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Plumage Coloration in Belted Kingfishers (*Megaceryle alcyon*) at a Mercury-contaminated River

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Abstract.—Because Belted Kingfishers (*Megaceryle alcyon*) eat a diet comprised primarily of fish they are a useful indicator species for aquatic contaminants such as mercury. Monitoring efforts generally compare nesting success or tissue contaminant concentrations from contaminated sites with reference sites. In contrast, this study examined subtler potential effects of mercury accumulation by quantifying plumage coloration (structural and melanin based) of nesting adult Belted Kingfishers and relating it to individual mercury concentrations. Mercury exposure was associated with increased brightness of plumage color consistent with the hypothesis that mercury slows the production of melanin. Clear sex differences in the chroma and hue of blue body feathers identified during this study suggest that Belted Kingfishers possesses cryptic dimorphism beyond the rufous “belt,” and thus mercury-induced alterations in blue plumage could reduce fitness. *Received 11 May 2012, accepted 23 January 2014.*

Key words.—Belted Kingfisher, *Megaceryle alcyon*, mercury, plumage, reverse dimorphism.

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Ornamental traits such as plumage coloration have been associated with health in numerous species and are an important component of reproductive effort (Siefferman and Hill 2005). Relative to less costly traits, ornamental traits are at a greater risk of perturbation by adverse environmental conditions (Hill 1995). In this study, we examined the relationship between exposure to mercury, an environmental pollutant known to affect avian health, and plumage coloration in male and female Belted Kingfishers (*Megaceryle alcyon*).

Belted Kingfishers have been recognized as an important target species for monitoring aquatic pollution because they feed at the top of lengthy aquatic food chains, and thus can accumulate high concentrations of biomagnifying contaminants such as mercury (Baron *et al.* 1997; Zamani-Ahmadmohammoodi *et al.* 2009). The male and female plumages are ostensibly similar in Belted Kingfishers; both possess blue back feathers, white chest feathers, and a ventral band of blue. Females have an additional ventral “belt” of rufous feathers that has given rise to the species’ common name and is a classic example of reverse sexual plumage dimorphism.

Mercury has the potential to affect plumage coloration in birds in several ways. At high levels of exposure, mercury impacts

many aspects of physiology, including the immune system (Scheuhammer *et al.* 2007; Lewis *et al.* 2013). If a molting bird is expending extra energy on immune defense, it may have less to invest in plumage structure and coloration. Mercury could also affect feather color indirectly by disruption of the endocrine system. For example, mercury contamination has been correlated with altered estradiol and testosterone concentrations in wading birds, as well as reduced reproductive effort and altered pairing behavior (Jayasena *et al.* 2011). Analogous situations occur with polychlorinated biphenyl (PCB) contaminants, which are well-known endocrine disruptors. When exposed to PCBs, sub-adult female Tree Swallows (*Tachycineta bicolor*) prematurely developed adult coloration (McCarty and Secord 2000) and American Kestrels (*Falco sparverius*) produced duller carotenoid-based ceres and lores (Bortolotti *et al.* 2003).

Alternately, mercury might affect feather coloration by directly disrupting the biochemical processes through which color is produced. Tyrosinase is a catalytic enzyme essential for melanin production (McGraw 2006). *In vitro* studies suggest that mercury inhibits tyrosinase availability by binding to tyrosinase in place of the catalytic cofactor copper (Lerner 1952). Because of this property, mercury has long been used as an active

ingredient in human skin-lightening creams (Al-Saleh *et al.* 2004). Thus, mercury could alter the concentration of melanin in growing feathers. Additionally, mercury binds to keratin molecules within the structure of feathers and could, in theory, alter the microstructure that produces coloration in some feathers (Appelquist *et al.* 1984).

To evaluate the relationship between mercury and plumage coloration, both were measured in feathers from Belted Kingfishers living in an area with high level of mercury contamination (Cristol *et al.* 2008; Jackson *et al.* 2011). Our objective was to determine whether feather mercury concentration is associated with altered plumage coloration in Belted Kingfishers.

METHODS

Study Area and Tissue Sampling

The South, Middle and North Rivers are the main tributaries forming the South Fork of the Shenandoah River. Between 1929 and 1950, a textile manufacturing facility in Waynesboro, Virginia (38.06° N, 78.88° W) deposited mercuric sulfate into the South River (Carter 1977), while the other two tributaries have remained free of significant mercury pollution. The South River was heavily contaminated and a fish consumption advisory remains in effect on it, as well as downstream on the South Fork of the Shenandoah River (Virginia Department of Environmental Quality 2014). Feathers and blood from Belted Kingfishers breeding on the heavily contaminated South River were compared to samples from Belted Kingfishers nesting on the moderately contaminated South Fork of the Shenandoah River as well as the two "reference" tributaries, which have no history of mercury contamination (Middle and North Rivers).

Belted Kingfisher nests were located by surveying all riverbanks using canoes early in the breeding season (April-May). Birds were caught from 23 May to 18 June 2005 and 22 April to 6 June 2006, either by placing a mist net in front of the nest or by excavating the nest from the back during brooding and then repairing the excavation. Blood and feathers were collected from both males ($n = 23$) and females ($n = 32$). Of the 55 adults banded, 27 were captured at 19 nests on the two reference rivers, 21 were captured at 13 nests on the heavily contaminated South River, and nine were captured at seven nests downstream on the moderately contaminated South Fork of the Shenandoah River. Two individuals were captured in both years, at different sites, and treated as independent samples because mercury concentrations had changed and plumage had molted.

Blood was sampled from the brachial vein using a 25-gauge needle. Blood samples were immediately

placed on ice and then frozen at -25 °C within 8 hr of collection. From each individual, we sampled nine feathers from the blue back, nine from the blue chest band, nine from the white area immediately dorsoventral to the blue chest band, and, in females, nine from the rufous "belt" across the abdomen. Age of adult Belted Kingfishers (second year or after second year) was determined by inspecting the blue chest band for the presence of juvenile rufous feathers (Bent 1940; Pyle 1997; Kelly *et al.* 2009).

Color Quantification

Each of the nine feathers of similar type were placed directly on top of each other, corresponding to the way they lay on a bird, and taped to a sheet of black construction paper (Siefferman and Hill 2003). An Ocean Optics USB 2000 UV-VIS spectrometer (Range = 250-880 nm) with a PX-2 light source was used to measure the color of the feathers. Feathers were measured with a probe that both sent and received light signals at a 90° angle to the feathers. The probe was set so that a 3 mm diameter region of feather surface was illuminated, the level at which the maximum pixel count occurred for a white standard (WS-1). Color measurements were recorded using the software OOIRrad (Ocean Optics 2006). An individual measurement was composed of the mean of 20 reflectance curves taken at 100 millisecond intervals. Five repeated measurements were performed on each sample of feathers from a single individual, and this was repeated for blue back, blue chest, and white chest plumage, and rufous feathers for females. To ensure independent measurements, between each measurement the probe was removed and replaced, aiming for the same location on the feathers.

Overall variation in color was evaluated using three commonly used colorimetric variables for each type of feather: brightness or reflectance across the entire spectrum, hue or dominant color, and chroma or the proportion of reflectance concentrated around the peak. To reduce the effects of variation in the data, each curve was first smoothed by calculating the median value of reflectance for every 81 readings. Hue was analyzed for colors that peaked within the visible range of birds (blue and white) by taking the mean value of the wavelength where the percent reflectance was at its maximum. For colors such as browns and reds, which have a much greater reflectance at the high end of the avian visual range and peak beyond 700 nm, a different measurement of hue was calculated as the wavelength at the point on the spectrum where the slope of the reflectance curve was the greatest (Siefferman and Hill 2003). Chroma was calculated for blue feathers as the proportion of total reflectance occurring from 300-500 nm and for rufous feathers as the proportion of total reflectance occurring from 500-700 nm. In white feathers, which had the most variation in the ultraviolet range, chroma was calculated as the proportion of total reflectance occurring from 300-400 nm. For all feather types, brightness was the mean reflectance over the visible range (300-700 nm) (Montgomerie 2006).

Mercury Analysis

Mercury was measured in the same feathers that had been used for colorimetric analysis, but afterward, because mercury analysis is destructive. In preparation for mercury analysis, feathers were washed with deionized water and dried in a low-humidity chamber. They were then homogenized with scissors to pieces of approximately 1 mm² to permit destructive analysis of total mercury.

Mercury was measured using the atomic absorption spectroscopy method in a direct mercury analyzer (Milestone DMA80). Nearly all of the mercury in feathers is in the form of highly bioavailable methylmercury, so measuring total mercury, which is less expensive, is an accurate estimate of methylmercury content (Wada *et al.* 2009). After every 20 samples, one duplicate sample, three method blanks, and two samples of standard reference materials (DORM-2 and DOLT-3, homogenized fish tissues purchased from the National Research Council of Canada) were run for quality control. Distributions of feather and blood mercury values were positively skewed and were therefore log normalized before analysis. All mean values are reported \pm SD and all mercury concentrations are parts per million (ppm) wet (blood) or fresh (feather) weight of tissue.

Statistical Analyses

Mercury concentrations in blood and feathers were evaluated separately using ANOVAs with age and sex as treatment groups. Comparisons between mates were conducted using paired *t*-tests. Pearson's product-moment correlations were used to determine the relationship between feather and blood mercury concentrations. Each colorimetric variable was analyzed in an ANCOVA using feather mercury concentration as a covariate and sex as treatment group to evaluate plumage response to mercury concentration and color differences between sexes. All analyses were tested using the statistical program R (R Development Core Team 2010).

RESULTS

Mercury Loads

Blood mercury concentrations, which reflect recent diet, were significantly higher in Belted Kingfishers breeding along the South River (3.35 ± 0.58 , $n = 21$) than those breeding on the South Fork of the Shenandoah River (0.56 ± 0.11 , $n = 9$) or the reference rivers (0.25 ± 0.03 , $n = 27$; $F_{2,54} = 22.99$, $P < 0.01$). At nests where both adult birds were captured, blood mercury concentrations did not differ between the sexes (paired $t_{16} = 0.096$, $P > 0.05$).

Feather mercury concentrations were strongly correlated among the different

feather types within individuals ($X^2 > 0.95$, $P < 0.05$ in all correlations), and therefore feathers of each individual were averaged to produce a single feather mercury score. Feather mercury concentrations from the South River (26.25 ± 7.27 , $n = 21$) tended to be higher, on average, than those from the South Fork of the Shenandoah River (9.44 ± 3.18 , $n = 9$) and reference (Middle and North) rivers (11.54 ± 5.07 , $n = 25$), but these differences were not significant ($F_{2,52} = 2.06$, $P > 0.05$).

The relationship between blood and feather mercury concentrations was evaluated using age and sex as cofactors. There was a significant positive covariance between blood and feather mercury concentrations ($F_{1,47} = 18.20$, $P < 0.01$), as well as a significant interaction between sex and the covariance of blood and feather mercury concentrations ($F_{1,47} = 5.94$, $P < 0.05$). When further evaluated, the blood and feather mercury concentrations in male kingfishers were significantly correlated ($X^2 = 0.75$, $r^2 = 0.57$, $P < 0.01$; Fig. 1A), while those of females were not ($X^2 = 0.24$, $r^2 = 0.06$, $P > 0.05$; Fig. 1B). A few males and many females had feather mercury values that did not match their blood mercury concentration.

Color and Feather Mercury Concentrations

Feathers containing melanin tended to be brighter at higher mercury concentrations (Table 1). An ANCOVA showed that regardless of sex, Belted Kingfishers with higher mercury had brighter blue chest feathers (Table 1), and a relationship, albeit non-significant, was also suggested for blue back and rufous "belt" feathers (P -values < 0.1 ; Table 1). This is apparent from the significant linear relationship between brightness of the blue chest feathers (structural color containing melanin pigment) and mercury concentration of both sexes (Female: $t_{29} = 2.113$, $r^2 = 0.13$, $P = 0.04$; Male: $t_{21} = 2.477$, $r^2 = 0.23$, $P = 0.02$; Fig. 2i). The linear relationships for blue back feathers and rufous "belt" feathers (females only, color produced by melanin) were also positive but did not differ significantly from the null hypothesis of

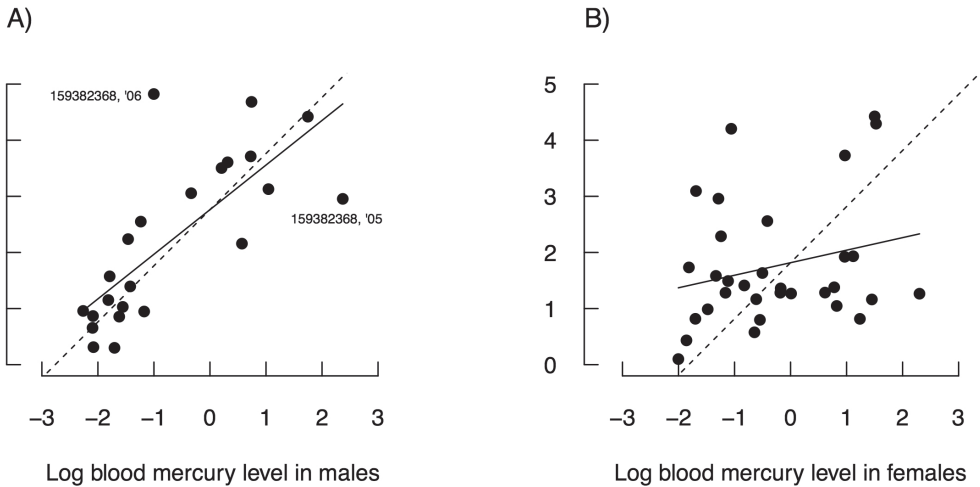


Figure 1. Correlation between blood and feather mercury levels in male (A) and female (B) Belted Kingfishers (*Megasceryle alcyon*). Solid line indicates slope and dashed line shows the 1:1 ratio for comparison.

no relationship with mercury concentration (Female blue back: $t_{30} = 1.78$, $r^2 = 0.10$, $P = 0.08$; Male blue back: $t_{21} = 0.45$, $r^2 = 0.01$, $P = 0.66$; Fig. 2c; Female rufous chest: $t_{29} = 1.16$, $r^2 = 0.044$, $P = 0.25$; Fig. 2l).

Blue chroma, or purity (i.e., the proportion of reflected color represented by blue wavelengths), tended to decrease in females as mercury increased, but this relationship was only significant in female blue back feathers ($t_{29} = -2.24$, $r^2 = 0.14$, $P = 0.03$;

Fig. 2b). The linear relationship between chroma and mercury was also negative in white feathers of both sexes combined ($t_{53} = -2.08$, $r^2 = 0.06$, $P < 0.05$; Fig. 2e). An ANCOVA revealed a significant effect of mercury on white chroma regardless of sex (significant F -value for mercury concentration; Table 1), such that Belted Kingfishers with higher mercury had less purity of white in their chest feathers. While the rufous feathers of females also showed a negative linear relationship with increasing mercury concentrations, it was not significant (Fig. 2k). An ANCOVA confirmed the non-significant effect of mercury on chroma of female rufous chest feathers. No other linear trends between mercury and brightness or chroma were suggestive of biological relationships (Fig. 2f, h). Finally, there was no detectable relationship between mercury and hue, which is a measure of the dominant color (Fig. 2a, d, g, j).

Sex Differences

Significant differences between male and female colors were found in the blue chest feathers, where males had significantly lower hue and higher chroma than females (Table 1). A very similar pattern was present in the blue back feathers, but differences were not significant.

Table 1. F -ratios among colorimetric scores with log normalized feather mercury concentrations, and sex, as factors in Belted Kingfishers (*Megasceryle alcyon*). Mercury concentration interactions with sex are not included as none were significant. Degrees of freedom were $F_{1,51}$ in all cases but rufous chest, which was $F_{1,30}$ as only females possess this plumage color. * indicates significance $P < 0.05$.

Feather Region	Color Variable	Sex	Mercury
Blue Back	Hue	1.35	0.46
	Brightness	0.12	3.28
	Chroma	1.74	1.13
White Chest	Hue	2.19	0.04
	Brightness	2.21	0.02
	Chroma	0.93	4.28*
Blue Chest	Hue	12.25*	0.81
	Brightness	0.63	9.50*
	Chroma	7.75*	0.35
Rufous Chest	Hue	—	0.48
	Brightness	—	3.16
	Chroma	—	3.01

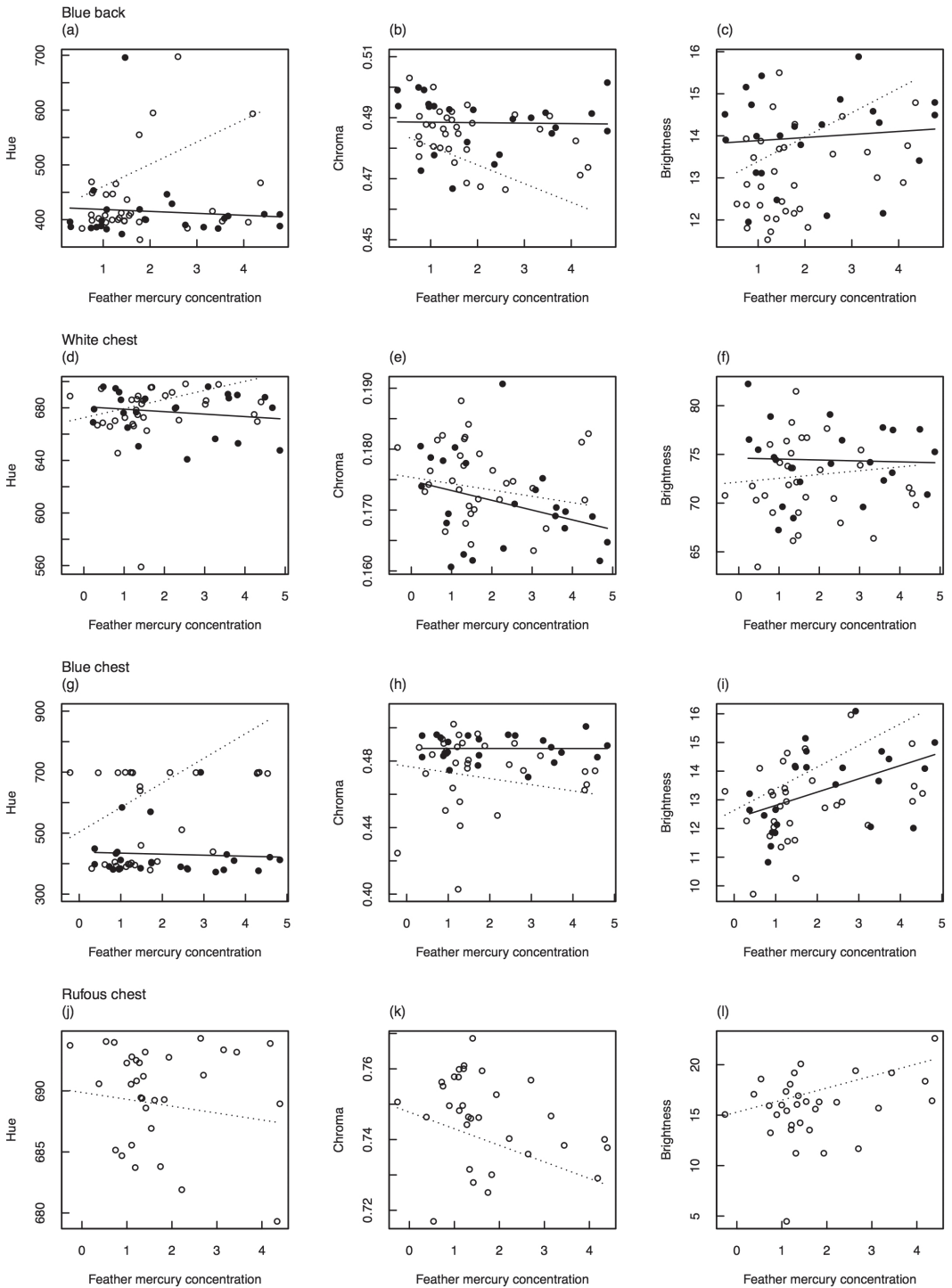


Figure 2. Relationship between feather mercury concentration and colorimetric variables (hue, chroma, and brightness) in male (solid line and filled circles) and female (dashed line and unfilled circles) Belted Kingfishers (*Megasceryle alcyon*). Four plumage regions were sampled: blue back feathers (a-c), white chest feathers (d-f), blue chest feathers (g-i), and rufous chest feathers possessed only by females (j-l).

DISCUSSION

Several aspects of plumage coloration in Belted Kingfishers appeared to be related to the concentration of mercury in feathers. Mercury added a significant or near-significant component of variation to aspects of blue coloration on chest and back and the ventral rufous coloration on females. The effects we found could be explained by one of at least three processes: 1) mercury is a potential endocrine disruptor and could alter the production of color by influencing sex-specific steroid hormones; 2) mercury could impact health and reduce the reserves available for investment in condition-dependent plumage coloration; or 3) mercury could directly disrupt pigment production or feather microstructure. Because no data on hormone concentrations were gathered, it is not possible to evaluate the role of the endocrine system in mediating the relationship between mercury and color.

The link between condition and plumage coloration has generally been explored in birds that, unlike Belted Kingfishers, have carotenoid-based pigments (McGraw *et al.* 2005a). In contrast, blue colors are almost always derived from the interactions between melanin and the nanostructure of the feathers (Prum 2006), and white colors are generally derived by physical variations within the feather structure that reflect all wavelengths of light. The rufous color of female Belted Kingfishers is likely derived mostly from phaeomelanin, a form of melanin (McGraw *et al.* 2005b). Because mercury has well-documented effects on condition in birds (Scheuhammer *et al.* 2007), it is possible that the body burden of mercury at the time of molt influences the reserves available for creating the pigments or microstructure of feathers. However, it was not possible to rigorously test whether mercury-induced reduction in condition was a mechanism underlying the relationship between mercury concentration and coloration because the study was conducted during the breeding season, approximately 5-10 months after molt. Condition measures taken at that time would have had little correlation with those

at the time the feathers were produced, especially in light of the observation that these Belted Kingfishers sometimes dispersed between contaminated and uncontaminated sites (White 2007).

The third hypothesized mechanism for the relationship between mercury and plumage coloration is direct disruption of the color production pathway. *In vitro* experiments (Lerner 1952) have shown that inorganic mercury could inhibit the production of melanin by competing with copper for binding sites on tyrosinase, the enzyme that catalyzes the initial step in melanin production. Tyrosinase requires a copper cofactor but will bind to other metals including mercury (McGraw 2003). Therefore, while copper facilitates the production of melanin, mercury slows it down. The presence of high concentrations of mercury during feather growth could reduce the amount of melanin available. Consistent with this theory, regenerated limbs of fiddler crabs exposed to methylmercury were devoid of melanin (Weis 1977).

The underlying melanin layer in structural colors increases the purity of a color (or chroma) by absorbing random scattering of light caused by the structure of the feathers (Shawkey and Hill 2006). If mercury inhibits melanogenesis and causes a decrease in the amount of melanin in the pigment layer, an increase in random scattering of white light may result, causing the feather to appear brighter. Consistent with this explanation, there was a tendency for all feather types containing melanin to become brighter with increases in mercury content regardless of sex. Additionally, the blue chest feathers of female Belted Kingfishers had lower chroma levels as mercury concentration increased (less concentrated blue coloration). Generally female Belted Kingfishers in their first breeding season have numerous residual rufous-tinged feathers in their blue chest band (Kelly *et al.* 2009). The lower chroma in birds with higher mercury may have resulted from an increase in the retention of these juvenile feathers in females in poorer condition. Rufous feathers in females with higher mercury concentrations tended to

have higher brightness and lower chroma scores and thus probably contained less melanin. The coloration of rufous feathers is not structurally based like blue feathers, but the same mechanism could occur because melanin is present in both. The potential role of mercury as an inhibitor of melanogenesis in feathers should be explored in controlled experiments where both mercury consumption and the dietary precursors to tyrosinase can be manipulated.

As expected, Belted Kingfishers sampled along a mercury-contaminated river during the breeding season had elevated mercury concentrations in blood relative to two uncontaminated rivers (Cristol *et al.* 2008). Feather mercury, which reflects body burden of mercury during the previous molt, was correlated with blood mercury for males but not for females. Like many other bird species, the Belted Kingfisher is a partial and a differential migrant; not all migrate and females disperse farther than males during the winter (Kelly 1998). In addition, it has been suggested that males, but not females, care for young after fledging (Davis 1980), and males are more likely to return to the same nesting territory than females (Albano 2000). Thus, a male nesting at a given contaminated site will be more likely to molt there and breed again at the same site in a subsequent year. Our finding that males had a closer correspondence between feather and blood mercury concentrations than females is consistent with this sex difference in kingfisher life history.

In a highly site-faithful species, one would expect close correspondence between feather and blood mercury, as found for Tree Swallows (*Tachycineta bicolor*) at this site (Brasso and Cristol 2008). In Belted Kingfishers, on the other hand, some individuals appear to have molted on a contaminated site after one breeding season, and then moved to a reference site for the next, or vice versa. Such transient individuals are the outliers to the dashed line in Fig. 1 (e.g., male 159382368 bred on a contaminated site in 2005 and a reference site in 2006 and its blood mercury dropped from 10.7 to 0.4 ppm).

We documented a previously unreported sex difference in Belted Kingfisher coloration, suggesting that sexual selection may be at work in this species. Male and female chest bands are different colors of blue, with females having more reflectance at higher wavelengths, and males having higher purity of blue at the dominant wavelengths. Due to the high reflectivity of white feathers and the low reflectivity of rufous feathers, the namesake rufous "belt" of female Belted Kingfishers decreases the reflectivity of the female's chest relative to the male. Typically, the female Belted Kingfisher is described as being a case of reverse sexual plumage dimorphism. The Belted Kingfisher is a sister species to the larger Ringed Kingfisher (*M. torquata*), which is similar in coloration to the Belted Kingfisher (Moyle 2006). In that species, both the males and females have a rufous chest, raising the possibility that the ancestral condition was rufous-chested and that male Belted Kingfishers evolved a white chest patch as a sexually-selected elaboration of plumage.

Belted Kingfishers breeding on a mercury-contaminated river exhibited altered plumage coloration, consistent with interference by mercury in the melanin-production pathway. Mercury concentrations in blood and feathers were not tightly correlated, suggesting that some birds, especially females, switched between contaminated and reference sites, thereby accumulating feather mercury at one site and blood mercury at another. The effects of altered plumage coloration in this species are unknown, but the finding that male and female blue plumage is more dimorphic than previously believed suggests that blue color could play a role in mate choice and altered plumage could have behavioral signaling and fitness consequences. The effects of mercury on Belted Kingfishers at this one contaminated river system are relevant nationwide; for example Belted Kingfishers living along approximately half the river miles on the Missouri, Ohio and Mississippi Rivers are at risk of adverse effects from mercury (Walters *et al.* 2010).

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