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Carotenoid concentration and coloration of American Kestrels (*Falco sparverius*) disrupted by experimental exposure to PCBs

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Summary

1. Bright coloration in birds is typically a sexually selected trait. Expression of such traits is sensitive to environmental factors, so they can function as bioindicators of environmental contamination. Of particular value may be carotenoid-based coloration because it is commonly used as a social signal and these pigments have important health functions.

2. American Kestrels (*Falco sparverius*) in captivity were exposed to dietary PCBs in March. Colour and plasma carotenoids of exposed and control birds were evaluated at pairing and courtship in April, and in winter in December. Juveniles produced by these birds (exposed to PCBs only *in ovo*) were examined at fledging and in winter.

3. The brightly coloured ceres and lores were evaluated by comparison to colour charts and quantified using digital photographs, and plasma carotenoid concentrations were quantified by spectrophotometry.

4. During breeding, PCB-exposed kestrels differed from controls for both colour and carotenoids, although the nature of effects was sex-specific. Carotenoids of juveniles were not related to treatment at fledging.

5. In winter, PCB-exposure resulted in patterns of colour/carotenoid variation opposite to controls; exposed adult males were duller, and juveniles of both sexes were brighter, than controls. PCB juveniles had higher plasma levels of carotenoids. Sexual dimorphism was apparent in colour and carotenoids of control adults, but not for PCB-exposed birds.

6. Our results are consistent with endocrine disruption. Modulation of both colour and carotenoids may have serious consequences to social behaviour and health.

Key-words: Endocrine disruption, integument coloration, plasma carotenoids, polychlorinated biphenyls, sexual selection

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Introduction

Sexual selection, whereby traits evolve to repel rivals or attract mates, has been a powerful force in the evolution of avian coloration (Savalli 1995; Badyaev & Hill 2000). Particularly good examples are bright reds, oranges and yellows of the skin and feathers of birds (Badyaev & Hill 2000). Like other sexually selected traits, these colours are typically sexually dimorphic, and develop in the breeding season and upon sexual maturity. These colours are predominantly derived from carotenoid pigments; molecules synthesized by plants and available to birds only through their diet (Brush 1990). These same molecules also play important roles in maintaining health, e.g. through antioxidant activity, immunological enhancement, and as precursors for vitamin A (Olson & Owens 1998; Møller *et al*. 2000).

Sexually selected traits are commonly condition-dependent, so their expression may be a useful measure of environmental quality as it represents the sum of environmental pressures on the animal (Hill 1995). Carotenoid-based coloration is particularly interesting from this perspective as it may not only be useful

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as a bioindicator of contaminant exposure, but disruption of the trait may indicate negative effects on physiological processes related to health. Recent field studies have shown perturbations in avian coloration along pollution gradients (Eeva, Lehikoinen & Rönkä 1998; Camplani, Saino & Møller 1999; McCarty & Secord 2000). In this study we investigate how experimental exposure to polychlorinated biphenyls (PCBs) may modulate colour and plasma concentration of carotenoids during breeding and in winter, in a wild species in captivity.

Our model is the American Kestrel (*Falco sparverius* L.), a small falcon. Kestrels have conspicuously coloured faces, for bare areas of skin above the bill and anterior to the eye, the cere and lores, respectively, vary from dull yellow to bright orange depending on carotenoid concentration. Males are brighter than females, and colour is most developed at sexual maturity and during the breeding season, consistent with predictions of sexual selection theory (Bortolotti *et al*. 1996; Negro *et al*. 1998).

Materials and methods

This work was conducted at the Avian Science and Conservation Centre of McGill University, Canada, where an outbred, captive colony of kestrels has been maintained since 1974. Beginning on 18 March 1998, kestrels were exposed to a dietary mixture of a 1 : 1 : 1 Aroclor 1254, 1248 and 1260. Aroclor 1242 and 1260 were obtained from US EPA, Research Triangle Park, NC, USA. Aroclor 1254 was obtained from Supelco, Mississauga, Ontario, Canada. PCBs were dissolved in safflower oil at a concentration of 4·85 mg g^{-1} total PCB and injected as 100-μL aliquots into the brain of dead, day-old cockerels, the standard food in this colony. Kestrels prefer eating the heads of the cockerels, but consumption probably varied among individuals and thus so did exposure. Control (CTL) birds were fed cockerels injected with safflower oil only. The levels of PCBs to add to the diet were determined from body burdens found in small mammals around contaminated sites (Environment Canada, unpublished data). In addition, we reviewed the literature for the proportions of different congeners of PCBs found in bird eggs and hence combined Aroclors to reflect those proportions (see also Smits & Bortolotti 2001). The calculated dose of approximately 7 mg kg^{-1} body mass day^{-1} resulted in environmentally relevant levels in eggs (Fernie *et al*. 2000). Drouillard *et al*. (2001) present a toxicokinetic model of PCBs in these birds.

Initially, kestrels were kept in large indoor aviaries at ambient temperature separated by sex and treatment. On 21 April 1998, 50 pairs (half controls) were created and placed in outdoor breeding pens 3·6 m (l) × 0·9 m (w) × 2·3 m (ht). Dietary exposure continued for about 100 days until the first sign of hatching of an egg (within each nest), and so the offspring were not exposed to dietary PCBs (see also Fernie *et al*. 2000, 2001a,b; Smits & Bortolotti 2001).

To quantify total carotenoids in plasma, 1 mL of blood was taken from the jugular vein using a heparinized syringe. Samples were taken at the time of pairing (21 April) and again 7 days later in the mid-courtship period (Fisher *et al*. 2001). For a winter sample, adult birds were bled on 2 February 1999. Blood was taken from juveniles at 22 days of age, just before fledging, and again on 15 or 16 December 1998. Following Bortolotti *et al*. (1996), 0·1 mL of plasma was diluted with acetone (1 : 10) and the flocculant protein was precipitated by centrifugation at 1500 ***g*** for 10 min. The supernatant was examined with a spectrophotometer with the optical density of the carotenoid peak at 476 nm, and carotenoid concentration (μg mL^{-1}) was estimated using a standard curve of lutein (alpha-carotene-3,3′-diol, Sigma-Aldrich Canada Ltd, Oakville, Canada).

Previous work with this colony and on wild birds used a six-step colour scale from dull yellow to bright orange, and each bird was subjectively assigned a score (Bortolotti *et al*. 1996; Negro *et al*. 1998). Unlike feathers which are permanently pigmented, cere and lore coloration can change over a matter of days. Colour scores were previously demonstrated to correlate with the concentration of plasma carotenoids in April (Bortolotti *et al*. 1996); however, given the time lag for colour to appear, the association may not be consistent during times of change. Kestrels were evaluated for colour prior to exposure to PCBs on 18 March, then again at pairing and at courtship. At the time of winter blood sampling, approximately 5 months after dietary exposure was terminated, colour was again evaluated using the scoring method as well as a more objective system of quantification using a digital camera (Villafuerte & Negro 1998). Birds were photographed with a Nikon 900 CoolPix with an external flash for consistent lighting. Red, green and blue (RGB) values for the ceres and lores were determined using Adobe PhotoDeluxe software. The mean value for RGB, standardized using reference colours (Villafuerte & Negro 1998), of each area per bird (i.e. six variables) was entered into a principal component analysis. The first component (PC1) represented 59% of the variance and is referred to here as an overall colour value. Red for both cere and lore had high negative loadings, while all other variables had high positive loadings on PC1; individuals with high negative values were considered brightly coloured.

Because both colour and concentrations of carotenoids vary with age in kestrels (Bortolotti *et al*. 1996), two age classes were considered: juveniles (hatched during this study) and adults (2 or more years old at breeding). Nonparametric statistical tests were used for analysis of colour scores. ANOVA/ANCOVA were used for all other variables, and non-significant interactions were removed iteratively and analyses repeated to obtain the most parsimonious model that explained

variation in the dependent variable. We examined whether body mass explained a significant portion of the variance because condition may be a factor explaining carotenoids and colour (Bortolotti *et al*. 1996). As all results were non-significant, mass was dropped from further analysis. Sample sizes vary somewhat among analyses as plasma volumes were not always sufficient for all components of the project.

Results

BREEDING COLOUR AND CAROTENOIDS

PCB-effects on colour scores in each of the sampling periods were investigated using Mann–Whitney tests (n = 49 males, n = 50 females). In the pre-dose sample in March, there were no differences between treatments as expected from our random selection of individuals (males: $Z = -0{\cdot}912$, $P = 0{\cdot}362$; females: $Z = -0{\cdot}489$, $P = 0{\cdot}625$). However, 1 month later at pairing, CTL males were significantly brighter than PCB males ($Z = -1{\cdot}991$, $P = 0{\cdot}046$), but there was no treatment effect for females ($Z = -1{\cdot}143$, $P = 0{\cdot}253$). In the courtship period, 7 days after pairing, no difference between PCB and CTL birds could be detected for males ($Z = -0{\cdot}831$, $P = 0{\cdot}406$) or females ($Z = -0{\cdot}230$, $P = 0{\cdot}818$). How individuals changed as they prepared for breeding, i.e. both from March to April pairing, and from pairing to courtship, was investigated using Wilcoxon Paired Tests (n = 49 males, n = 50 females). In the first month of exposure CTL males maintained their colour ($Z = -0{\cdot}905$, $P = 0{\cdot}366$) but PCB males significantly declined in brightness ($Z = -2{\cdot}810$, $P = 0{\cdot}005$). CTL females became significantly duller ($Z = -2{\cdot}049$, $P = 0{\cdot}040$) whereas colour of PCB females did not change significantly ($Z = -1{\cdot}507$, $P = 0{\cdot}132$). In the short period between pairing and courtship, male colour became brighter for PCB birds ($Z = -3{\cdot}500$, $P < 0{\cdot}001$), but the trend was not significant for controls ($Z = -1{\cdot}667$, $P = 0{\cdot}096$). For females, PCB-exposed birds went significantly duller ($Z = -2{\cdot}106$, $P = 0{\cdot}035$) but controls showed no significant change ($Z = -1{\cdot}155$, $P = 0{\cdot}248$).

For plasma carotenoids at pairing, an ANOVA resulted in an interaction between sex and treatment. When the sexes were subsequently analysed separately, CTL females had significantly lower concentrations ($F_{1,38} = 5{\cdot}27$, $P = 0{\cdot}027$) and CTL males had significantly higher concentrations ($F_{1,48} = 4{\cdot}20$, $P = 0{\cdot}046$) than birds exposed to PCBs (Fig. 1). During courtship, plasma levels of males did not vary with treatment ($F_{1,43} = 0{\cdot}032$, $P = 0{\cdot}858$), but there was a suggestion that CTL females had lower values ($F_{1,45} = 3{\cdot}12$, $P = 0{\cdot}084$) (Fig. 1). There were considerable differences in carotenoids between male and female controls at pairing ($F_{1,38} = 18{\cdot}01$, $P = 0{\cdot}001$) and courtship ($F_{1,45} = 7{\cdot}16$, $P = 0{\cdot}010$), yet there was no suggestion of dimorphism in PCB-exposed birds at pairing ($F_{1,48} = 0{\cdot}02$, $P = 0{\cdot}88$) or courtship ($F_{1,43} = 0{\cdot}01$, $P = 0{\cdot}92$) (Fig. 1).

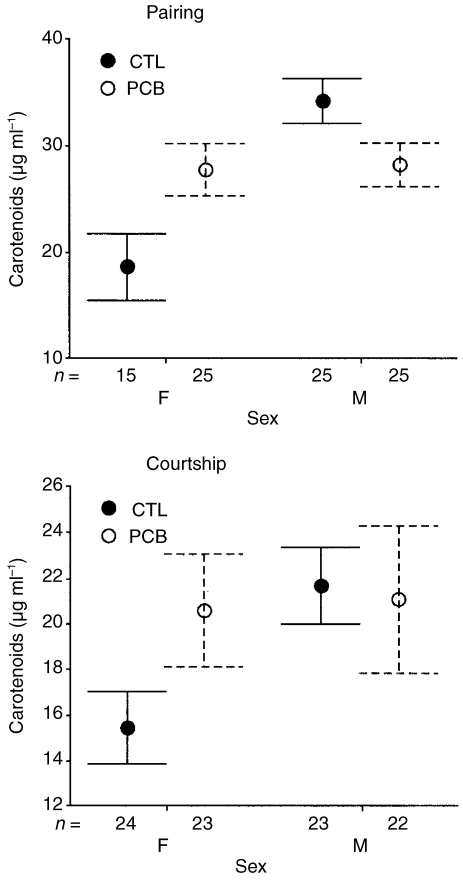

Fig. 1. Plasma carotenoid concentrations (mean ± SE) of male and female adult American Kestrels in PCB-exposed and control (CTL) groups at the time of pairing (21 April) and courtship (28 April).

CAROTENOIDS OF JUVENILES AT FLEDGING

It was not possible to assess the carotenoid-based colour of the cere or lores. In the wild and captivity, the ceres and lores of nestlings are a pale whitish-blue and only develop a significant amount of colour after fledging. Plasma carotenoids at fledging did not vary with respect to treatment for either males (mean CTL = 17·9, SE = 1·2, mean PCB = 16·6, SE = 1·3, $F_{1,22} = 0{\cdot}446$, $P = 0{\cdot}51$) or females (mean CTL = 17·7, SE = 1·6, mean PCB = 16·1, SE = 1·7, $F_{1,18} = 0{\cdot}376$, $P = 0{\cdot}55$).

WINTER COLOUR AND CAROTENOIDS

There was good agreement between colour scores perceived by eye and PC1 colours derived from photographs (Spearman correlation, $r_s = 0{\cdot}652$, $P < 0{\cdot}001$, $n = 188$, Fig. 2), as found in similar comparisons of techniques (Villafuerte & Negro 1998; Wiebe & Bortolotti 2001). The advantage of PC1 is that it varied in a continuous manner, and so was a better descriptor than scores in capturing subtle variation.

ANOVA for the PC1 colour value was run with age class, sex and treatment as factors. Because of a significant interaction between age class and treatment,

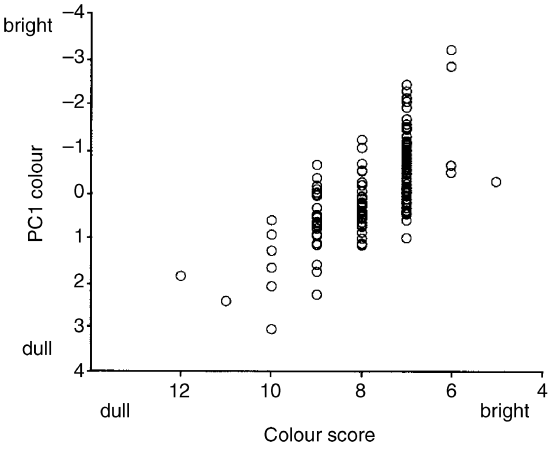

Fig. 2. Scatterplot of colour of the ceres and lores of American Kestrels as determined by colour chart (colour score) and analysis of digital photographs (PC1 colour).

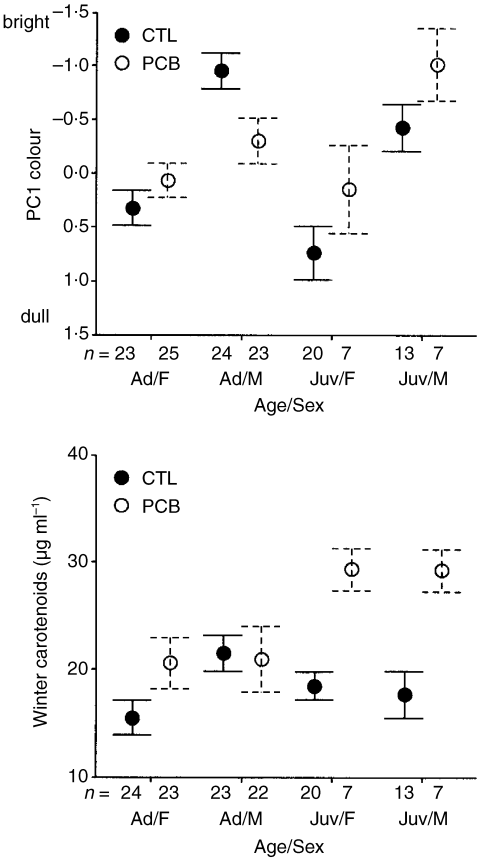

Fig. 3. Mean (± SE) colour values by digital photographs (top) and mean ± SE) plasma carotenoid concentration (bottom) as determined for different age/sex classes of PCB-exposed and control (CTL) American Kestrels in winter.

ANOVAs were performed on each age separately. For juveniles there was a significant effect of sex ($F_{1,44}$ = 15·68, P < 0·0001) and treatment approached significance ($F_{1,44}$ = 3·46, P = 0·070) (Fig. 3). However, for adults a significant interaction between sex and treatment required separate analyses by sex. Males exposed to PCBs were significantly duller than controls ($F_{1,45}$ = 6·15, P = 0·017), but females in the two groups were not significantly different ($F_{1,46}$ = 1·34, P = 0·25) (Fig. 3).

As carotenoids were sampled at different times for adults and juveniles, ages were analysed separately. PCB-exposed juveniles had levels significantly higher than controls ($F_{1,44}$ = 31·37, P < 0·0001) but males and females did not differ ($F_{1,44}$ = 0·09, P = 0·76). For adults there was no significant effect of either treatment ($F_{1,89}$ = 1·00, P = 0·32) or sex ($F_{1,89}$ = 2·12, P = 0·15). The lack of sexual dimorphism in the latter analysis is largely a result of males and females exposed to PCBs having the same levels of carotenoids ($F_{1,43}$ = 0·01, P = 0·92), whereas males had higher levels than females in the control group ($F_{1,45}$ = 7·16, P = 0·01).

Further evidence of sex-specific effects is revealed by a breakdown of the relationship between plasma carotenoids and colour. There is normally a positive relationship between the two, and it is generally particularly strong in male birds (Hill *et al.* 1994; Bortolotti *et al.* 1996; Saino *et al.* 1999). The association could be examined only for juveniles as photographs and blood sampling were done on the same day. Analysis of control kestrels confirmed that males with more carotenoids were more colourful (r = –0·663, P = 0·013, n = 13), but there was no relationship for females (r = –0·066, P = 0·782, n = 20). In contrast, there was no suggestion of any relationship between carotenoids and colour for PCB-exposed males (r = 0·166, P = 0·722, n = 7). The near significant results for contaminated females (r = 0·722, P = 0·067, n = 7) are perhaps further evidence of a disrupted physiology as the trend was in the opposite direction: birds with high concentrations of carotenoids tended to be dull.

Discussion

PCBs disrupted both colour and carotenoids in the breeding season (Fig. 1). Birds in the two treatments were the same in the pre-dose period, but differences appeared 1 month later, and again just 7 days after that during courtship when colour should have been at its maximal functional significance. Observations of these birds revealed that PCB-exposed males, despite being duller, exhibited more sexual behaviours during courtship than controls (Fisher *et al.* 2001). Contaminated males also contributed less to incubation (Fisher 2002). PCB-exposed breeding females also differed from controls. From March to April, control females lost colour as has been observed elsewhere (G. Bortolotti, unpublished data, and see Negro *et al.* 1998). Such a pattern is expected as carotenoids are diverted to developing ovaries (Surai 2002). PCB females did not lose colour and so may not have been as prepared for reproduction. Similarly, and perhaps related causally, these same contaminated females had a significant delay in egg laying (Fernie *et al.* 2001a).

When the adults were re-examined in winter, control birds showed the expected pattern of age and sex differences with carotenoids varying in a similar fashion

(Fig. 3). As found for carotenoids at breeding, sexual dimorphism in winter adults did not exist for PCB-exposed birds. More striking was that the brightest individuals with the most carotenoids were PCB juveniles (Fig. 3). The latter effects are particularly dramatic given PCB-exposure occurred only *in ovo* and at environmentally relevant concentrations. No contaminant-induced effect on carotenoids was found for juveniles at fledging perhaps because of physiological immaturity, or because there were few demands for carotenoids as pigments at that time. The effects of PCBs on growth of first and second generation offspring were also sex-specific, with either enhanced or delayed development (Fernie, Smits & Bortolotti 2003a; Fernie *et al*. 2003b). In addition, there was an increased incidence of teratogenic and developmental abnormalities in offspring of contaminated pairs (Fernie, Bortolotti & Smits 2003c), but such individuals were not used in this study. When these *in ovo* exposed kestrels were paired with clean, experienced breeders in the spring following colour analysis, they showed decreased reproductive success compared to controls (Fernie *et al*. 2001b).

Our study adds to a growing body of evidence for disruption of avian coloration by environmental contaminants. McCarty & Secord (2000) investigated plumage of Tree Swallows (*Tachycineta bicolor*) in an area contaminated by PCBs. Similar in part to our study (Fig. 3), they found an unusually high proportion of sub-adult females with bright, adult-like feathers even though such colours were not carotenoid-based. Eeva *et al*. (1998) found nestling Great Tits (*Parus major*) living in proximity to a metal smelter to have paler yellow feathers than birds further away. The difference in colour between populations was most probably explained by diet as the abundance of carotenoid-rich food items was associated with location (Eeva *et al*. 1998). Camplani *et al*. (1999) and Møller & Mousseau (2001) reported that male, but not female, Barn Swallows (*Hirundo rustica*) exposed to radiation at Chernobyl had a reduction in colour in the red (carotenoid-based) facial feathers. Additionally, partial albinism caused by germline mutation was disproportionate in the red facial area. These authors speculated that the physiological mechanism for coloration of feathers had broken down and that males may be more limited in carotenoid availability than females perhaps because of a greater need for carotenoids for colour, immune function and scavenging free-radicals.

Documentation of colour variation in parts of birds other than feathers is uncommon. Bortolotti, Smits & Bird (2003) found iris colour of kestrels (same individuals as this study) varied with exposure to PCBs. Irides were less red in contaminated birds, but the pigments were not carotenoids, and the effect was only observed in older birds actually fed PCBs.

While these recent studies raise concerns over the phenotypic effects of contaminants, little has been resolved as to mechanisms or consequences. Causality is particularly difficult to sort out without experimentation, which in field studies is logistically or ethically problematic (McCarty & Secord 2000). Our laboratory study has controlled for dietary availability of pigments which is particularly problematic in studies of wild birds with carotenoid-based coloration. Similarly, components of health, including immune function (Lochmiller, Vestey & Boren 1993), are influenced by diet and so our study controlled for other confounding variables. Despite the disparity in species, pigments and contaminants among studies, sex-specific effects were common (Camplani *et al*. 1999; McCarty & Secord 2000; Møller & Mousseau 2001; this study). Sexually selected characters, such as colour patterns, are more sensitive to environmental influences than are traits under natural selection and hence have the potential to be bioindicators of contamination (Hill 1995). Because sexually selected traits have evolved for social reasons, disruption by xenobiotics may have significant consequences. Bright colours may be an honest signal of good health because the bearer has access to abundant carotenoids (Olson & Owens 1998; Møller *et al*. 2000). However, as the normal correlation between plasma carotenoids and colour in male kestrels failed to appear in contaminated birds, an inaccurate assessment of an individual's phenotype may result. Camplani *et al*. (1999) also found that for swallows exposed to radiation, independent signals of phenotypic quality (of which colour was one) no longer were positively correlated, again suggesting that inaccurate or at least mixed signals could be received. Increased costs of social behaviour and reproduction, delays in breeding, incompatible pairing behaviour and so on are not implausible consequences, all of which may have negative effects on reproductive success. Such costs do not even consider any failings of physiological systems that may be directly necessary for reproduction or health.

If carotenoid levels decrease, it may reduce a bird's ability to scavenge free radicals, mount an effective immune response, or provide important antioxidants for eggs. Much of the toxicological literature (review by Rolland 2000) has not dealt with carotenoids directly, but rather with retinol (vitamin A, see Peakall 1992 for terminology) – a crucial vitamin for many physiological processes derived directly from carotenoid precursors (Surai 2002). While several studies of xenobiotics in birds have reported diminished concentrations of retinol, dietary availability of carotenoid precursors have never been controlled for (Rolland 2000). Retinol and thyroid hormones are transported on the same carrier protein complex, and both are known to decline with PCB exposure (Rolland 2000). Our work on both nestling (Smits *et al*. 2002) and adult kestrels (T. Marchant, G. Bortolotti, K. Fernie & J. Smits, unpublished data) showed PCB exposure diminishes levels of thyroxine (T4) and triiodothyronine (T3). In similar studies of American Kestrels, Hoffman *et al*. (1996) found PCB 126 to cause degenerative lesions of

the thyroid in nestlings, and Quinn *et al*. (2002) found Aroclor 1242 to suppress levels of T4 in adults. Thyroid hormones have significant influences on feather development, so Quinn *et al*. (2002) predicted plumage colour of kestrels would be affected by PCBs. Although their experimental dosing with Aroclor 1242 depressed hormone levels, it failed to have any impact on the colour of secondary or tail feathers. Quinn *et al*. (2002) concluded that timing or dose of exposure, or a genetic control over colour, could explain their lack of positive results; however, none of the colours was carotenoid-dependent, and plumage colour may be controlled by steroids (Owens & Short 1995).

Thyroid hormones are also functionally important in the immune system (Smits *et al*. 2002) and several avian studies have linked carotenoids, or carotenoid-based coloration, to aspects of health and immune function (e.g. Olson & Owens 1998; Saino *et al*. 1999; Lindstrom & Lundstrom 2000; Møller *et al*. 2000). The pattern of colour/carotenoids we found in kestrels in winter mirrors the sex-specific antibody response of PCB-exposed adult kestrels when vaccinated with a novel antigen (DNP-KLH) during the breeding season: males were immunosuppressed while females were immunostimulated (Smits & Bortolotti 2001). Hoffman *et al*. (1996) dosed nestling kestrels with PCB 126 and found lymphocyte depletion of the spleen and bursa, reduced immune organ weight, and a higher susceptibility to oxidative stress.

While thyroid hormones have many important functions, hormones of particular concern for both feather colour and reproductive effects of PCBs should be sex steroids. The expression of bright plumage colour may be dependent on oestrogen or testosterone depending on the species (Witschi 1961; Owens & Short 1995). Far less is known of the endocrine control of non-feather parts of birds, but both sex steroids can influence the colour of bills (Witschi 1961). Oestrogen supposedly influences bill colour of the Red-billed Quelea (*Quelea quelea*); it fades to orange during breeding, or remains red in ovarectomized birds (Witschi 1961; Owens & Short 1995). However, that colour change may merely be caused by the allocation of carotenoids to yolks, and is not evidence for a direct endocrine effect on colour. Nevertheless, oestrogen may be a good candidate for future studies as some xenobiotics are known endocrine disruptors, and their oestrogenic effects are of particular concern to human and non-human animal health (Crews, Willingham & Skipper 2000). Studies of domestic fowl have shown oestrogen stimulates the redistribution of lipoproteins, the main carrier molecules of carotenoids (Kudzma, Swaney & Ellis 1979; and see Bortolotti *et al*. 2003a).

Endocrine disruption in our kestrels was confirmed as we detected depressed corticosterone, T3 and T4 concentrations (Smits *et al*. 2002; Love *et al*. 2003; Marchant *et al*. unpublished data) but these effects, and behavioural abnormalities (Fisher *et al*. 2001; Fisher 2002), were overwhelmingly more common in males. No changes were detected in circulating levels of oestrogen or testosterone in breeding females and males, respectively (Fisher *et al*. 2001; Marchant *et al*. unpublished data); however, oestrogen mimics may impact endocrine function by a variety of mechanisms (Crews *et al*. 2000).

Clearly the mechanisms and potential consequences of colour disruption are many, and unlikely to be independent. The effects reported here, and for other birds (Eeva *et al*. 1998; Camplani *et al*. 1999; McCarty & Secord 2000; Bortolotti *et al*. 2003b), suggest a widespread phenomenon that has not previously been recognized. The fact that these results are recent may be a product of current emphasis on objective quantification of colour, combined with advances in our understanding of both the evolutionary, social and health implications of variation in both colour and carotenoids.

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