[a] in eggs of breeding pairs and plasma (ng/ml) of unpaired male American kestrels at the time of testes extraction.

	α-HBCD		β-HBCD		γ-HBCD		ΣHBCD	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Breeding pairs (eggs)								
Control eggs	0.3	0.4	0.2	0.1	0.2	0.1	0.6	0.5
HBCD-exposed eggs	163.5	75.1	13.9	5.6	2.6	3.8	179.9	80.6
Unpaired males (plasma)								
End depuration period								
Control	0.02	0.02	0.03	0.00	0.03	0.02	0.09	0.04
HBCD-exposed	1.97	0.95	0.06	0.06	0.81	0.6	2.83	1.49

[a] Letcher and Fernie, Environment Canada, unpublished data. Breeding males: $n_C = 11$, $n_{HBCD} = 20$. Unpaired males: $n_C = 12$, $n_{HBCD} = 10$.

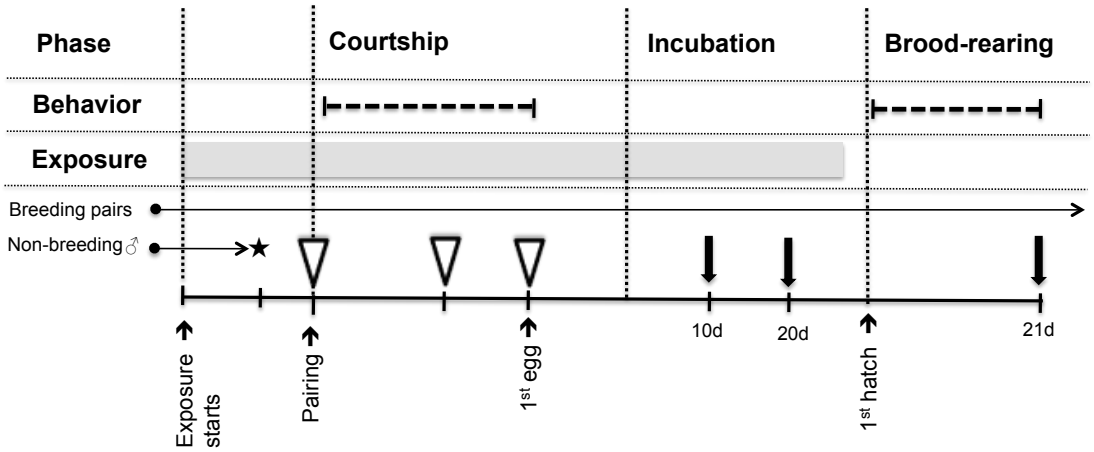

Fig. 1: Timeline for data collection during each reproductive phase of American kestrels exposed via diet to Hexabromocyclododecane (HBCD). Unpaired males (n_C = 12, n_{HBCD} = 10) were exposed for three weeks and then sacrificed for testes collection (★). Breeding males and females were exposed 4 weeks prior to pairing (n_C = 11, n_{HBCD} = 20). For the breeding males from these pairs, body mass was recorded and a blood sample was taken for hormone analysis (T, T_4) 3 times during courtship at the biological reference points of pairing, the week before laying and the week the first egg was laid (▽). Body mass was additionally recorded twice during incubation and once during brood rearing (⬇). Behavioral data were collected during the courtship and brood-rearing periods, the timing of which is marked by the dashed lines.

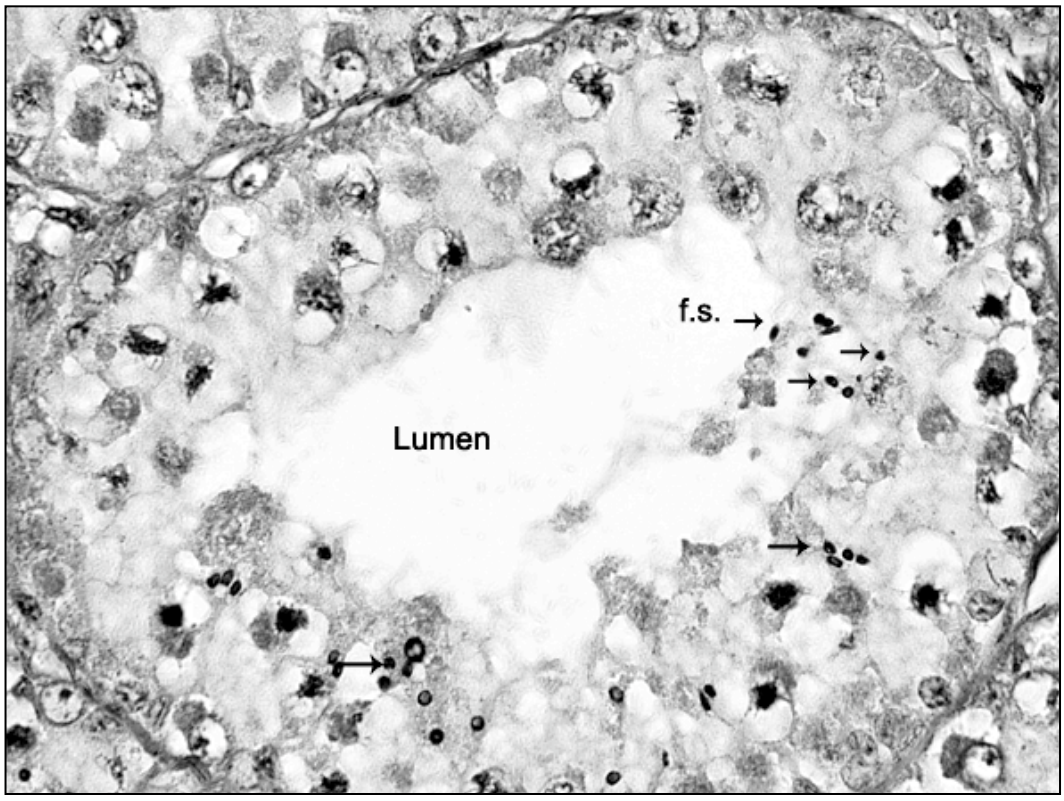

Fig. 2: Cross-section of a seminiferous tubule from the left testis of an unpaired American kestrel at the onset of spermatogenesis. The lumen is visible as are final (elongated) spermatids (f.s. ⇨), early spermatids (e.s. ▲) and dividing spermatocytes (s.c.: Δ) in the epithelium.

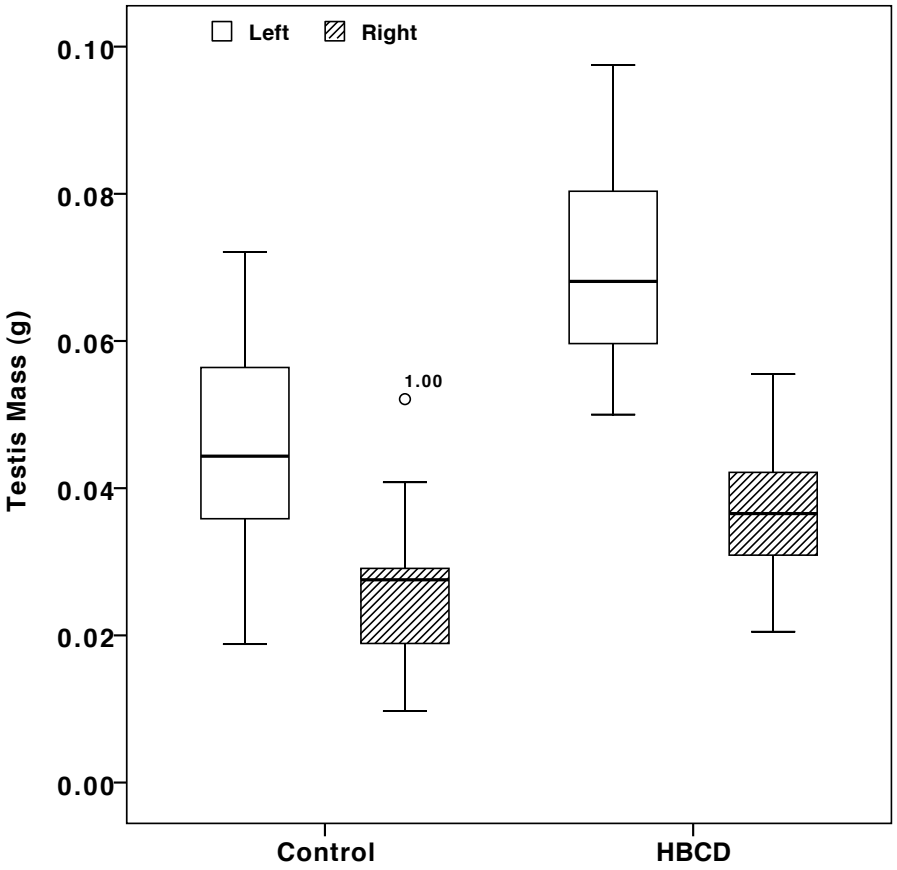

Fig. 3. Left and right testis mass for male American kestrels exposed to technical hexabromocyclododecane (HBCD) or control vehicle. The left testis of HBCD-exposed males were larger than those of controls ($t_{(19)}$ = -3.50, p = 0.002).

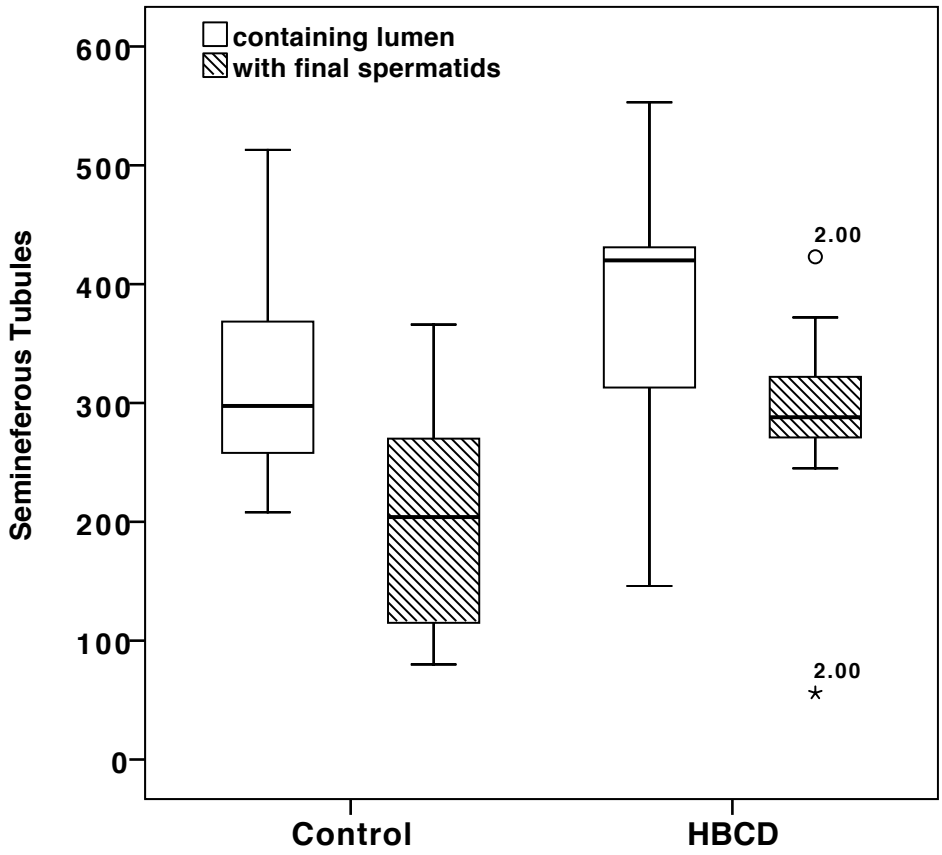

Fig. 4: Seminiferous tubule counts from testes cross-sections of the left testis of hexabromocyclododecane (HBCD)-exposed and control male American kestrels (n_C = 12, n_{HBCD} = 10).The number of tubules containing a lumen and number of tubules with final spermatids are depicted. HBCD-exposed males had more seminiferous tubules containing final spermatids than controls ($t_{(19)}$ = -2.07, p = 0.052).

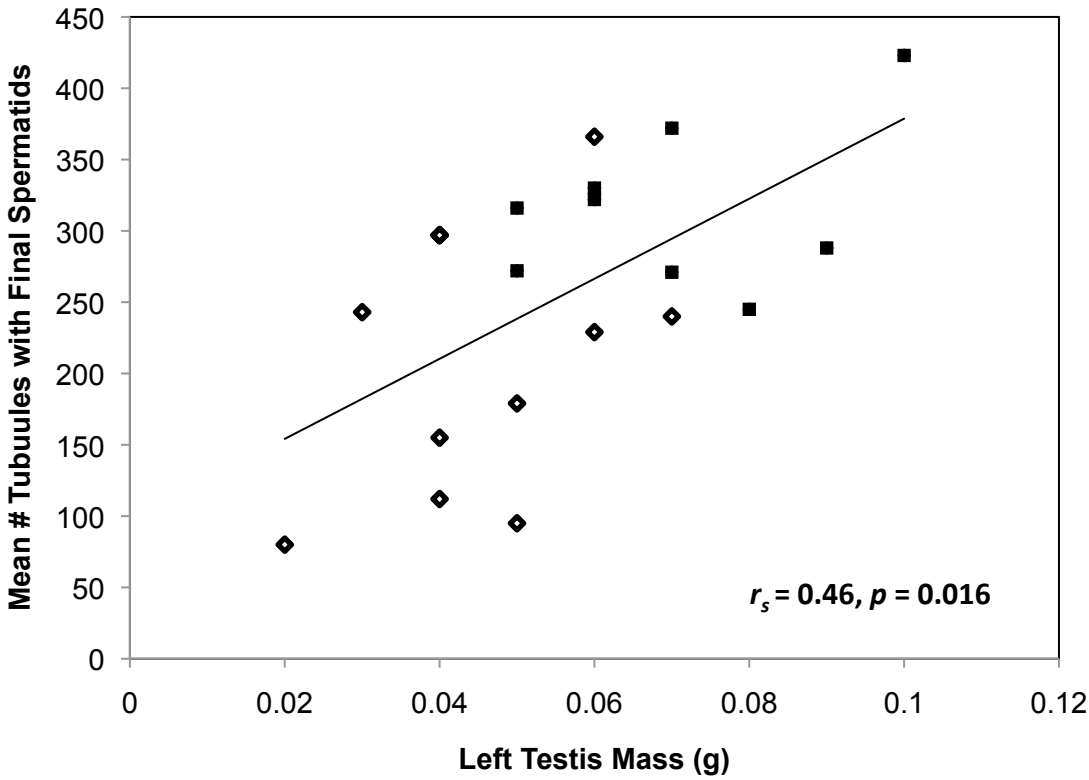

Fig. 5: The mean number of seminiferous tubules containing final spermatids as a function of left testis mass in hexabromocyclododecane (HBCD) exposed (■) and control (◇) American kestrels (Pearson's correlation).

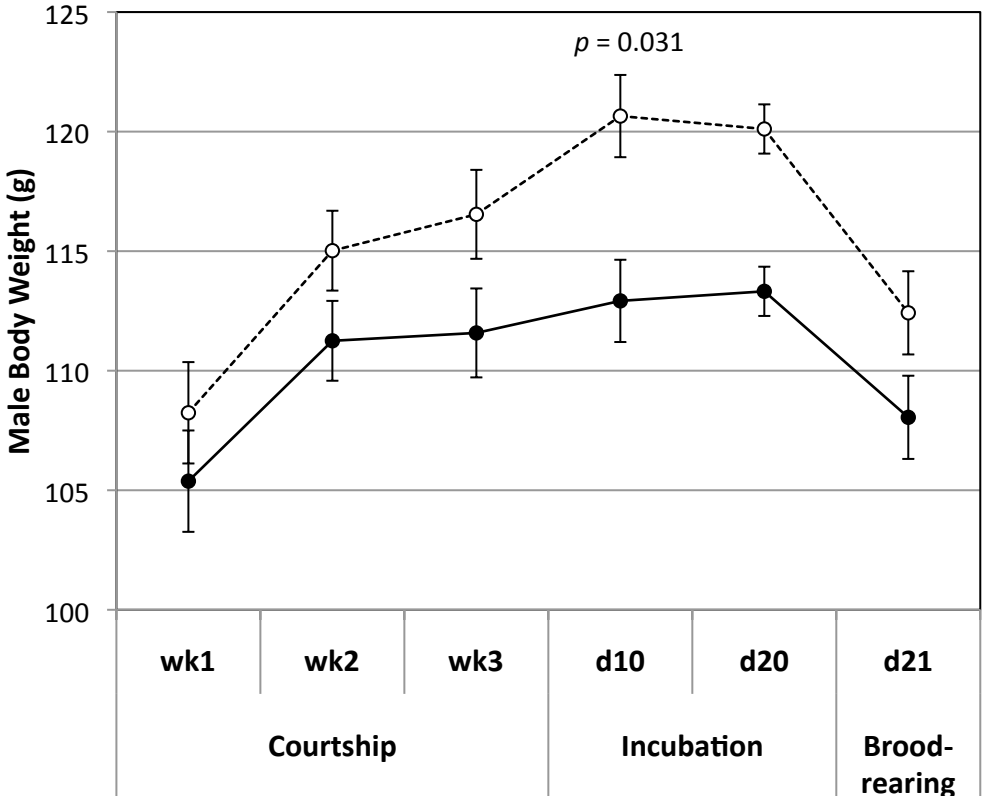

Fig. 6: Male mean body mass of American kestrels throughout the courtship, incubation and brood-rearing periods for hexabromocyclododecane (HBCD) exposed (○) and control (●) males.

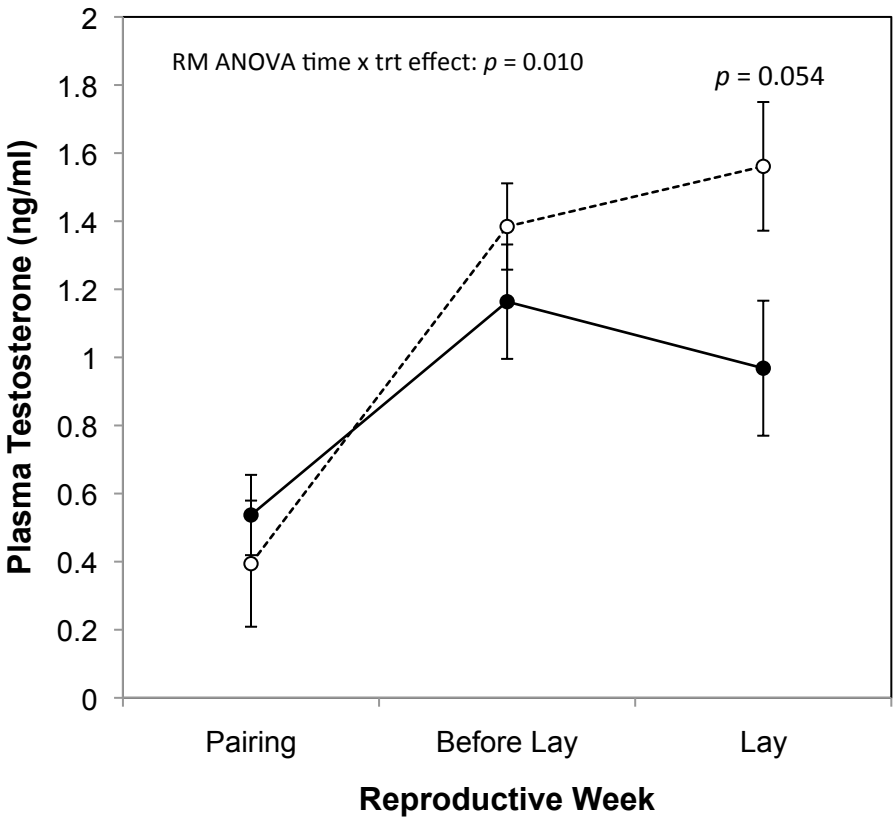

Fig. 7: Plasma testosterone concentrations for breeding male American kestrels exposed to technical hexabromocyclododecane (HBCD) (○) or control vehicle only (●) via diet (n_C = 10, n_{HBCD} = 20). Means for the biological reference points of pairing, the week before egg-laying and the week the first egg was laid, are displayed (t-test, RM ANOVA).

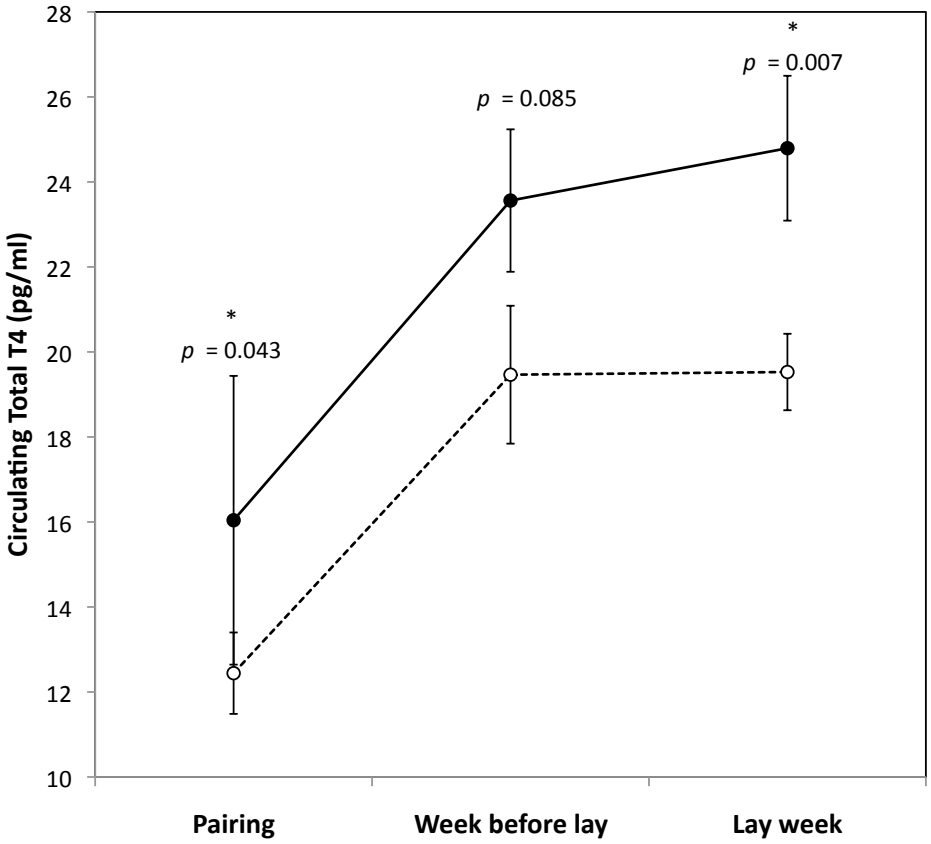

Fig. 8. Plasma total T_4 concentrations for breeding male American kestrels exposed to technical hexabromocyclododecane (HBCD) (○) or control vehicle only (●) via diet. Means for the biological reference points of pairing, the week before egg laying and the week the first egg was laid, are displayed (GLM with body mass as a covariate, p values are for the treatment main effect).

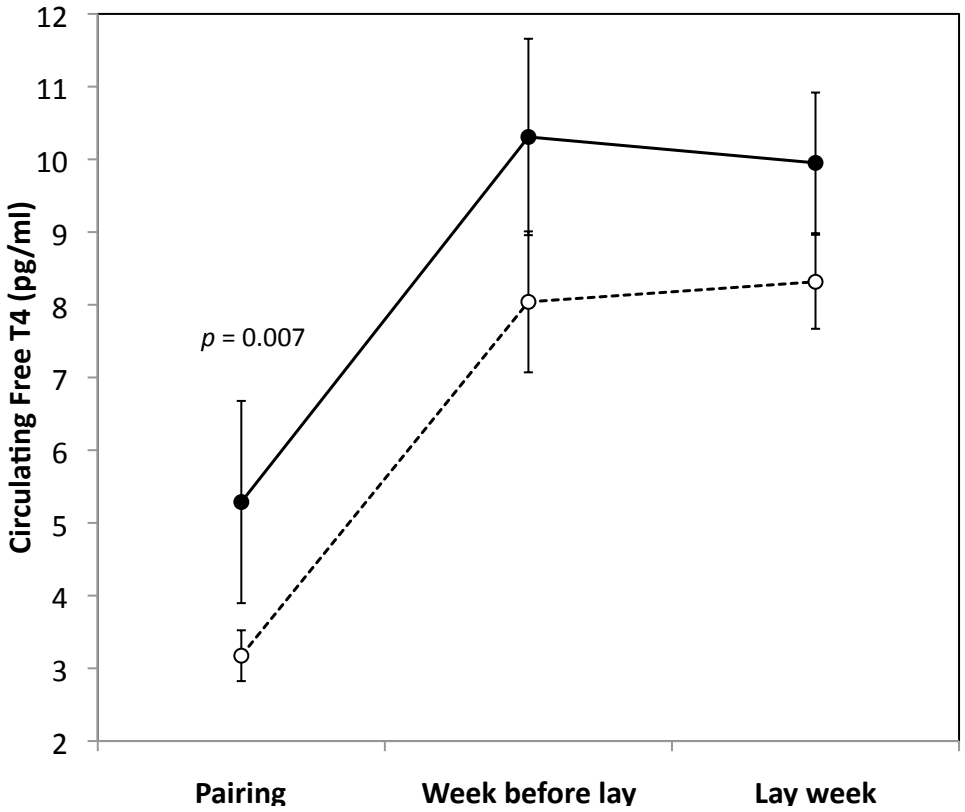

Fig. 9. Plasma free T_4 concentrations for breeding male American kestrels exposed to technical hexabromocyclododecane (HBCD) (○) or control vehicle only (●) via diet. Means for the biological reference points of pairing, the week before egg-laying and the week the first egg was laid, are displayed (GLM with body mass as a covariate, p values are for the treatment main effect).

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CHAPTER 6

SUMMARY AND CONCLUSIONS

In this thesis the reproductive effects of two brominated flame retardants at environmentally relevant exposure levels were assessed in captive American kestrels. In the first experiment, males were exposed embryonically to the polybrominated diphenyl ether (PBDE) mixture, DE-71. In the second experiment, male and female pairs were exposed via their diet to hexabromocyclododecane (HBCD) during gonadal recrudescence. The effects on reproductive success, behavior and male physiology of these two chemicals in kestrels as described in this thesis, may be summarized as follows and are presented in detail in Appendix 1. Additionally, the results for dietary exposure to PBDEs in kestrels as determined previously by Fernie et al. [1-3] are summarized to emphasize the multigenerational effects of DE-71 exposure, and to compare with the effects of HBCD and DE-71 by the same route of exposure (via diet).

Regarding reproductive success, pairs exposed via diet to HBCD had earlier lay dates and larger clutch sizes but had no associated increase in reproductive success (Ch. 4). Conversely, DE-71 exposed pairs had delays in laying and reduced fertility both with diet-exposure to the pair [1] and *in ovo* exposure to the male only, when compared to their respective controls (Ch. 2, [4]). Despite these differences, females exposed via diet to DE-71 or HBCD, and unexposed females paired with in ovo DE-71-exposed males, displayed a reduction in egg mass and volume when compared to their respective controls.

Regarding reproductive behavior, exposure to DE-71 altered copulation frequencies both in diet-exposed pairs [2] and *in ovo* exposed males. In the latter, copulation frequency was dramatically reduced when compared to controls (Ch. 2, [4]). Exposure to HBCD via diet did not affect copulatory behaviour of the pair (Ch. 4).

However, exposure to either chemical in all three studies caused reductions in other courtship behaviors that are important for the pair-bond, including vocalizations, nest-inspections and/or bonding-displays in both sexes compared to their respective controls (Ch. 2, 4, [2, 4]). Exposure to HBCD or in ovo to DE-71 also caused reductions in male parental behaviors in exposed males compared to controls, particularly with diet exposure to HBCD (Ch. 2, 4, [4]).

Regarding reproductive physiology, males receiving either in ovo high-exposure to DE-71 or diet-exposure to HBCD, demonstrated increased testis mass at the onset of spermatogenesis (Ch. 3, 5). The testes of males exposed to HBCD had more seminiferous tubules containing final spermatids and elevated testosterone levels during courtship compared to controls (Ch. 5). Conversely, males receiving high in ovo exposure to DE-71 had more seminiferous tubules containing lumen but with fewer tubules containing final spermatids, lower testosterone levels during courtship, but similar circulating thyroid hormones to control males (Ch. 3). Furthermore, though testes were larger in the high DE-71 in ovo exposure males, epididymal sperm numbers were similar among the three groups (Ch. 3). These contrasting findings between the two studies suggest that HBCD and DE-71 caused testis mass increases by different mechanisms: a disruption of fluid balance with in ovo exposure to DE-71, and deregulation of the thyroid axis in HBCD-exposed males are likely explanations (Ch. 3, 5). Perivitelline sperm numbers were increased with low and high in ovo exposure to DE-71 (Ch. 3) but were not altered by HBCD exposure (Ch. 5), which may be reflecting their respective copulation frequencies (Ch 2, 4) (Table 1).

The concentrations of unintentional HBCD exposure in the DE-71 experiment may have had some impact on the reproductive measures examined [1, 2, 4]. In these studies, concentrations of HBCD were statistically correlated with decreased eggshell thickness and the delayed laying dates in diet-exposed females [1]. However in the HBCD diet exposure study, eggshell thinning did not occur, and females initiated their clutches earlier. For males receiving in ovo exposure to DE-71 their depressed copulatory behavior, reduced fertility and smaller clutch sizes were also correlated with in ovo HBCD concentrations [4]. However, in the HBCD diet-exposure experiment, the opposite was found for the timing of breeding and number of eggs produced, while copulation frequency and egg fertility were not affected. For other endpoints, results from the two studies paralleled one another, including the reduction in other courtship behaviors, decreased egg size and the depressed brood-rearing behavior of males. With respect to the male reproductive tract and endocrinology, those exposed to HBCD via diet and DE-71 in ovo displayed increased testis mass, however histological findings differed as did testosterone and thyroxin levels during breeding. Additionally, in the in ovo DE-71 experiment, the unintentional exposure concentrations of HBCD were not related to any male physiological parameters measured, suggesting that the effects noted in this research were a result of the developmental exposure to DE-71 only. Overall, synergistic or additive effects of the combined exposure to HBCD and PBDEs in the DE-71 experiment cannot be ruled out entirely, however, since many of the findings differ, and because unintentional HBCD exposure levels were very low, it appears as though the effects of exposure to DE-71 were successfully isolated.

Laboratory studies that identify the endpoints affected by toxicants are useful in identifying which contaminants may be important for wildlife species [5] and estimating the risks to their populations [6]. For DE-71 it was demonstrated that several reproductive parameters were affected, ultimately leading to reduced reproductive success. For HBCD, it was demonstrated that laying dates were earlier and clutch size was increased, however, despite these reproductive advantages, no subsequent increase in reproductive output followed.

For both chemicals, several of the endpoints may have been affected in ways that may ultimately be detrimental for the reproductive success of wild predatory birds. These include 1) the timing of breeding, which, in temperate species has evolved to be precisely timed with peaks in food availability [7]; 2) egg size, where larger eggs are consistently associated with better reproductive success in birds [8]; 3) courtship behavior which is critically important for solidifying the pair bond and inducing readiness to breed, especially for females [9, 10]; 4) parental behavior and provisioning of the young are critical, particularly for altricial and semi-altricial species like the American kestrel; 5) testosterone, which is a key regulator of male courtship behavior [11], can influence parental behavior [12] and is linked to several physiological characters related to overall health [13-15]; 6) testicular histology, which can indicate a chemical's potential for affecting male fertility.

Because exposure levels were environmentally relevant [16-19], it is highly probable that birds receiving similar exposure to HBCD and/or PBDEs in the wild are subject to comparable reproductive alterations. Free-living birds are also subject to additional stressors that may further exacerbate such outcomes including predation, food

limitations, disease and parasitism and habitat loss as well as exposure to a complex mixture of contaminants for which BFRs make up only a small proportion (e.g. [20]). It is beyond the scope of this thesis to determine exactly how these two chemicals may affect the stability of wildlife populations. However, the research presented herein has demonstrated that concentrations of DE-71 and HBCD currently measured in bird eggs are reproductive toxicants in birds of prey and should be considered as a risk factor for their populations in the wild.

Recommendations for Future Research

Further investigation of the reproductive effects of HBCD and PBDEs (the DE-71 mixture or otherwise) in kestrels or other wildlife species is warranted. Both HBCD and the BDE-209 mixture are still in current use, and the products containing these BFRs, as well as the DE-71 mixture are, and will continue to be, sources of environmental contamination for an undetermined span of time. For both chemicals, specific recommendations for the direction of further research as a direct follow up from the research presented in this thesis include the following.

1) A more detailed analysis of the effects of exposure during the whole period of testicular recrudescence would be useful for determining how both of these chemicals affect the developing testis.
2) For both of these chemicals it would be highly valuable to conduct testicular histology at the time of peak spermatogenesis in birds. At this stage, the different cell types should be examined to determine what stage(s) of spermatogenesis

might be affected. Before this would be possible, the staging of spermatogenesis for kestrels would have to be characterized as well.

3) The analysis of sperm quality would also be useful, particularly for PBDEs, where a reduction in fertile eggs was seen despite the elevated perivitelline sperm numbers. To further investigate the effect on sperm quality, the number and types of abnormal sperm, computer assisted sperm analysis (CASA) of motility parameters, and fertilization ability could be assessed.
4) A more detailed analysis of the endocrine outcomes in males for both chemicals would be advantageous and should include the examination of circulating levels of other hormones such as the gonadotropin-releasing hormone, luteinizing hormone and follicle-stimulating hormone, which would give further insight into some of the reproductive effects presented in this thesis.
5) The alteration in reproductive parameters such as the timing of breeding, egg size, and clutch size demonstrates that female reproductive physiology is also affected by exposure to either of these BFRs, and further investigation is warranted. Some useful endpoints to examine include circulating hormone levels, egg composition and mass and histology of the ovary.
6) Since multigenerational effects were strongly noted in the DE-71 study, it would be highly beneficial to conduct a reproductive assessment in kestrels receiving in ovo exposure to HBCD as well.

Finally, since kestrels are shown to be reproductively sensitive to these two BFRs, the need to investigate the reproductive effects of other more recently introduced BFRs and/or those that are now emerging as environmental contaminants in birds' eggs

becomes apparent. These include hexabromobenzene (HBB), pentabromoethylbenzene (PBEB), 1,2bis(2,4,6-tribromophenoxy)ethane (BTBPE), decabromodiphenyl ethane (DBDPE), 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (TBECH) [21, 22] for several of which there is little to no data on their in vivo effects in the literature.

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APPENDIX 1: SUMMARY OF RESULTS OF EXPOSURE TO TWO BROMINATED FLAME RETARDANTS IN CAPTIVE AMERICAN KESTRELS.

	PBDEs (DE-71)		HBCD
Exposure rout	Diet-exposure to Pairs	In ovo exposure to male	Diet-Exposure to Pairs
Exposure concentration	H: 0.658 µg/µl L: 0.140 µg/µl safflower oil daily	H: 1130.59 ± 95.34 ng/gww L: 288.60 ± 33.35 ng/g ww C: 3.01 ± 0.46 ng/g ww	0.544 µg/µl daily
Sample	n_C= 19, n_L= 17, n_H= 15	n_C= 7, n_L= 7, n_H= 7	n_C= 11, n_{HBCD}= 20
REPRODUCTIVE SUCCESS			
Lay date	Delayed (H: $p = 0.030$)	N.S. delay	Earlier ($p = 0.009$)
Clutch size	N.D.	↓ (H: $p = 0.020$)	↑ ($p = 0.008$)
# Fertile eggs	N.D.	↓ (H: $p = 0.007$)	N.D.
# Hatchlings	↓ ($p = 0.038$)	N.S. ↓	N.D.
# Fledglings	↓ ($p = 0.019$)	N.S. ↓	N.D.
FEMALE PHYSIOLOGY			
Egg weight	↓ (L&H: $p < 0.001$)	↓ (L&H: $p < 0.001$)	↓ ($p < 0.001$)
Egg volume	↓ (L&H: $p < 0.001$)	↓ (L&H: $p < 0.001$)	↓ ($p < 0.001$)
Eggshell thickness	↓ ($p = 0.008$)	N/A	N.D.
Body mass	N/A	N/A	N.D.
MALE BEHAVIOR			
Courtship Copulation	↓ initially (L&H: $p < 0.001$) then ↑	↓ (L&H: $p = 0.002$)	N.D.
Interest in nest	↓ (L&H: $p = 0.030$)	↓ (L&H: $p = 0.052$)	N.D.
Vocalizations		↓ (L&H: $p = 0.020$)	↓ ($p = 0.046$)
Food-transfers	N/A	↓ (L&H: $p = 0.067$)	N.D.
Bonding-display	N/A	N.D.	N.D.
Brood-rearing Enter-nest	N/A	↓ ($p = 0.069$)	↓($p = 0.034$)
Bonding behaviours	N/A	N.D.	↓ ($p = 0.036$)
Food-retrieval	N/A	N.D.	↓ ($p = 0.028$)
FEMALE BEHAVIOUR			
Courtship Nest-inspection	N.S. ↓ (H: $p = 0.070$)	N.D.	N.D.
Vocalizations	N.D.	↓ (H: $p = 0.012$)	↓ ($p = 0.050$)
Bonding-display	N.D.	N.D.	↓ ($p = 0.013$)
Solicit food transfer	N.D.	N.D.	N.D.
Brood rearing Enter-nest	N/A	N.D.	↑ ($p = 0.034$)

(Appendix 1 Contd.)

		PBDEs (DE-71)	HBCD
MALE PHYSIOLOGY			
Testes mass	N/A	↑ (H: $p \leq 0.055$)	↑ ($p = 0.019$)
Body mass	N/A	N.D.	↑ ($p < 0.013$) then ↓
Gonadosomatic index	N/A	↑ (H: $p = 0.046$)	N.D.
# Tubules with lumen	N/A	↑ (H: $p = 0.030$)	N.D.
# Tubules with final spermatids	N/A	N.D.	↑ ($p = 0.052$)
Epididymal sperm	N/A	N.D.	N/A
Perivitelline sperm	N/A	↑ (L&H: $p = 0.028$)	N.D.
Testosterone during breeding	N/A	↓ at egg-laying (H: $p = 0.056$)	↑ at egg-laying ($p = 0.054$)
Testosterone at testes extraction	N/A	N.D.	N.D.
T_4 during breeding	N/A	N.D.	↓ ($p < 0.05$)

N/A: not, applicable i.e. was not collected. N.D.: no significant difference. N.S.: non-significant trend. For the PBDE studies, H: high-exposure, L: low exposure, C: control. ↑: increase , ↓: decrease in exposed individuals compared to their respective controls. Data on reproductive parameters and behavior in diet-exposed kestrel are referenced from previous research by Fernie et al. 2009 and Fernie et al. 2008. Data from their in ovo exposed male offspring are referenced from research by Marteinson et al. 2010, and Chapters 2 and 3, this volume. Data from HBCD exposed pairs referenced from Chapters 4 and 5, this volume.