Does 2D:4D predict fitness in a wild mammal?

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Abstract: Environmental pressures present during critical periods in fetal development can have a strong influence on the long-term fitness of an individual. Susceptibility to parasites, immune function, and future reproductive success are all vulnerable to stressful events in utero. The causes and consequences of prenatal environmental stress are often difficult (if not impossible) to evaluate, especially in wild populations. Digit ratio, the ratio of 2nd digit to 4th digit length (2D:4D) has been identified as an index of fetal androgen exposure. Current techniques for assessing digit ratio pose severe limitations to the accuracy of digit measurement of wild animals owing to the constant movement of the feet and inaccessibility of laboratory equipment. Our study attempts a new indirect technique wherein subjects had an imprint taken of each foot that was then photographed and scanned for digital measurement. Using red squirrels (*Tamiasciurus hudsonicus* (Erxleben, 1777)) as a model species, we hypothesized that higher degrees of fetal testosterone exposure (assumed from digit ratios) would result in reduced reproductive success and increased susceptibility to parasite infection. Digit ratio was negatively correlated with reproductive output and positively correlated with parasite loads. Although this may indicate that exposure to excess testosterone in utero may jeopardize future fitness, the relationships are inconsistent.

Résumé : Les pressions environnementales présentes durant les périodes critiques du développement fœtal peuvent avoir une forte influence sur la fitness de l'individu à longue échéance. La vulnérabilité aux parasites, la fonction immunitaire et le succès reproductif futur sont tous sensibles aux événements de stress dans l'utérus. Il est souvent difficile (voire impossible) d'évaluer les causes et les conséquences des stress environnementaux avant la naissance, particulièrement dans les populations sauvages. On a identifié le rapport digital, soit le rapport de la longueur du 2ᵉ doigt sur celle du 4ᵉ doigt (2D:4D) comme indice de l'exposition fœtale aux androgènes. Les techniques actuelles de mesure du rapport digital imposent des limites sévères à la précision de la mesure des doigts chez les animaux sauvages à cause du mouvement continuel des pieds et l'inaccessibilité d'appareils de laboratoire. Notre étude essaie une nouvelle technique indirecte par laquelle on prend une empreinte de chaque pied qui est ensuite photographiée et numérisée pour alors mesurer les doigts. En utilisant des écureuils roux (*Tamiasciurus hudsonicus* (Erxleben, 1777)) comme espèce modèle, nous posons comme hypothèse que les niveaux plus élevés d'exposition fœtale à la testostérone (d'après le rapport digital) entraînent une diminution du succès reproducteur et une vulnérabilité accrue à l'infection par les parasites. Le rapport digital est en corrélation négative avec le rendement reproductif et en corrélation positive avec la charge parasitaire. Bien que cela puisse indiquer que l'exposition à un excès de testostérone dans l'utérus peut mettre en péril la fitness future, les relations restent ambiguës.

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Introduction

Environmental pressures present during critical periods in development can have a strong influence on the long-term fitness of an individual (Ellison 2005). The susceptibility to disease and parasites, immune function and future reproduction are all vulnerable to stressful events in utero (Ellison 2005). Although hormones are essential for developing the brain and body (Möstl and Palme 2002; Müller et al. 2005; Touma and Palme 2005), excessive exposure can have high fitness costs on a developing fetus (Müller et al. 2009). High levels of fetal testosterone in utero may contribute to initially poor immune performance because testosterone is also a known immunosuppressant (Hamilton and Zuk 1982; Folstad and Karter 1992; Derting and Virk 2005). Compromised immune function can significantly decrease life expectancy and reproductive success (reviewed in Norris and Evans 2000; Sadd and Schmid-Hempel 2009). Furthermore, infection early during development may have irreversible consequences

as young bodies may be less able to recover from severe infection (Fitze et al. 2004; Puchala 2004). The causes and consequences of prenatal environmental stressors are often difficult (if not impossible) to evaluate, especially in wild populations. Therefore, indicators of prenatal stress (i.e., excessive hormonal exposure) have valuable application for understanding the impacts on future fitness.

Digit ratio, the ratio of 2nd digit to 4th digit length (2D:4D), has been identified as an index of fetal androgen exposure (Manning et al. 1998; Manning and Bundred 2000). Although most work has involved humans, there is evidence that digit ratio has application across taxa as a marker of prenatal development (Manning et al. 1998, 2003; McMechan et al. 2004). The use of 2D:4D stems from the discovery that the development of digits and the urogenital system are controlled by the same genes, *homeobox A* and *D* (Krumlauf 1994; Kondo et al. 1997; Roubolini et al. 2006). These genes are influenced by prenatal androgens that also contribute to

the shaping of the reproductive organs in utero (Kondo et al. 1997). The impact androgens have on digit ratio has been experimentally established in recent studies (Ring-necked Pheasant (*Phasianus colchicus* L., 1758): Romano et al. 2005; Saino et al. 2007; Painted Dragons (*Ctenophorus pictus* (Peters, 1866)): Tobler et al. 2011).

Few investigations have examined the relationships among digit ratio and specific fitness traits, especially in nonhuman species. Existing evidence suggests that digit ratios can be used as a meaningful biological marker. For example, male House Sparrows (*Passer domesticus* (L., 1758)) with larger digit ratios expressed weaker T-cell immunity than males with smaller digit ratios (Navarro et al. 2007). As androgens are known immunosuppressants (Folstad and Karter 1992; Mougeot et al. 2006), this may suggest that larger digit ratios indicate higher levels of androgens. Digit ratio has also been correlated with various measures of reproductive ability in humans, including reproductive success (Manning et al. 2000; Manning and Fink 2008), sperm counts (Manning et al. 1998), and sexual attractiveness (Bogaert et al. 2009).

However, there are inconsistent patterns among taxa. Male mammals consistently display lower digit ratio values than females (Brown and Brown 2002; Roney et al. 2004), whereas male birds have shown higher values than females (Burley and Foster 2004); however, there is also evidence that digit ratios do not differ between the sexes (reviewed in Lombardo et al. 2008). With these inconsistencies, predictions about the relationships between digit ratio and fitness traits are difficult to make. The variation in results may be partly explained by the accuracy of current and past measurement techniques (reviewed in Kemper and Schwerdtfeger 2009; Lilley et al. 2009). Both direct (ruler or callipers) and indirect (photocopies or X-rays and digital measurement software) techniques have been used to assess digit ratio (reviewed in Kemper and Schwerdtfeger 2009; Lilley et al. 2009). Current techniques pose severe limitations to the accuracy of digit measurement for live-trapped wild animals. Direct measurements can be inaccurate as captured animals are highly active when being handled and indirect methods require laboratory equipment that cannot be brought into the field. Here we describe a new indirect technique whereby subjects have an imprint taken of each foot that is then photographed and scanned for digital measurement.

The purpose of our study was to assess the relationship among digit ratio and correlates of individual fitness using digit ratios in North American red squirrels (*Tamiasciurus hudsonicus* (Erxleben, 1777)). Red squirrels are size monomorphic, thus differences in digit ratio may indicate sex differences in hormone levels rather than sex differences in size. We hypothesized that higher degrees of fetal testosterone should result in reduced reproductive success and compromised immune function that may also increase the susceptibility to parasite infection. Thus, we predicted that those individuals with lower digit ratios would have lower reproductive success and higher parasite burdens. To our knowledge, few attempts have been made to investigate the implications of fetal testosterone on multiple factors of individual fitness in a wild population.

Materials and methods

All methods and procedures were conducted according to an animal use protocol approved by the Laurentian University Animal Care Committee (2009-03-01).

Field methods

A marked population of red squirrels was sampled within a 23 ha grid of mixed deciduous forest at the Wildlife Research Station (WRS) in Algonquin Provincial Park, Ontario (45°30′N, 78°40′W), between late April to late August 2009. Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, Wisconsin, USA) were set 40 m apart and approximately 1.5–2 m from the ground on a wooden platform (60 cm × 15 cm × 1.5 cm). Each trap was equipped with a sheet of aluminum covering the top and sides and polyester bedding inside to discourage predators and to reduce exposure of trapped animals to inclement weather (Gorrell and Schulte-Hostedde 2008). Traps were set at dawn 5–6 days each week, baited (10 g mixture of peanut butter and oats with a thin slice of apple), and checked twice daily (early and late morning).

Upon capture, each red squirrel was transferred into a handling bag where it was sexed, aged, weighed (Pesola scale; accuracy ±0.1 g) and marked with a numbered ear tag for future identification and long-term tracking. All tools and tissue were disinfected prior to tagging. Age category was determined based on mass (g) (adults greater than ~150 g) and sex was verified by the presence or absence of testes, as well as by the distance between genitals and anus when males were not scrotal (distance between male genitalia and anus is longer than in females).

DNA and parasite sample collection and analysis

Parentage analysis

Reproductive success was determined by parentage assignment using molecular techniques to identify the number of offspring per adult. Tissue samples for genetic analyses were taken with dissecting scissors from the distal tip of the ear (~1 mm), placed in an Eppendorf tube, and preserved on ice immediately after collection to prevent tissue degradation. Ear tissue was transferred to a –20 °C freezer until DNA extraction could be performed.

DNA was extracted from ear tissue using a QIAGEN DNeasy tissue kit (QIAGEN Inc. Mississauga, Ontario, Canada). Eight microsatellite loci (*Thu00*, *Thu02*, *Thu09*, *Thu10*, *Thu21*, *Thu23*, *Thu36*, *Thu70*) were used to determine maternity and paternity from tissue samples using primers developed for red squirrels (Gunn et al. 2005; Bonanno and Schulte-Hostedde 2009). The polymerase chain reaction (PCR) protocol, including cocktail, PCR cycling reactions, and annealing temperatures for microsatellites, was followed as noted in Bonanno and Schulte-Hostedde (2009). PCR products were sent to MOBIX, the McMaster Institute for Molecular Biology and Biotechnology, at McMaster University for genotyping. Parentage was assigned using the likelihood-based approach and simulation procedures of CERVUS version 3.0 (Schulte-Hostedde et al. 2002; Kalinowski et al. 2007; Bonanno and Schulte-Hostedde 2009). Simulations were run for 10 000 cycles with the following parameters:

number of candidate parents, the proportion of candidate parents sampled for mothers (1) and for fathers (0.90), the proportion of loci typed (0.99), and the rate of typing error (0.01). All assignments were based on 80% (relaxed) to 95% (strict) confidence criteria (Kalinowski et al. 2007; Bonanno and Schulte-Hostedde 2009). Reproductive success was assigned based on matches of 80% confidence.

Ectoparasite identification

Ectoparasites were collected with a metal flea comb (teeth spacing <300 μm, which is one-tenth the size of the smallest fleas; Burgham Ltd., Toronto, Ontario, Canada) by combing five times down the mid-back to the base of the tail, five times along each hind leg, and five times down the tail from base to end. Ectoparasites collected were preserved in 70% ethanol. Sample collections were limited to once every 4 weeks to minimize the manipulative effects of parasite removal on fitness (Gorrell and Schulte-Hostedde 2008). Each individual parasite was weighed (accuracy ±0.000001 g; Sartorius MC21S).

Endoparasite analysis: fecal egg counts

Helminth worm eggs from fecal samples were collected either from the animal directly during processing or from the trap platform. The platform was cleaned after each capture to ensure that each fecal sample was from the most recently captured red squirrel. Each fecal sample was stored in 70% ethanol and then frozen for later quantification of intestinal helminth egg load (Pauli et al. 2004; Gorrell and Schulte-Hostedde 2008; Hillegass et al. 2008).

An index of endoparasite load was determined by counting helminth eggs per gram (EPG) of feces using a modified centrifuge McMaster technique (Rossanigo and Gruner 1991; Pauli et al. 2004; Hillegass et al. 2008). Feces were blended into 1:10 dilution of Fecasol (9650-32; RICCA Chemical Company, Arlington, Texas, USA) liquid zinc sulphate floatation solution. The mixture was strained through a sieve to separate matter, poured into a 1.5 mL Eppendorf tube, and centrifuged at 3000 rev/min for 5 min. Each tube was left for 10 min to allow for eggs to float to the top layer of tube, after which 1 mL of solution was extracted from the tube and pipetted into McMaster slide chambers. The slide was then examined under a microscope (10× magnification; Olympus BX41), where the eggs were counted in two 1 cm grids. EPG was calculated by multiplying the sum of the number of eggs observed within each chamber by 100 (Pauli et al. 2004; Gorrell and Schulte-Hostedde 2008; Hillegass et al. 2008). Endoparasite eggs were identified to genera using two online archives and identification guides; *Diagnosing Medical Parasites: A Public Health Officers Guide to Assisting Laboratory and Medical Officers* (http://www.afpmb.org/sites/default/files/whatsnew/2009/Diagnosing_Medical_Parasites.pdf; accessed 18 December 2011) and *RVC/FAO Guide to Veterinary Diagnostic Parasitology* (http://www.rvc.ac.uk/review/Parasitology/Index/Index.htm; accessed 15 October 2009).

Digit ratio

Each individual captured had both hind feet pressed into Play-Doh™ (23845; Hasbro) mounted onto sheets of cardboard (approxiamtely 7 cm × 5 cm). Imprints were collected as needed for each individual in no particular order. A digital photograph (Canon Powershot SD1100 mounted on a tripod) was taken shortly afterwards with a ruler for calibration. Each imprint was equidistant (~4 cm) from the camera. Digit measurements were taken using the image analysis program Image J version 1.43 (National Institutes of Mental Health (NIMH), Bethesda, Maryland, USA). Second and fourth digits were measured from distal tip of each digit to the proximal crevice of the palm excluding the nail (Fig. 1; Manning et al. 1998; Chang 2008; Helle and Lilley 2008). Each individual had 2–3 imprints made and each imprint measurement was repeated three times for accuracy. The digit ratio for each individual's left and right hind foot was calculated using the mean imprint measurements for the second and fourth digit.

Data handling and statistical analyses

All statistics were conducted using Statistica 7 (StatSoft, Inc., Tulsa, Oklahoma, USA). Analyses of covariance (ANCOVA) were conducted to examine the relationship among digit ratio, fitness variables, and parasite burdens of adult females and males using sex as a categorical variable. Separate analyses were used for left and right hind foot digit ratios, as well as the mean digit ratio. Data were $\log_{10}(x + 1)$-transformed to improve normality for variables that were not normally distributed (i.e., reproductive success: $p = 0.002$; ectoparasite intensity: $p = 0.00008$). All data were within three standard deviations of the mean, suggesting that the analysis of outliers was not necessary.

Measures of parasite load

The seasonal mean values of parasite loads (ectoparasite and endoparasite) were used in all analyses. Five measures of parasite load were calculated for each individual. Ectoparasite load was calculated by intensity (total mean count), mean richness (presence of fleas and (or) mites), and biomass (total mean biomass). Similarly endoparasite load included intensity (total mean number of intestinal eggs found per fecal sample) and richness (total mean number of different species found per sample).
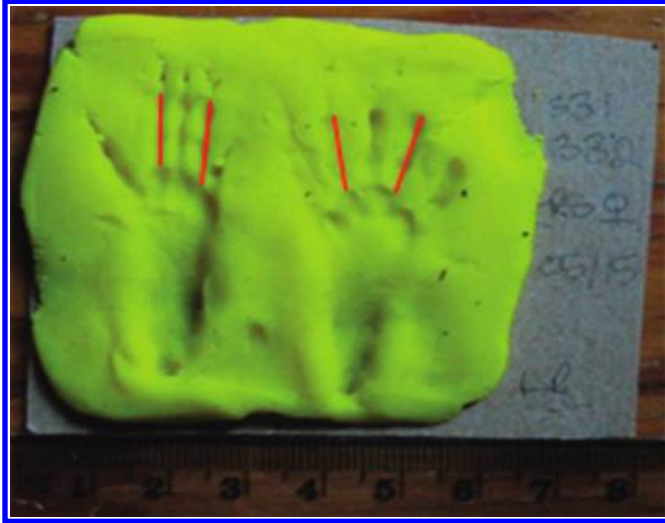
Digit ratio

Left and right hind foot digit ratios were calculated by dividing the length of the 2nd digit by the length of the 4th digit on each foot (Manning et al. 1998; Chang 2008; Helle and Lilley 2008). The mean digit ratio was also calculated for all adults (left + right 2D:4D/2). Measurement error (ME; the difference between the actual value and the value obtained by a measurement) was calculated for the variance within each set of measurements per imprint, as well as for the variance among measurements for each individual (Bailey and Byrnes 1990; Schulte-Hostedde and Millar 2000). Percent ME was calculated using the formula %ME = $(s^2 \text{ within})/(s^2 \text{ within} + s^2 \text{ among}) \times 100$ (Bailey and Byrnes 1990; Schulte-Hostedde and Millar 2000).

Results

Descriptive statistics for morphological measurements and digit ratios can be found in Table 1. Twenty-nine adult red squirrels (females: $N = 17$; males: $N = 12$) were included in the analyses. Measurement error (ME) within digit ratios for each imprint was relatively low, supporting the reliability of

Fig. 1. An illustration of digit ratio measurements taken from the hind foot imprint of a red squirrel (*Tamiasciurus hudsonicus*). Second and fourth digits were measured from distal tip of each digit to the proximal crevice of the palm. Each individual had two to three imprints made and each imprint measurement was repeated three times for accuracy using the image analysis program Image J version 1.43.

this new technique (left foot: 2nd digit = 5%, 4th digit = 5%; right foot: 2nd digit = 4%, 4th digit = 4%). Digit ratios did not differ between the sexes ($F_{[2,24]} = 1.13$, $p = 0.34$). No sex differences were found among parasite measures (endoparasite intensity: $Z = 1.003$, $p = 0.32$; endoparasite richness: $Z = 1.620$, $p = 0.11$; ectoparasite intensity: $Z = 0.247$, $p = 0.79$; ectoparasite richness: $Z = –0.118$, $p = 0.86$; ectoparasite biomass: $Z = –0.186$, $p = 0.84$). There was no difference (males: $t_{[22]} = 1.251$, $p = 0.22$; females: $t_{[30]} = –1.217$, $p = 0.23$) or correlation (males: $r^2 = 0.006$, $p = 0.81$; females: $r^2 = 0.04$, $p = 0.46$) between left and right hind foot digit ratios.

Reproductive success

Reproductive success was measured by the number of offspring from each individual assigned through parentage analyses. Fifty-nine North American red squirrels (adult females = 17 and adult males = 12; juvenile females = 12 and juvenile males = 18) were genotyped. Three loci were not in Hardy–Weinberg equilibrium owing to high occurrence of homozygotes. These loci were retained in the analysis because they were scored reliably. All 30 offspring were assigned to their respective mother at a 95% confidence level. Known mothers were then used to increase the confidence in paternity assignment. Twenty-two of the 30 offspring were assigned to their respective father at a 95% confidence level and all 30 were assigned at an 80% confidence level.

Log-transformed reproductive success was negatively related to left hind foot 2D:4D ($F_{[1, 22]} = 5.005$, $p = 0.04$; Fig. 2), thus higher digit ratios were associated with lower reproductive success. No relationship was observed with right hind foot ($F_{[1,22]} = 0.016$, $p = 0.90$) or mean digit ratio ($F_{[1,21]} = 2.578$, $p = 0.12$). No interactions between sex were observed with either hind foot or mean digit ratio (left:

$F_{[1,22]} = 0.060$, $p = 0.81$; right: $F_{[1,22]} = 0.300$, $p = 0.59$; mean: $F_{[1,21]} = 0.445$, $p = 0.51$).

Parasite load

Mites and two species of fleas (*Orchopeas caedens* (Jordan, 1925) and *Opisodasys pseudarctomys* (Baker, 1904)) were found on red squirrels. Mites were not identified to species. Ectoparasite measures were highly correlated in both females ($N = 16$; intensity to richness: $r_S = 0.98$, $p < 0.001$; intensity to biomass: $r_S = 0.94$, $p < 0.001$; richness to biomass: $r_S = 0.93$, $p < 0.001$) and males ($N = 11$; intensity to richness: $r_S = 0.98$, $p < 0.001$; intensity to biomass: $r_S = 0.76$, $p = 0.01$; richness to biomass: $r_S = 0.75$, $p = 0.01$), thus only intensity was used in the analyses. No relationships were found between log ectoparasite intensity and digit ratio (right 2D:4D: $F_{[1,24]} = 0.194$, $p = 0.66$; left 2D:4D: $F_{[1,24]} = 0.545$, $p = 0.47$; mean 2D:4D: $F_{[1,23]} = 0.084$, $p = 0.78$). There were no interactions with sex (right 2D:4D: $F_{[1,24]} = 0.285$, $p = 0.60$; left 2D:4D: $F_{[1,24]} = 0.290$, $p = 0.60$; mean 2D:4D: $F_{[1,23]} = 0.422$, $p = 0.52$).

Helminth eggs were identified to genus (*Strongyloides* Grassi, 1879; *Tricuris* Roederer, 1761; *Toxocara* Stiles and Hassall, 1905; and *Ascaris* L., 1758). Seven unknown genera were also found in fecal samples. Endoparasite intensity and richness were uncorrelated in both females ($N = 8$, $r^2 = 0.45$, $p = 0.07$) and males ($N = 6$, $r^2 = 0.001$, $p < 0.96$) and therefore their potential relationships to digit ratio were considered independently. Endoparasite intensity was positively related to right hind foot 2D:4D ($F_{[1,11]} = 6.436$, $p = 0.03$; Fig. 3), but no relationship was observed with left hind foot ($F_{[1,10]} = 0.423$, $p = 0.53$) or mean digit ratio ($F_{[1,11]} = 1.574$, $p = 0.24$). Endoparasite richness was positively related to left hind foot 2D:4D ($F_{[1,16]} = 5.156$, $p = 0.04$; Fig. 4), but no relationship was observed with right hind foot ($F_{[1,17]} = 0.022$, $p = 0.88$). There was a trend of endoparasite richness increasing with higher mean digit ratios ($F_{[1,16]} = 3.053$, $p = 0.10$). No sex interactions were observed for endoparasite intensity (right 2D:4D: $F_{[1,11]} = 0.359$, $p = 0.56$; left 2D:4D: $F_{[1,10]} = 1.808$, $p = 0.21$; mean 2D:4D: $F_{[1,11]} = 2.375$, $p = 0.15$), or endoparasite richness (right 2D:4D: $F_{[1,17]} = 0.79$, $p = 0.39$; left 2D:4D: $F_{[1,16]} = 1.005$, $p = 0.33$; mean: $F_{[1,16]} = 0.351$, $p = 0.56$).

Discussion

The main results from our study revealed negative relationships among left hind foot digit ratio, reproductive success, and endoparasite richness, but a positive relationship between right hind foot digit ratio and endoparasite intensity. Regardless of which foot exhibited the relationships, individuals with higher digit ratios had lower reproductive success than those individuals with lower digit ratios (Fig. 2). Similarly, higher endoparasite intensity and species richness was associated with higher digit ratios (Figs. 3, 4).

Our results show evidence that higher digit ratios may indicate negative impacts of prenatal exposure to maternal androgens (Manning et al. 1998; Roney et al. 2004). However, the inconsistencies within this study, as well as within the literature, may also suggest there is no link between digit ratio and adult fitness. There are several lines of evidence that support this latter hypothesis. For instance, the pattern of higher

Table 1. Descriptive statistics for variables of ectoparasite intensity, endoparasite intensity and richness, reproductive success, and digit ratios of male and female adult red squirrels (*Tamiasciurus hudsonicus*).

Variable	Male			Female		
	N	Mean	95% CI	N	Mean	95% CI
Digit ratio						
Left hind foot	12	0.98	0.95–1.02	17	0.97	0.95–1.00
Right hind foot	12	0.96	0.93–0.99	17	0.99	0.97–1.01
Reproductive success	9	1.11	0.30–1.92	16	1.19	0.79–1.59
Ectoparasite intensity	11	0.27	0.04–0.50	16	1.56	0.12–0.64
Endoparasite intensity	7	6 077	707 – 11 447	8	12 514	5 711 – 19 315
Endoparasite richness	8	1.30	0.88–1.72	12	1.56	1.20–1.92

Note: Sample sizes (*N*), mean, and 95% confidence intervals (CI) are displayed.

Fig. 2. Scatter plot of log-transformed reproductive success and left hind foot digit ratio (2D:4D) for male and female red squirrels (*Tamiasciurus hudsonicus*) collected in Algonquin Park, 2009. Log-transformed reproductive success decreases with increasing digit ratio ($F_{[1,22]} = 5.005$, $p = 0.04$). No effect of sex was observed ($F_{[1,22]} = 0.060$, $p = 0.81$).
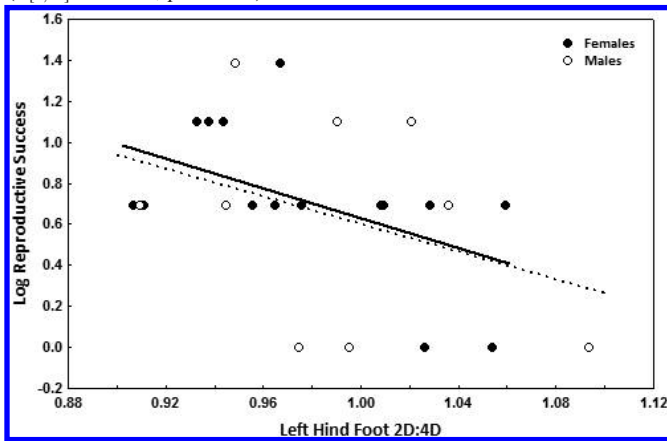

Fig. 3. Scatter plot of total endoparasitic eggs (endoparasite intensity) and right hind foot digit ratio (2D:4D) for male and female red squirrels (*Tamiasciurus hudsonicus*) collected in Algonquin Park, 2009. Increasing endoparasites levels were positively related to increasing right hind foot 2D:4D ($F_{[1,11]} = 6.436$, $p = 0.03$). No sex interactions were observed ($F_{[1,11]} = 0.359$, $p = 0.56$).
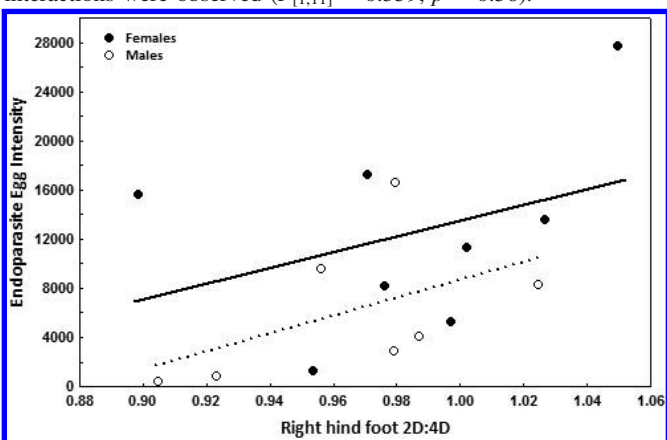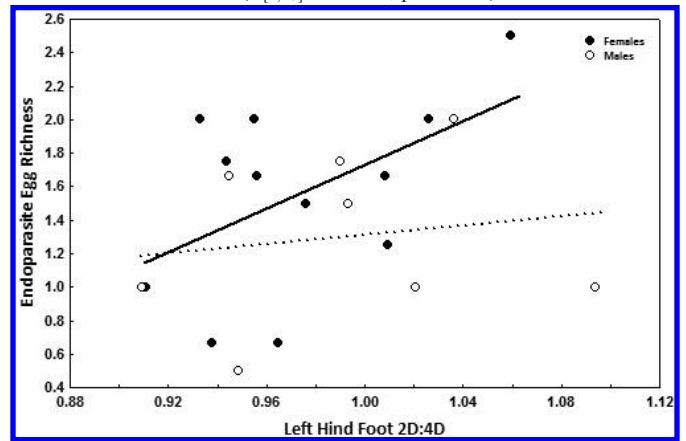

Fig. 4. Scatter plot of endoparasite species richness (endoparasite richness) and left hind foot digit ratio (2D:4D) for male and female red squirrels (*Tamiasciurus hudsonicus*) collected in Algonquin Park, 2009. Higher levels of endoparasite richness were positively related to increasing 2D:4D ($F_{[1,16]} = 5.156$, $p = 0.04$). No sex interaction was observed ($F_{[1,16]} = 1.005$, $p = 0.33$).

digit ratios that we found is opposite to our original predictions that lower digit ratios would indicate higher prenatal testosterone in mammals as found in previous studies (Brown and Brown 2002; Manning et al. 2003; McMechan et al. 2004).

In addition, previous work has found relationships with one foot and not the other as in our study (right foot: Manning et al. 1998; McFadden and Shubel 2002; Burley and Foster 2004; McMechan et al. 2004; Roney et al. 2004; left foot: Romano et al. 2005; Roubolini et al. 2006). Past studies have investigated sexual dimorphism and proposed that one limb may be more sexually dimorphic than the other. However, this does not explain results (as also observed in our study) where no sexual dimorphism was found (Romano et al. 2005; Malas et al. 2006; Lombardo et al. 2008).

On the other hand, these inconsistencies may be merely reflecting undetectable patterns that need more exploration or greater statistical power. Low sample size was a limitation of the current study and could be a factor in masking relationships between digit ratio and fitness. Asymmetrical limbs could biologically explain relationships that are found in one hand and not the other. Stressful events in utero can cause developmental instability that compromises an individual's

ability to combat future environmental stressors and can lead to asymmetry of limbs and organs (reviewed in Møller 1996; Dongen 2006; Polak 2008).

Few studies have looked at the relationships among parasites and digit ratio. However, available evidence supports the hypothesis that infection is associated with higher prenatal testosterone exposure (e.g., toxoplasmosis: Flegr et al. 2005; human papillomavirus: Brabin et al. 2008). Indicators of low immunity have also been associated with higher digit ratio (Navarro et al. 2007). Thus, an increased susceptibility to parasites may be due to a compromised immune system from excess exposure to prenatal stress (reviewed in Møller 2006; Polak 2008). However, recent evidence also suggests that prenatal testosterone may even stimulate future immunity (Tobler et al. 2010).

It is also possible that other hormones may impact fetal development other than testosterone. For instance, maternal corticosterone was positively related to offspring digit ratio in offspring of field voles (*Microtus agrestis* (L., 1761)) (Lilley et al. 2010). Glucocorticoids (cortisol and corticosterone) are secreted by the adrenal cortex through activation of the hypothalamic–pituitary–adrenal axis in response to a stressful event (Romero 2002; Touma and Palme 2005), and although it is well established that stress is often necessary in the initiation or facilitation of many behaviours (defence, copulation, hunting, etc.), chronic stress (prolonged periods of high glucocorticoids) can cause immunosuppression and lead to severe fitness costs (Möstl and Palme 2002; Romero 2002; Belden and Kiesecker 2005). Specific deleterious effects include suppression of T-cell and antibody production (a key aspect of immune defence; Ottaviani and Franceschi 1996). Therefore, stress hormones could be an alternate or additional environmental pressure during development (Weinstock 2008; Lilley et al. 2010).

Furthermore, the inconsistencies found within the literature may be attributed to a lack of information (many studies only report results for one foot; Burley and Foster 2004) or differences between hind feet and fore feet. Human studies have typically used hand digit ratios, whereas most other nonhuman models use measurements of hind feet (Manning et al. 1998; Dongen 2006; Polak 2008). The explanations for this may be more obvious when using birds but less when using mammals or reptiles. Particularly for rodents, it may simply be too difficult to access fore feet during live capture (but see Roubolini et al. 2006). For humans, even if both the hands and feet are measured, there can still be inconsistencies. McFadden and Shubel (2002) reported that feet digit ratio measurements of human subjects were unreliable because of curving of the toes. Unfortunately, for these reasons few studies have investigated digit ratio differences between hands and feet directly (but see McFadden and Shubel 2002).

Investigations using digit ratio have grown exponentially within the past 10 years (reviewed in Dongen 2006), but the accuracy of such measures has been often called into question (Voracek et al. 2007; Kemper and Schwerdtfeger 2009). The number of different techniques used to assess digit ratio may also vary in the degree of accuracy achieved (reviewed in Dongen 2006; Kemper and Schwerdtfeger 2009). Repeatability for many studies was often low, making it difficult to draw sound conclusions (i.e., Lombardo et al. 2008). Kemper and Schwerdtfeger (2009) reviewed the three most common

measurement methods for digit ratio: ruler, calliper, and digital software. The highest precision estimates were found with software but this method also took the most time (Kemper and Schwerdtfeger 2009). For our study, direct measurements using callipers or a ruler would have decreased accuracy significantly because of subjects constantly moving their feet while in the handling bag. Thus, indirect methods were the ideal option. To our knowledge no studies have used this imprint method to assess digit ratio.

Clearly, more investigations including measurements of front and hind feet are needed, as well as further experimental investigation into the impacts of hormones on limb development (see Romano et al. 2005; Saino et al. 2007). Future digit ratio work should take an integrative approach, including aspects of immunology, endocrinology, and reproduction. Although clear conclusions cannot be drawn without an established (quantitative) relationship between digit ratio and hormone levels, future studies should consider measures of immunity that accurately assess overall immunocompetence. Additionally, manipulation of fetal androgens may provide clearer links between hormones and digit ratios and such studies need to be conducted across more taxa (see Saino et al. 2007; Tobler et al. 2011). Similar improvements can be made to studies investigating the influence of fetal testosterone and future reproduction. A clearer understanding of these relationships in a variety of species will lead to more comprehensive ecologically relevant work involving wild populations.

Acknowledgements

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