Cod: 8.2014

ENDOCRINE DISRUPTION OF SEX AND THYROID HORMONES BY 1,2-DIBROMO-4-(1,2-DIBROMOETHYL)CYCLOHEXANE (DBE-DBCH) IN AMERICAN KESTRELS

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Introduction

Brominated flame retardants (BFRs) are a group of industrial compounds that have been in use since the 1970s. 1,2-dibromo-4-(1,2-dibromoethyl)cyclohexane (DBE-DBCH - formerly abbreviated as TBECH) is an emerging BFR that is now increasingly detected in the environment and biota globally. Particularly noteworthy is its recent prominence relative to other BFRs in indoor and urban air in Europe (e.g. Newton et al. 2015), suggesting a possible increase in its use. DBE-DBCH has the unique endocrine disrupting ability of androgen potentiation, including in bird cells (Asnake et al. 2013). Despite this ability, few studies have been conducted to determine the potential effects of exposure to DBE-DBCH on the endocrine system in vivo. Brown trout (Salmo trutta) exposed to various levels of β-DBE-DBCH showed changes in circulating concentrations of testosterone (TT), estradiol (E_2) and total thyroxine (TT_4) (Gemmill et al. 2011; Park et al. 2011), and female American kestrels (Falco sparverius) exposed to β-DBE-DBCH deposited increased concentrations of estradiols in their eggs (Marteinson et al. 2012). Furthermore, the same kestrels demonstrated changes in androgen-related behaviors during the breeding season (Marteinson et al. 2015). The objective of the current study was to determine possible effects of β-DBE-DBCH, the isomer found in highest concentrations in animal tissue, on circulating sex steroid and thyroid hormone concentrations for the first time in birds.

Materials and Methods

Captive American kestrels with known pedigrees were used for this study at McGill University (Montreal, Canada). Breeding pairs were formed in April (2010) using young females (1-2 years old) and slightly older males (3-4 years old). Pairs were housed in breeding pens that allowed the birds to express their natural courtship behavioural patterns. They were fed an ad libitum diet of frozen-thawed day old cockerels (Gallus domesticus). A formulation of β-DBE-DBCH was prepared using an ultra high-purity form (neat; > 97% purity) obtained from Wellington Laboratories (Guelph, ON, Canada). Sixteen kestrel pairs in the treatment group were exposed by diet to 0.239 ng β-DBE-DBCH /g kestrel/day (an environmentally relevant concentration), while 14 control pairs ingested the safflower oil vehicle. Dietary exposure occurred for an average of 82 d, beginning 4 weeks prior to pair formation through until the end of incubation (Marteinson et al. 2012, 2015). Courtship behavior of the pair was recorded daily following Marteinson and her colleagues (2015).

Prior to morning feeding, male and female birds were blood sampled at the time of pairing and at the end of the breeding season; additionally, the males were blood sampled the week before egg-laying began, the week that their mate laid the first egg of the clutch, and at mid-incubation. Plasma was stored at -80°C until the time of analysis. Free (F) T_4 and TT_4 were assessed using enzyme immunoassay (EIA) kits (Inter-Medico: FT4 cat# CAN-fT4-4340 lot #150090; TT4 cat# CAN-T4-4240 lot #150540). Sex steroid hormones were analysed using EIA kits (Salimetrics LLC: High Sensitivity Salivary 17β-Estradiol Enzyme Immunoassay kit: cat# 1-3702-5 lot #1412507; Expanded Range Salivary Testosterone Enzyme Immunoassay kit: cat# 1-2402-5 lot #1411504). The LOQ for E_2 was 3.0 pg/mL, and for TT, 0.03 ng/mL.

Statistical analysis

For males, we report the multivariate results of Repeated Measures Analysis of Variance (RM MANOVA) that were conducted to determine the overall effects of treatment, time, and time-treatment interactions (time*trt) on concentrations of TT, TT_4, and FT_4. Analysis of Covariance (ANCOVA) for hormone concentrations at individual sampling times with body mass as a covariate was applied when significant. Plasma concentrations of thyroid hormones at the end of the breeding season were compared

separately using ANCOVAs (excluded from the RM analysis) because they were analyzed in a different lab. For females, concentrations of FT_4, TT_4, E_2, TT, and the E_2:TT ratio, at pairing were compared between control and exposed birds using t-tests or ANCOVAs with body mass as a covariate when significant. Females with TT and E_2 measurements below the method limit of detection were assigned a value equivalent to that limit, and this occurred for only 4% of samples. To account for the possible effects of other factors that can impact TT concentrations in males, a series of Generalized Linear Mixed Models (GLMM) with repeated measures (across the same biological time points) were conducted with data categorized by treatment and ranked using Akaike's Information Criterion corrected for small sample sizes (AICC). Fixed factors included time, body mass, sampling date, FT_4 and TT_4 concentrations, and two specific behaviors (i.e., mean copulation rates for the pair, female vocalization frequencies) in the 5 d prior to hormone assessment. All comparisons are made within each sex between control birds and birds exposed to β-DBE-DBCH.

Results and Discussion

Compared to control male kestrels, males exposed to β-DBE-DBCH demonstrated significantly higher plasma concentrations of FT_4 over the course of the breeding season (RM MANOVA treatment effect: $F1,19 = 5.31$, $p = 0.033$; Fig. 1). The temporal patterns of FT_4 significantly changed over the breeding season (RM MANOVA time: $F3,23 = 8.77$, $p < 0.001$), but there was no influence of the β-DBE-DBCH on these temporal patterns (RM MANOVA time*trt: $p = 0.760$). There was also no significant influence of body mass on FT_4 or TT_4 concentrations.

The exposed male kestrels had significantly lower TT_4 plasma concentrations overall (RM MANOVA treatment effect: $F1,18 = 4.57$, $p = 0.046$). The TT_4 concentrations of the males changed showed significant temporal changes overall (RM MANOVA Time: $F3,23 = 8.77$, $p < 0.001$), but as with FT_4, the exposure to β-DBE-DBCH had no significant influence on these temporal TT_4 patterns (RM MANOVA time*trt: $p = 0.948$).

Conversely for female kestrels, there were no significant differences between the control and exposed females in plasma concentrations of FT_4 or TT_4 at pairing (p-values > 0.969). There were also no differences in circulating TT_4 in either males or females at the end of the breeding season, approximately 35 d after exposure to the β-DBE-DBCH had stopped.

Reductions in circulating levels of T_4 in the exposed male kestrels during the first half of the breeding season may indicate that thyroid gland production and/or release of this hormone from the gland were impaired by the immediate exposure of the birds to β-DBE-DBCH. Circulating concentrations of the protein-bound TT_4 was reduced in the β-DBE-DBCH-exposed males, while the unbound form, FT_4, was increased, suggesting that there may also have been some disruption of transthyretin and/or albumin, the thyroid hormone transport proteins. FT_4 is converted to triiodothyronine (T_3) by various deiodinase enzymes, mostly in the liver. The excess of circulating FT_4 in the male kestrels exposed to β-DBE-DBCH suggests that the deiodination and/or conjugation pathways involved with FT_4 and T_3 may have also been altered, possibly resulting in changes in circulating T_3 (that was not investigated). The possible impacts of β-DBE-DBCH on these various parameters of thyroid function in the male kestrels were not assessed, and further research is warranted to test this hypothesis.

The male kestrels exposed to β-DBE-DBCH also demonstrated a weak trend towards increased overall levels of TT compared to control males (RM ANOVA: treatment effect $F1,17 = 3.03$, $p = 0.095$). The GLMM analysis demonstrated a significant time*trt interaction between the two groups where exposed males showed considerably higher concentrations of TT in the week before egg-laying compared to controls (time*trt $F5,76 = 22.26$, $p < 0.001$ Fig: 2). The model including time*trt only was also the unequivocal top model with a weight of 0.76. Though subsequent models had negligible weights compared to the top model, several factors significantly affected the variation in TT: body mass ($F1,80 = 7.94$ $p = 0.006$), FT_4 ($F1,77 = 6.47$, $p = 0.013$), sampling date ($F1,80 = 53.49$, $p < 0.001$), copulation frequency ($F1,80 = 13.67$, $p < 0.001$) and female vocalization frequency ($F1,80 = 34.48$, $p < 0.001$) alone and in combination with other variables.

Compared to controls, female kestrels exposed to β-DBE-DBCH demonstrated significantly lower concentrations of TT ($t24 = 2.10$, $p = 0.047$), and slightly lower concentrations of E_2 ($t24 = 0.96$, $p = 0.347$). These slight reductions in E_2 were correlated with the significant decreases in TT ($r = 4.00$, $p = 0.030$), although the E_2:TT ratio did not differ between the groups. None of the male kestrels had detectible plasma levels of E_2 (LOQ = 3.0 pg/mL) at any time.

Typically, exposure to an androgen agonist results in a reduction in TT (as well as estrogen) since the increased negative feedback within the hypothalamic-pituitary-gonadal axis alters TT production and possibly the conversion of TT to E_2 through aromatase and/or reductase pathways (Clark et al. 1997 and references therein). This pattern was observed in the female kestrels, and indeed, there were altered concentrations of estradiols in their eggs (Marteinson et al. 2012). However, the endocrine response of males is contrary to what would be expected by the action of an agonist acting on the androgen receptor. This suggests that other mechanisms may also be involved with the steroid response of the male kestrels, suggesting that DBE-DBCH has multimodal actions on the steroid pathway including through the observed concurrent thyroid disruption (described above), and changes in sexual behaviors (Marteinson et al. 2015), observed in the same kestrels. Further research into the effects of β-DBE-DBCH on the steroid pathway is also warranted to test this hypothesis.

Acknowledgements
For their support and assistance, we thank I. Ritchie, D.M. Bird, L. Bardo and M. Gagnon from McGill University, and for chemical analysis, L. Gauthier (Letcher laboratory, Environment Canada). We thank the following for funding: Environment Canada (Ecotoxicology and Wildlife Health Division and the Chemicals Management: K.J. Fernie, R.J. Letcher), and the Natural Sciences and Engineering Research Council of Canada (239250-07, R.J. Letcher).

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Figures 1 and 2. Circulating concentrations of FT_4 (pg/ml; raw means) and TT (ng/ml; estimated marginal means) throughout the reproductive phases of courtship and incubation in captive male American kestrels (*Falco sparverius*). Comparisons are made between control males and those males exposed to β-DBE-DBCH by diet.