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Original Article

Testosterone production, sexually dimorphic morphology, and digit ratio in the dark-eyed junco

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The hormonal environment an individual experiences during development can have lasting effects on behavior, morphology, and physiology. However, measuring endogenous hormone exposure during early embryonic development and relating it to adult phenotype has proved to be challenging. The relative length of digit 2 to digit 4 (2D:4D) in adults is thought to reflect the relative concentration of androgens and estrogens during development and has been used as a morphological proxy of developmental hormone exposure, enabling the exploration of the relationships between individual differences in hormone exposure and phenotype. Here, we use this approach and ask whether 2D:4D relates to the responsiveness of the adult hypothalamic-pituitary-gonadal axis and to sexually dimorphic structures (wing, tail, and tarsus length) in a common songbird, the dark-eyed junco (*Junco hyemalis*). Among males, we found a negative relationship between 2D:4D and the ability to elevate testosterone in response to a physiological challenge, an injection of gonadotropin-releasing hormone. We also found age- and sex-specific relationships between 2D:4D and wing, tail, and tarsus length. We found a positive relationship between 2D:4D and body size measures in females, and second-year and older males, but a negative relationship in first-year males. We conclude that individual variation in exposure to developmental hormones, as reflected by 2D:4D, is correlated with adult hormone production ability and sexually dimorphic morphology in adulthood, suggesting that endogenous variation in steroid hormone exposure may have long-term consequences similar to those seen in experimental manipulations. *Key words:* digit ratio, gonadotropin-releasing hormone, junco, maternal hormones, testosterone, 2D:4D. *[Behav Ecol]*

INTRODUCTION

Variation in elements of the early developmental environment (e.g., temperature, location, resource levels) can have dramatic and long-lasting effects, acting as an important source of phenotypic variation (Mousseau and Fox 1998; West-Eberhard 2003). Among the most potent early environmental effects in vertebrates are steroid hormones and understanding how these developmental hormones influence adult phenotype is an important challenge for biologists (Dufty et al. 2002; Gil 2003; Uller 2008). At laying, avian eggs contain the full complement of maternally derived hormones, unlike developing mammals, which may experience fluctuations in hormone concentrations throughout gestation, making birds an ideal system (Gil 2003; Groothuis et al. 2005; Groothuis and Schwabl 2008). Experimental manipulation of steroid hormones during development can heavily influence offspring phenotype (e.g., Hayward and Wingfield 2004; von Engelhardt et al. 2004; Navara et al. 2005; Paitz et al. 2011; Ruuskanen et al. 2012). Testosterone, for example, can have short-term anabolic effects on offspring growth and behavior (Schwabl 1993; Lipar and Ketterson 2000; Groothuis and Schwabl 2008; Cox et al. 2009), as well as long-term effects on morphological, physiological, and behavioral traits in adults (Schwabl 1993; Gil 2003; Groothuis and Carere 2005; Groothuis et al. 2005; Rubolini et al. 2006).

One powerful way that developmental hormones may influence adult traits is via alteration of the adult hormonal phenotype, which mediates a variety of morphological, behavioral, and physiological traits (Ketterson et al. 1992; Trainor and Marler 2001; Brown et al. 2005; McGlothlin et al. 2008; Mills et al. 2009; Cain and Ketterson 2012). Recent work indicates that experimentally elevating testosterone in the egg alters plasma testosterone levels later in life (Muller et al. 2007; Pfannkuche et al. 2011). These findings suggest that a portion of the individual variation in adult hormonal phenotype may be attributable to differences in hormone exposure during development, possibly via effects on the hypothalamic-pituitary-gonadal (HPG) axis, which regulates the production and secretion of gonadal steroids (Pfannkuche et al. 2011). However, it is currently unclear whether variation in endogenous hormone exposure has similar consequences.

Elucidating the relationship between adult hormonal phenotype and endogenous variation in developmental hormone exposure is currently hampered by methodological difficulties. For instance, in many species, yolk hormones vary according to position in lay order; however, relating phenotype to lay order is of limited use in understanding the extent to which endogenous hormone concentrations are a source of individual variation because females vary substantially in the amount of steroid hormone they deposit naturally in eggs (Gil 2003; Burley and Foster 2004; Groothuis et al. 2005; von Engelhardt and Groothuis 2005; Jawor et al. 2007). This issue can be avoided by measuring

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offspring hormone environment directly, sampling individual yolks prior to development. However, sampling the yolk is difficult and can destroy the developing embryo, particularly in species with small eggs (Schwabl 1993; Bowden 2001; Garamszegi et al. 2007), and hormone concentrations vary substantially according to depth of yolk that is sampled, for example, testosterone is concentrated in the center of yolk and drops precipitously in the outer layers, limiting the usefulness of yolk biopsies (Lipar et al. 1999; Bowden 2001; Burley and Foster 2004; Groothuis and von Engelhardt 2005). Finally, the hormonal environment is dynamic, hormones are metabolized during development (Groothuis and Carere 2005; Paitz and Bowden 2011; Paitz et al. 2011), and an integrated measure of its impact on individual variation in phenotype is needed.

Consequently, some researchers have argued for the use of a morphological proxy for overall hormonal environment during development, which would enable investigation of the long- and short-term consequences of variation in hormone exposure in free-living individuals of a variety of species (Burley and Foster 2004; Navarro et al. 2007; Dreiss et al. 2008; Tobler et al. 2011). In mammals, the relative length of digit 2 to digit 4 (2D:4D) has been shown to reflect the ratios of estrogen:androgens experienced during ontogeny (Zheng and Cohn 2011). The relationship between 2D:4D and hormone exposure appears to be due to differences in androgen and estrogen receptor density in the digits combined with the consequences of steroid activation of those receptors, that is, stimulating or inhibiting growth (Manning 2011; Zheng and Cohn 2011). Specifically, in lab mice, digit 4 is relatively richer in both types of receptors; the presence of high levels of androgens stimulates digit growth, whereas the presence of relatively high levels of estrogens inhibits growth. As a result, 2D:4D in mice reflects the relative concentrations of androgens to estrogens during development, suggesting 2D:4D may be a useful proxy for examining the effect that variation in developmental hormone exposure has on phenotype (Manning 2011; Zheng and Cohn 2011). Experimental elevation of testosterone in utero in lab rats produced males and female with lower 2D:4D, supporting this possibility (Talarovičová et al. 2009). The mechanisms underlying this relationship are highly conserved (Burley and Foster 2004; Manning 2011; Tobler et al. 2011), and researchers have begun using 2D:4D as a proxy for developmental hormones in birds, reptiles, and amphibians (Burley and Foster 2004; Forstmeier 2005; Chang 2008; Dreiss et al. 2008; Forstmeier et al. 2010; Tobler et al. 2011). Interestingly, in birds, greater androgen:estrogen exposure is generally associated with higher 2D:4D (Burley and Foster 2004; Saino et al. 2007), the reverse of what is seen in mammals. However, recent work in zebra finch (*Taeniopygia guttata*) suggests that variation in 2D:4D may be due to a receptor polymorphism rather than solely hormonal exposure (Forstmeier et al. 2010), indicating that 2D:4D may reflect how an individual experiences the developmental hormonal environment.

Here, we use 2D:4D as a morphological proxy for overall developmental hormone exposure (concentration and sensitivity) in order to explore potential relationships between individual variation in endogenous hormone exposure and adult phenotype. We examine the relationship between male 2D:4D and 2 endogenous measures of adult hormonal phenotype, production of testosterone in response to a physiological stimulation of the HPG axis (a standardized injection of gonadotropin-releasing hormone [GnRH]), and testosterone levels prior to the challenge. Individual ability to respond to GnRH is repeatable (Jawor et al. 2006) and estimates the individual physiological maximum (McGlothlin et al. 2007). The strength of an individual's response to a GnRH challenge is related to important, fitness-relevant, morphological, physiological, and behavioral traits (McGlothlin et al. 2007, 2008, 2010; Cain and Ketterson 2012), and HPG responsiveness seems particularly powerful at predicting life-history tradeoffs (Goymann et al. 2007). Muller et al. (2007) found that experimental elevations of yolk testosterone produced elevated baseline testosterone levels in juvenile starlings (*Sturnus unicolor*), which would predict a positive relationship between 2D:4D and adult hormonal phenotype. However, a similar study on 14-day-old chicks (*Gallus gallus domesticus*) found reduced baseline testosterone (Pfannkuche et al. 2011), suggesting the opposite pattern as an alternative prediction. If 2D:4D is unrelated to developmental hormonal exposure, or if endogenous variation has only very weak effects on HPG function, then we would predict no relationship between adult hormonal phenotype and 2D:4D. Because experimental alterations of developmental hormones can also affect morphology, we also examined the relationships between 2D:4D and sexually dimorphic measures of body size (wing, tail, and tarsus). If developmental hormones are mediating trait expression or growth, and high 2D:4D reflects greater androgen exposure, as previous experimental manipulations suggest, this would predict a positive relationship between morphology and 2D:4D.

METHODS

Study system/general methods

Study subjects were free-living dark-eyed juncos of the white-winged subspecies (*Junco hyemalis aikeni*) breeding in Black Hills National Forest near Pringle and Custer, SD. Juncos are a common, mildly dimorphic songbird; males are slightly larger and more ornamented than females (Nolan et al. 2002). The study was conducted from 15 May to 15 August 2007. Individuals were captured using baited mist nets and Potter traps. In some instances, we used playback of song for short periods. All individuals were banded with serially numbered USFWS metal bands and a unique combination of color bands for later field identification. All captured individuals were measured for mass (to the nearest 0.1 g), tarsus, wing, and tail length (to the nearest 0.5 mm) and aged using a combination of mark-recapture data from a previous year and the color of the primary wing coverts and iris (Nolan et al. 2002). Males were then administered a GnRH challenge (see "Endogenous testosterone production") and 2D:4D measurements were taken (see "Digit ratios") just before birds were released at the capture site.

Digit ratios

The relative length of 2D:4D was measured by stamping the foot as detailed in Burley and Foster (2004). Briefly, the open foot was pressed onto a nontoxic, felt stamp pad, and then onto a blank note-card until at least 3 clear and complete images were achieved. The cards were then digitized and imported into NIH Image, a software program for image processing (http://rsbweb.nih.gov/ij/, Last accessed 23 Oct 2012). We measured 2D:4D on the right foot of 71 birds (16 females and 55 males). Digit lengths were measured by first drawing a line from the hallux through digit 3, bisecting the pad of the foot. From this central point on the pad, closest to the hallux, a line was drawn to the tip of the second and fourth digit and the length of these lines was recorded. We calculated 2D:4D by dividing the length of digit 2 by digit 4. Measures were taken on the 3 best images and then averaged to calculate a composite average 2D:4D for each bird. This method of digit measurement yielded

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repeatabilities of R^2 = 0.91 for digit 2 (N = 96, F = 31.89, P < 0.0001) and R^2 = 0.93 for digit 4 (N = 97, F = 38.56, P < 0.0001) (repeatability sensu Lessells and Boag 1987).

Endogenous testosterone production

Adult testosterone levels are dynamic, changing across seasons, within season, and in response to social interactions. The production of testosterone by the gonads is indirectly regulated by GnRH, a neuropeptide produced in the hypothalamus, which leads to the release of sex steroids by stimulating the pituitary to release luteinizing hormone, which then acts on the gonad (Wingfield et al. 1991; Moore et al. 2002). A surge of GnRH stimulates the gonad to transiently increase circulating testosterone levels to the physiological maximum and providing a repeatable bio-assay of HPG responsiveness (Jawor et al. 2006; Goymann et al. 2007). The specifics of the GnRH challenge are detailed elsewhere and are described only briefly here (see Jawor et al. 2006). For every adult male captured, an initial blood sample (initial testosterone) was taken, followed by an intramuscular injection of 50 µL of a solution containing 1.25 µg of chicken GnRH-I (Sigma L0637; American Peptide 54-8-23). After exactly 30 min had elapsed, a second blood sample was taken immediately for the response hormone measure (postchallenge testosterone). Capture time and handling time were recorded. Samples were centrifuged, the plasma drawn off, frozen, and stored at −20 °C until assayed.

Hormone assays

Plasma samples were assayed for testosterone using an EIA kit (Assay Designs, Inc., #901-065) as described previously (Clotfelter et al. 2004; Jawor et al. 2006). Approximately 2000 cpm of tritiated testosterone (H_3T) were added to each sample for determination of sample recovery prior to 2 rounds of diethyl ether extractions. Extracts were dried down using N_2 and resuspended in 50 µl ethanol and 300 µl assay buffer. Samples were run in duplicate, and testosterone concentrations were determined with a 4-parameter logistic curve-fitting program (Microplate Manager; Bio-Rad Laboratories, Inc.). Concentration values were corrected to reflect incomplete recovery; average recovery of H_3T after extraction was 95%. Because capture method may affect testosterone levels, we excluded from the analysis 2 birds that were caught using atypical methods (targeted feeding fledglings or after >30 min of song playback).

Statistical analysis

All statistical analyses were performed using JMP 9 for Mac (SAS Institute Inc.). Because the number of males and females sampled was highly asymmetrical (59 males and 16 females), the relationships between body size and 2D:4D were examined separately according to sex. However, examining them together does not qualitatively change the results. For parametric analysis of testosterone values, both measures (initial and postchallenge) were square root transformed to improve normality. Postchallenge testosterone was normal after transformation but initial testosterone remained non-normal (Shapiro–Wilk test: initial testosterone, W = 0.92, P = 0.0007; postchallenge testosterone, W = 0.98, P = 0.855). Regression is generally robust to violations of non-normality in data (Box and Watson 1962). Transformed values were used for both initial testosterone and postchallenge testosterone in analysis; using untransformed data produced similar results. To determine the relationship between 2D:4D and endogenous testosterone production, we used forward stepwise regression (0.25 probability to enter and 0.10 to leave). Previous studies have shown that initial testosterone and postchallenge testosterone have distinct relationships with behavior (aggression and parenting) (Goymann et al. 2007; McGlothlin et al. 2007); therefore, we analyzed each measure separately. The measures of testosterone (initial testosterone and postchallenge testosterone) were used as dependent variables, allowing us to control for other factors that may affect hormone levels prior to examining the relationship with 2D:4D (Jawor et al. 2006). The full models included the date of the challenge, mass, age, and the amount of time that elapsed between catching and the initiation of the challenge (handling time). Because initial testosterone was correlated with postchallenge testosterone (r_{59} = 0.61, P < 0.0001), we also included initial testosterone in the full model when examining postchallenge testosterone, allowing us to hold initial variation in initial testosterone constant while exploring the differences in the response to GnRH. To enable evaluation of the approach taken, we report the results of both the full (beginning) model and the minimal (full) model (Forstmeier and Schielzeth 2011; Simmon et al. 2011).

To examine the relationship between morphology and 2D:4D, we used forward stepwise regression analysis (0.25 probability to enter, 0.10 to leave). To explore possible interactions between 2D:4D and dimorphic structures (wing, tail, and tarsus), we began with a model that included age, 2D:4D, and a 2D:4D by age interaction term. To permit visualization of hormone and morphology relationships, individual leverage effect pairs from leverage plots were calculated. Leverage pairs are made up of the actual residual from the best-fit line and the residual error without the effect in the model, and they are akin to partial correlations (Sall 1990). For traits that showed nonsignificant relationships, we also calculated the observed effect size (Thomas and Krebs 1997).

RESULTS

Endogenous testosterone production and 2D:4D

Mean initial testosterone (±1 SE) was 1.01 ± 0.13 ng mL^{-1} and decreased with date (R^2 = 0.16, P = 0.0010). Mean postchallenge testosterone was 3.30 ± 0.33 ng mL^{-1} and also decreased with date (R^2 = 0.39, P < 0.0001). In the full model, including all predictors, there was no relationship between initial testosterone and 2D:4D. In the final model exploring the relationship between initial testosterone and 2D:4D, we found no detectable relationship with age, mass, handling time, 2D:4D, or the age by 2D:4D interaction term (P = 0.83, see Table 1 for details).

We found a significant negative relationship between postchallenge testosterone and 2D:4D, controlling for the date of the challenge, initial testosterone, and age (Figure 1, final model: Adj. R^2 = 0.67, $F_{4,55}$ = 28.13; 2D:4D: b = −7.57, P = 0.0137, see Table 1 for details). Mass and handling time were not significant predictors and were removed from the model. First-year males showed slightly higher levels of postchallenge testosterone, but the difference was not significant (b = 0.12, P = 0.0760). Removing initial testosterone from the model did not qualitatively change the results (full model: Adj. R^2 = 0.50, $F_{3,55}$ = 19.02; 2D:4D: b = −7.88, P = 0.0348), however, the age difference became statistically detectable (b = 0.16, P = 0.0424).

Dimorphic morphology and 2D:4D

In males, the relationship between 2D:4D and morphology was strongly dependent on age. Among second-year or older males, tail, wing, and tarsus lengths were all positively related

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Table 1
Beginning (full) models and final (minimal) models examining the relationship between individual endogenous hormone production ability and a morphological proxy for developmental hormone exposure (2D:4D) in male dark-eyed juncos

Trait	Model results	Explanatory variables	*b*; *P*
Initial testosterone (full model)	Adj. $R^2 = 0.14$; $F_{5,53} = 1.48$, $P = 0.2133$	Date of challenge	−0.0000001; 0.0127
		Age	0.032; 0.627
		Mass	−0.004; 0.9360
		Handling time	0.00007; 0.6675
		2D:4D	−0.636; 0.8346
Initial testosterone (minimal model)	Adj. $R^2 = 0.20$; $F_{2,53} = 7.70$, $P = 0.0012$	Date of challenge	−0.019; 0.0003
		2D:4D	0.521, 0.8322
Postchallenge testosterone (full model)	Adj. $R^2 = 0.67$; $F_{6,53} = 18.21$, $P < 0.0001$	Date of challenge	−3.10e-7; <0.0001
		Age	0.074; 0.28
		Mass	−0.015; 0.78
		Handling time	−0.001; 0.54
		Initial testosterone	0.931; <0.0001
		2D:4D	−6.647; 0.0403
Postchallenge testosterone (minimal model)	Adj. $R^2 = 0.67$; $F_{4,53} = 28.13$, $P < 0.0001$	Date of challenge	−0.003; <0.0001
		Age	0.115; 0.0760
		2D:4D	−7.571; 0.0137
		Initial testosterone	0.819; <0.0001

to 2D:4D (Figure 2 and Table 2). However, among first-year males, the relationships between 2D:4D and morphology were all negative (Figure 2 and Table 2). All females were second-year or older and showed similar relationships between 2D:4D and morphology to those seen in second-year and older males. Female 2D:4D was positively related to tarsus (Figure 2; $R^2 = 0.51$, $F_{1,17} = 15.56$, $P = 0.0013$). There was a positive but nonsignificant relationship with wing length (see Table 2 for details, $P = 0.0611$, $\delta = 1.31$). The relationship with tail length was in the same direction (positive) but was also not significant (see Table 2 for details, $P = 0.2000$, $\delta = 0.85$).

DISCUSSION

Endogenous hormone production

We found a negative relationship between a morphological proxy for developmental hormone exposure, 2D:4D, and individual ability to produce testosterone (Figure 1), but no relationship with initial testosterone. The relationship between 2D:4D and endogenous hormone production ability suggests that to the extent that this morphological proxy reflects the developmental hormone milieu, variation in exposure to hormones during development may shape adult HPG responsiveness to a challenge. In passerine birds, a high 2D:4D has been reported to reflect greater exposure to relatively higher androgens levels and lower estrogen levels (Burley and Foster 2004; Romano et al. 2005; Saino et al. 2007). If this pattern holds for the junco, the results presented here would indicate that exposure to high levels of androgens, or low levels of estrogens, results in reduced HPG responsiveness in adulthood. This possibility is supported by recent experimental work. Experimentally elevating testosterone in the egg can alter plasma testosterone, though the direction of this effect can depend on age at evaluation or species. In 2-week-old chickens, individuals that were treated in the egg have lower plasma testosterone levels (Pfannkuche et al. 2011); in juvenile starlings, birds hatched from androgen-treated eggs have elevated plasma testosterone levels (15–17 days posthatch) (Muller et al. 2007).

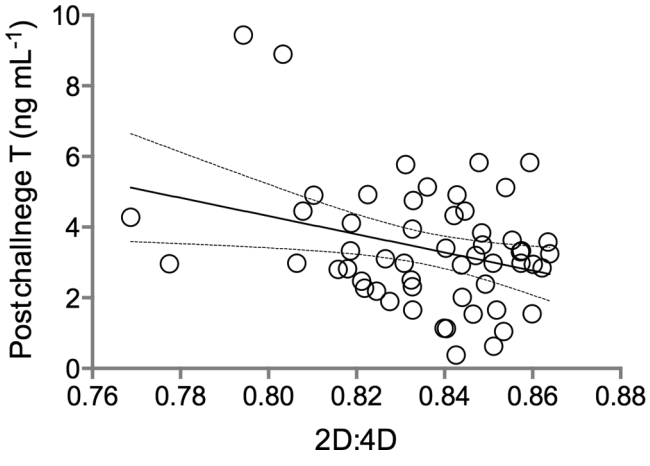

Figure 1
Scatterplot illustrating the relationship between a morphological proxy for developmental hormone exposure (2D:4D) and amount of testosterone produced in response to physiological challenge with GnRH. Points are leverage plot pairs (see "METHODS") showing the relationship between variables after controlling for other predictors in the model, akin to a partial correlation. Curved lines are 95% confidence interval.

Interestingly, we failed to find a relationship between 2D:4D and initial testosterone. This may be a product of our methodology: birds were brought back to a central processing station before being bled and hormone levels may have changed during transport. However, similar results were found in Japanese quail (*Coturnix japonica*); experimentally elevating yolk androgens had no effect on fecal testosterone 3 weeks after hatching, though there was an effect on behavior (Daisley et al. 2005). This suggests that although natural variation in developmental hormonal exposure may alter individual ability to produce testosterone in response to stimulation of the HPG axis, the effect such variation has on testosterone levels in the absence of stimulation (i.e., baseline) is attenuated.

There are multiple nonexclusive pathways by which developmental exposure to steroid hormones may alter adult hormone production ability but not baseline testosterone. Steroids may have an organizational effect of the HPG axis, mediating the responsiveness of the HPG axis to future stimuli (Pfannkuche et al. 2011). Early steroid exposure could also mediate how the gonads respond directly or indirectly to signals from the hypothalamus or pituitary in adulthood, while having little effect on baseline levels, possibly via regulation of the Hox genes

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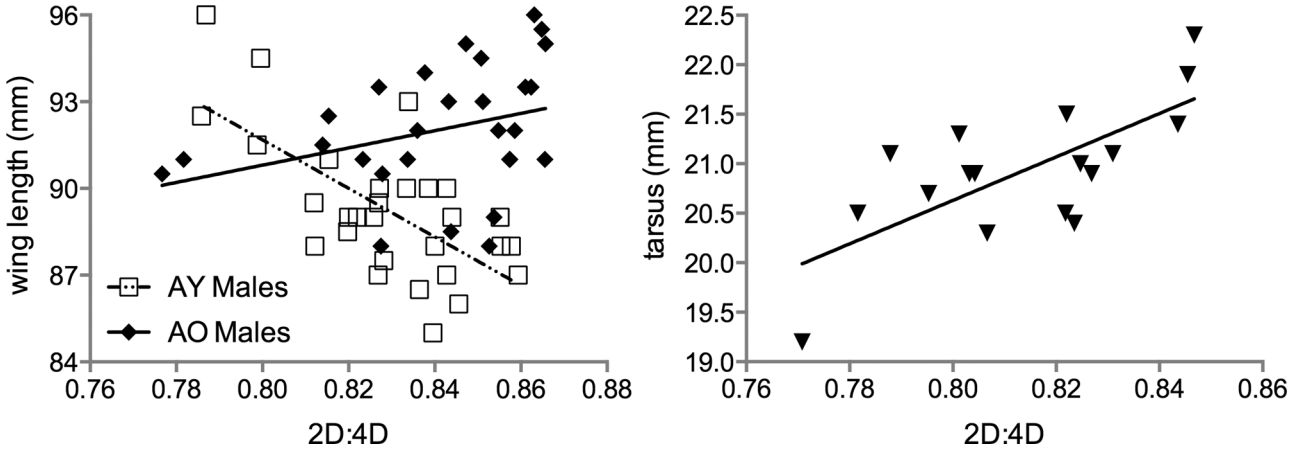

Figure 2
Scatterplots with regression lines illustrating the age- and sex-specific relationships between a morphological proxy for developmental hormones (2D:4D) and phenotypic traits. The left panel shows male wing length and 2D:4D. Closed diamonds and solid line denote second-year or older males; open squares and dashed line denote first-year males. The relationships between 2D:4D, tail, and tarsus length (not shown) are similar. The right panel shows female 2D:4D and tarsus length. The relationships between wing and tail length in females (not shown) are similar to the relationship with tarsus, but were not significant.

orchestrating urogenitial development (Manning et al. 2003; Forstmeier et al. 2010; Pfannkuche et al. 2011). An additional possibility is that 2D:4D reflects developmental sensitivity to steroids as much, or more, than it reflects steroid hormone concentration during development per se. Recent research on zebra finches suggests that variation in 2D:4D is attributable to some extent to variation in estrogen receptor function (Forstmeier et al. 2010). Thus, variation in 2D:4D may instead reflect how individual digits respond to hormone exposure rather than concentration. This also suggests that maternally derived estradiol may play a more important role in 2D:4D than previously recognized (Forstmeier et al. 2010; Manning 2011; Zheng and Cohn 2011).

At present, it is unclear whether individual variation in the tendency to elevate testosterone in response to GnRH is due to differences in sensitivity of the pituitary to stimulation by the hypothalamus, the sensitivity of the gonad in response to stimulation by the pituitary, to negative feedback of testosterone on the hypothalamus and pituitary, or all of these sources of variation (Adkins-Regan 2005; Ball and Balthazart 2008; McGlothlin et al. 2010). Recent work in the junco addressing several of these alternatives suggests that a substantial portion of the variation in individual testosterone responses may be due to differences in the steroidogenic machinery of the gonad (Bergeon Burns CM, unpublished data). However, differences at any of these levels would have important consequences for individual hormone profiles and the expression of hormone-mediated traits (Williams 2008). Individual testosterone production ability is related to numerous morphological, physiological, and behavioral traits, as well as fitness measures, in birds, mammals, and fish (Hofmann 2006; McGlothlin et al. 2007, 2010; Ball and Balthazart 2008; McGlothlin et al. 2008; Williams 2008; Mills et al. 2009; Cain and Ketterson 2012). Consequently, if developmental hormone exposure is affecting adult ability to respond to stimulation from the brain through any of these pathways, as our results suggest, it could have important consequences for adult behavior, physiology, and morphology and thus, fitness.

Digit ratios and sexually dimorphic structures

In addition to potential organizational effects on the HPG axis generally or the gonad alone, steroid exposure during development can also have direct effects on peripheral tissues (Gil 2003; Navara et al. 2005; Rubolini et al. 2006; Saino et al. 2007; Cox et al. 2009). We found that 2D:4D was related to wing, tail, and tarsus length in males (though in age-specific

Table 2
Final models examining the age-specific relationships between morphological traits and a morphological proxy for developmental hormone exposure (2D:4D) in male and female juncos

Sex	Trait	Minimal model results	Explanatory variables	*b*; *P*	δ, effect size
Males	Tail	Adj. $R^2 = 0.53$; $F_{3,53} = 10.36$, $P < 0.0001$	Age	−2.89; 0.0001	
			2D:4D	70.70; 0.0071	
			2D:4D × age	−119.46; 0.0047	
	Wing	Adj. $R^2 = 0.47$; $F_{3,53} = 14.62$, $P < 0.0001$	Age	−3.29; <0.0001	
			2D:4D	43.25; 0.0460	
			2D:4D × age	−106.86; 0.0027	
	Tarsus	Adj. $R^2 = 0.13$; $F_{3,52} = 2.43$, $P = 0.0769$	Age	−0.18; 0.1614	
			2D:4D	7.17; 0.1381	
			Age	−2.89; 0.0001	
Females	Tail	Adj. $R^2 = 0.11$; $F_{16} = 1.81$	2D:4D	24.5; 0.2000	0.85
	Wing	Adj. $R^2 = 0.22$; $F_{16} = 4.10$	2D:4D	56.2; 0.0611	1.31
	Tarsus	Adj. $R^2 = 0.51$; $F_{17} = 15.56$	2D:4D	21.9; 0.013	

For nonsignificant relationships, we also report effect size (δ); see "METHODS" for details.

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ways, Figure 2), and tarsus length in females. In birds and mammals, developmental testosterone often functions as an anabolic growth hormone (e.g., Navara et al. 2005; Crespi et al. 2006). However, ring-necked pheasants (*Phasianus colchicus*) hatched from eggs with experimentally elevated androgens had reduced tarsal spurs (Rubolini et al. 2006), which is similar to our finding that first-year males with larger 2D:4D also had shorter wings and tarsi. Increased testosterone during development can also affect the eventual expression of sexually selected ornaments, or sexually dimorphic traits, and other studies have reported relationships between 2D:4D and sexually dimorphic traits. Interestingly, the directions of the relationships are inconsistent. For instance, in barn swallows (*Hirundo rustica*) and collared flycatchers (*Ficedula albicollis*), 2D:4D is negatively related to tail length and wing length, respectively (Garamszegi et al. 2007; Dreiss et al. 2008). Conversely, 2D:4D was positively related to throat patch size (a melanin-based signal) in house sparrows (*Passer domesticus*) (Navarro et al. 2007). Further, house sparrow males hatching from eggs with experimentally elevated testosterone (which might be associated with larger 2D:4D) also developed larger throat patches (Strasser and Schwabl 2004). There are also a number of studies that report relationships between other digit ratios and traits of interest; painted dragon lizards (*Ctenophorus pictus*) with a bib have significantly larger 3D:4D than lizards without a bib, and experimentally elevating yolk testosterone significantly increases 3D:4D in hatchlings (Tobler et al. 2011).

In our study, females showed the same trends as older males; higher 2D:4D was related to longer wing, tail, and tarsus although the relationship was significant only for tarsus (Figure 2). Because all females measured were in the same age class (all second-year or older), we were unable to determine whether females would also have exhibited age-related relationships. Other recent research on female juncos in a different population found a positive relationship between ability to produce testosterone in response to GnRH and tarsus length, and between wing length and same-sex aggression, which was positively related to reproductive success (Cain and Ketterson 2012). Research on rhesus macaques (*Macaca mulatta*) found that higher ranking females had lower 2D:4D (Nelson et al. 2010). Together these findings suggest that if variation in developmental hormone exposure is contributing to female size, aggression, or testosterone production ability, it could have important evolutionary consequences for females as well as males.

A final possibility is that the relationships between 2D:4D and measures of body size might be driven solely by allometry (the growth trajectory of one character relative to another), due to an inherent relationship between body size, digit length, and the ratio of digits (Kratochvil and Flegr 2009). To examine this possibility, we looked at the relationship between the absolute lengths of digits 2 and 4. Using ordinary least squares (OLS) regression, we found a linear relationship between the two ($R^2 = 0.24$, $P < 0.0001$). However, the intercept for this relationship was significantly greater than zero; meaning that as digit lengths increase, the 2D:4D would necessarily decrease (Kratochvil and Flegr 2009). As a result, allometric scaling would predict that larger animals (with longer toes or tarsi) have smaller 2D:4Ds, whereas we found that birds with large tarsi had high digit ratios. This approach has recently been critiqued. Forstmeier (2011) argues that reduced major axis (RMA) regression is a more appropriate way of testing for allometric relationships than OLS regression. Therefore, we calculated the RMA regression lines and found that the RMA line intercept was also significantly greater than zero. Together, these findings suggest that allometric scaling is an unlikely explanation for the relationships reported here.

Age-specific relationships

In our study, second-year and older males had larger 2D:4D measures on average than first-year males, and the age by 2D:4D interaction was significant, indicating that the directions of the relationships between 2D:4D and morphology were age-specific. Second-year and older males showed a positive relationship with morphology, whereas younger males showed a negative relationship. Previous work in collared flycatchers also reported age differences (Garamszegi et al. 2007; Ruuskanen et al. 2011); however, it is not possible to determine whether the pattern is general because age effects on the relationship between morphology and 2D:4D are rarely examined. Junco wing and tail lengths often increase slightly between the first and second breeding seasons (Nolan et al. 2002), but variation in how much they increase might drive the age-specific relationships we observed. Skeletal measures in songbirds like digit and tarsus length are usually cemented by the time of fledging; however, some studies have reported changes in bone length with age (Wagner and Morton 1997; Møller and De Lope 1999). Alternatively, the observed age difference may indicate that males experience differential mortality according to body size or hormonal exposure, or it may reflect changes in hormone levels as the bird ages. Regardless, such results suggest that it is important to consider age when examining 2D:4D relationships (Garamszegi et al. 2007), and future studies should determine whether 2D:4D is changing within individuals and in a consistent manner.

CONCLUSION

In conclusion, our results support the possibility that endogenous variation in the developmental hormone milieu can have long-term effects on adult hormonal phenotype and morphology. This suggests that some of the observed individual variation in ability to produce testosterone (Jawor et al. 2006, 2007) and the morphological and behavioral traits that are related to that ability (McGlothlin et al. 2007, 2008; Cain and Ketterson 2012) may be attributable to individual differences in exposure to steroid hormones during development. Such consequences may, in turn, lead to transgenerational effects (Gorman and Williams 2005; Monaghan 2008; Dufty et al. 2002). Consequently, disentangling how endogenous individual variation in hormonal exposure during development mediates adult phenotype is an important goal for researchers interested in understanding the evolution of hormonally mediated phenotypes, and sexually dimorphic traits and behavior.

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