

# Female house wrens (*Troglodytes aedon*) increase the size, but not immunocompetence, of their offspring through extra-pair mating

ANNA M. FORSMAN,\* LAURA A. VOGEL,\* SCOTT K. SAKALUK,\* BONNIE G. JOHNSON,†  
BRIAN S. MASTERS,† L. SCOTT JOHNSON† and CHARLES F. THOMPSON\*

\*Department of Biological Sciences, Illinois State University, Normal, IL 61790, USA, †Department of Biological Sciences, Towson University, Towson, MD 21252, USA

## Abstract

House wrens are typically socially monogamous, but frequently engage in extra-pair matings leading to multisired broods. Because females do not appear to acquire direct material benefits from their extra-pair mates, we tested the hypothesis that female house wrens derive indirect genetic benefits, such as enhanced immunocompetence (cutaneous immune activity, humoral immunity, and plasma bactericidal activity) and condition (size and haematological traits) for their offspring, by mating polyandrously. We predicted that extra-pair young (EPY) should show greater immune responsiveness and better body condition than their within-pair maternal half-siblings (WPY). Contrary to our prediction, WPY had higher cutaneous immune activity than their EPY brood-mates in two of three years, and EPY and WPY did not differ in measures of innate and humoral immunity. WPY also had higher albumin to  $\gamma$ -globulin ratios than EPY; however, they were not in better condition based on other measures. EPY had consistently longer tarsi (a measure of long-bone size) than their WPY half-siblings, suggesting that females engage in extra-pair copulations with larger males. The benefits of large structural size in the study population is unknown, but based on evidence from other passerines, we suggest that structural size may be an important fitness-related trait in house wrens. We conclude that our results are not consistent with the hypothesis that females gain immune-related benefits for their offspring by engaging in extra-pair matings. Further study of the fitness consequences of differences in tarsus length is needed to determine whether females acquire size-related benefits for their offspring from extra-pair mates.

*Keywords:* extra-pair paternity, immune response, indirect genetic benefits, *Troglodytes aedon*

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## Introduction

Over 90% of all avian species are socially monogamous (Lack 1968; Ligon 1999). However, as a result of extra-pair fertilization only about 14% of such species are also considered genetically monogamous (Griffith *et al.* 2002). Males clearly benefit directly from additional fertilizations outside the social pair bond through increased number of offspring sired. Females, however, do not realize this benefit to the same extent because they are constrained by

the number of offspring they can produce and care for at any given time. Therefore, females may be selected to maximize the quality of the limited number of offspring they produce (Bateman 1948; Trivers 1972). By engaging in extra-pair mating (EPM), females may enhance the quality of their offspring by securing either direct material benefits or indirect genetic benefits from extra-pair males (Ligon 1999; Jennions & Petrie 2000). Material benefits have direct and positive effects on the reproductive success of females and include fertility assurance (Wetton & Parkin 1991), beneficial ejaculate compounds (Reynolds & Gross 1990), and access to additional food resources (Gray 1997), territory, or parental care. However, females in most bird species do not appear to acquire direct material benefits

Correspondence: Anna M. Forsman, Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, NY 14853, USA. Fax: +607 255 8088; Email: amf226@cornell.edu

from extra-pair mates (Gray 1997; Kempenaers *et al.* 1997; Ligon 1999). Thus, researchers have focused on finding evidence for indirect *genetic* benefits to females, i.e. the contribution of superior or complementary alleles for offspring from extra-pair mates (Jennions & Petrie 2000; Zeh & Zeh 2001; Griffith *et al.* 2002; Westneat & Stewart 2003; Akçay & Roughgarden 2007).

If females engage in EPMs with males whose gametic contributions enhance offspring quality, then offspring sired by extra-pair males [extra-pair young (EPY)] should be of higher quality than their maternal half-sibling brood-mates [within-pair young (WPY)]. Socially monogamous bird species are ideal study systems for investigating paternal genetic effects because they allow for comparison of maternal half-siblings present in mixed-paternity broods, while minimizing effects attributable to maternal genes, maternal effects, and rearing environment (Westneat *et al.* 1995; Kempenaers & Sheldon 1996; Griffith *et al.* 2002). Several studies have looked for evidence of indirect genetic benefits to female birds by comparing EPY and WPY in various measures of quality, including body size (Charmantier *et al.* 2004; Bouwman *et al.* 2007), survival (Charmantier *et al.* 2004), genetic heterozygosity (Foerster *et al.* 2003; Masters *et al.* 2003; Fossøy *et al.* 2008), and immune responsiveness (Johnsen *et al.* 2000; Kleven & Lifjeld 2004; Garvin *et al.* 2006; Kleven *et al.* 2006; Edler & Friedl 2008; Fossøy *et al.* 2008).

Immune responsiveness, or immunocompetence, is the ability of an organism to mount an effective immune response upon stimulation by a foreign antigen (Fairbrother *et al.* 2004). Immunocompetence and survival are positively correlated in some species of birds (Saino *et al.* 1997a; Tella *et al.* 2000; Møller & Saino 2004), suggesting that immunocompetence may be an important determinant of fitness. Because immunocompetence is also heritable (e.g. Brinkhof *et al.* 1999; Cichoń *et al.* 2006), females may be able to influence immunological quality of offspring by mating with males with high immunocompetence. However, the evidence for immune-based benefits of EPMs is mixed. Studies on two bird species have found evidence for enhanced immune responsiveness of EPY compared with their WPY half-siblings (Johnsen *et al.* 2000; Garvin *et al.* 2006) and three others have not (Kleven & Lifjeld 2004; Kleven *et al.* 2006; Edler & Friedl 2008). These studies each used a standardized phytohaemagglutinin (PHA) skin test to measure cutaneous immune activity as an indicator of nestling immunocompetence (Martin *et al.* 2006). However, concern has been raised recently as to whether single immune assays are sufficient to characterize overall immunocompetence (e.g. Adamo 2004).

We tested the hypothesis that female house wrens (*Troglodytes aedon*) engage in EPMs to secure indirect genetic benefits by comparing the quality of EPY and WPY within

broods. We predicted that EPY nestlings would be in better condition and mount more robust immune responses than their WPY maternal half-siblings. Our study was unique in that we examined multiple measures of offspring quality, including body size, physiological condition, and, in particular, multiple components of the immune system.

## Study species, materials and methods

### *Study species*

House wrens are small (10–12 g), drab, sexually monomorphic, cavity-nesting passerines (biology described in Johnson 1998). In spring, females visit male territories and choose mates at least partly based on nest-site quality (Johnson & Searcy 1993; Eckerle & Thompson 2006). The extent to which females may consider other territory attributes or male characteristics is unknown. In house wrens, most individuals form long-term pair bonds with only one individual at a time (i.e. they are socially monogamous). Pair bonds last through much or all of the reproductive cycle and both males and females provision the young. On occasion, a male may attract a second female to nest on his territory (Johnson & Kermott 1991). EPM is common in all populations examined to date (26.7% of broods, Soukup & Thompson 1997; 40% of broods, Masters *et al.* 2003; 28% of broods, Poirier *et al.* 2004). EPM seems to occur primarily when extra-territorial males intrude into the territories of their immediate neighbours; females do not routinely pursue EPMs off-territory or advertise their fertility (Johnson & Kermott 1989). There is no evidence that female house wrens derive direct material benefits from extra-pair mates.

### *Study site and general field methods*

We conducted this study during 2004–2006 (inclusive) breeding seasons on the 150-ha Mackinaw study area in McLean County, Illinois, USA (40°40'N, 88°53'W), where there are 700 protected, wooden nestboxes distributed 30 m apart along N-S transects that are 60 m apart in secondary deciduous forest. This area is described in more detail elsewhere (Drilling & Thompson 1988). The nestboxes used in this study ( $N = 301$ ) were located in three semi-isolated neighbourhoods within the study area.

We visited all territories every 1–3 days, noting nestbox contents, behavioural activities of any wrens present, and male identity as revealed by unique combinations of coloured leg rings. We trapped and individually marked unringed adults when incubating (i.e. females) or feeding young (i.e. males). To obtain DNA for paternity analyses, we collected a blood sample from adults upon capture and from all young 11 or more days after the day that eggs began hatching (i.e. on or after 'brood-day 11'). Data on the condition and

**Table 1** Mean values  $\pm$  1 standard error of immunological and condition measures for extra-pair (EPY) and within-pair (WPY) house wren maternal half-siblings.  $N$  = number of broods in the analysis. For parameters examined in multiple years, means shown are least-squares means controlling for the effect of year. Differences between EPY and WPY that remain significant after controlling for the false discovery rate ( $\alpha = 0.05$ ) are denoted with asterisks (see Methods, Statistical analyses)

Parameter	EPY mean	WPY mean	$N$	$F$	$P$
Mass (g)	9.8 $\pm$ 0.05	9.7 $\pm$ 0.05	117	0.76	0.384
Tarsus (mm)	18.6 $\pm$ 0.06	18.4 $\pm$ 0.06	73	6.08	0.016*
Haematocrit (%)	42.7 $\pm$ 0.4	41.5 $\pm$ 0.4	107	3.48	0.065
Albumin/ $\gamma$ -globulin	5.2 $\pm$ 0.1	5.6 $\pm$ 0.1	90	5.74	0.019*
Phytohaemagglutinin response (mm)	0.49 $\pm$ 0.02	0.54 $\pm$ 0.02	102	5.54	0.021*
Antibody response (optical density units)	0.073 $\pm$ 0.050	0.066 $\pm$ 0.050	15	0.01	0.921
Bactericidal activity (% <i>Escherichia coli</i> killed)	51.0 $\pm$ 2.7	49.1 $\pm$ 2.7	27	0.26	0.618

immune responsiveness of young were collected prior to DNA analyses; thus, fieldwork was conducted without knowing the paternity of young. This design necessitated that we assay every nestling within each brood to assure the collection of immunological data from both EPY and WPY broodmates for comparison. However, data from individual nestlings within broods were not analysed as independent observations to avoid pseudoreplication (see 'Statistical analyses' section below). Furthermore, we analysed only those broods in which we identified both WPY and EPY. Resultant sample sizes for each measure are shown in Table 1. Blood samples were stored at 4 °C until they were centrifuged later the same day at 1,610  $\times g$  for 60 s (Hemastat II, Separation Technologies) to separate cells and plasma for further analyses (see below). Permits to handle, bleed, and inject house wren young with non-pathogenic antigens (see below) were secured from appropriate federal and state agencies. Our protocol was approved by the Animal Care and Use Committees of both Illinois State University and Towson University (ISU #17-2003 and TU #SP0203RPR.02, respectively).

#### *Indices of physiological health and condition*

We recorded the mass (nearest 0.1 g) and tarsus length (nearest 0.1 mm) of nestlings on brood-day 11 as measures of growth and size. These measures have been shown to correlate positively with probability of survival after nest-leaving in passerine birds (mass: Perrins 1965; Smith *et al.* 1989; Tinbergen & Boerlijst 1990; Linden *et al.* 1992; tarsus length: Alatalo & Lundberg 1986). In our study population, the probability of recruitment into the breeding population correlates positively with mass on brood-day 11 (C. F. Thompson, unpublished data).

Nestling blood samples collected for paternity analyses were also used to obtain measurements of health and immunity (the latter described below). After centrifuging samples, we measured haematocrit as the percentage of

whole blood constituted by red blood cells, using the mean of three measurements. Haematocrit is an indicator of an individual's efficiency of oxygen uptake and ability to distribute oxygen to body tissues (Ots *et al.* 1998), and low haematocrit levels are correlated with decreased body condition in passerines (Chapman & George 1991; Richner *et al.* 1993; Ots *et al.* 1998).

We measured relative amounts of individual plasma protein classes for each nestling. Plasma albumin/ $\gamma$ -globulin ratios are routinely used as an indicator of health status in birds (e.g. Ots *et al.* 1998; Grasman *et al.* 2000; Grasman 2002; Hórak *et al.* 2002; Sheridan *et al.* 2004), and there is evidence that birds with higher albumin/ $\gamma$ -globulin ratios are more likely to return to their breeding grounds in the following year (Kilgas *et al.* 2006). Diseased individuals generally tend to have lower plasma albumin and higher  $\gamma$ -globulin levels than healthy ones (Kawai 1973; Ots *et al.* 1998). Plasma proteins were separated by gel electrophoresis using a kit including pre-prepared agarose gels and reagents (Helena Laboratories, Quick-Gel System, cat. no. 3550). We electrophoresed 3  $\mu$ L of each plasma sample at 400 v for 8.5 min and stained gels following the manufacturer's instructions. Stained gels were scanned with a densitometer to quantify the relative amounts of pre-albumin, albumin,  $\alpha$ -,  $\beta$ -, and  $\gamma$ -globulin proteins present in electrophoretic bands (QuickScan 2000 WIN version 2); we report albumin/ $\gamma$ -globulin ratios. In 2006, aliquots of fresh plasma were reserved for immediate use in a bactericidal activity assay (see below). We stored the cellular component of each nestling and adult blood sample in 500  $\mu$ L of Queen's lysis buffer (Seutin *et al.* 1991) until DNA extraction.

#### *Cutaneous immune activity*

We characterized cutaneous immune activity of young in all three years (i.e. 2004–2006) using a standardized PHA assay. PHA is a plant-derived mitogen that produces a measurable tissue swelling by stimulating the recruitment

of leucocytes, involved in both adaptive and innate immune responses, at the site of injection (McCorkle *et al.* 1980; Martin *et al.* 2006). Young were injected with 50  $\mu\text{L}$  (5 mg/mL; Sigma Aldrich, L8754) of PHA dissolved in sterile phosphate-buffered saline (PBS) (Smits *et al.* 1999) in the webbing of the left wing on brood-day 11. We used a digital thickness gauge (Mitutoyo no. 547–500) to measure wing-web thickness (mean of three measures) prior to and 24 h after PHA injection. We used change in wing-web thickness as a measure of generalized immune cell activity.

#### *Humoral immunity*

We assessed humoral immunity of young in 2005 by quantifying antigen-specific antibody production in response to immunization with tetanus toxoid. This toxoid, which is an inactivated (i.e. nonpathogenic) form of the neurotoxin tetanospasmin produced by the bacterium *Clostridium tetani*, does not affect survival in wild birds (Saino *et al.* 1997a; Råberg *et al.* 2000; Hanssen *et al.* 2004). We immunized young by intraperitoneal injection with 100  $\mu\text{L}$  of commercially available, alum-precipitated tetanus toxoid on brood-day 7 (Iowa Vet Supply, cat. no. 202773). Approximately 50  $\mu\text{L}$  of blood were collected from the brachial vein immediately prior to injection and 6 days postinjection (brood-day 13) to quantify pre- and postinjection antibody titres to tetanus toxoid. We quantified antibody titres by enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with tetanus toxoid in PBS (1 : 5 dilution) to capture only those antibodies with specific binding capacity to the injected antigen. We added one plasma sample to each tetanus-coated well at a 1 : 20 dilution with ELISA wash buffer (1 $\times$  PBS; 1% bovine serum albumin, BSA, Fisher Scientific, BP1605; 0.05% Tween-20, Fisher Scientific, BP337). Two aliquots of plasma were analysed for each nestling and mean antibody titre was used for statistical analysis. We removed unassociated reagents with wash buffer and blocked any unbound well surface with BSA to avoid nonspecific antibody interference. To detect nestling antibodies, we used a horseradish peroxidase-conjugated secondary antibody (1 : 1000 dilution; Bethyl Laboratories, A140-110P) that we had previously determined to cross-react with house wren immunoglobulin (A. M. Forsman, unpublished data). Antibody concentrations (post-minus pre-injection) were quantified by spectrophotometer (405 nm) and are reported in optical density units.

#### *Bactericidal activity*

We measured bactericidal activity of nestling plasma, which encompasses multiple components of the innate immune system, including natural antibodies, complement proteins, and lysozyme, in 2006 (Tieleman *et al.* 2005;

Matson *et al.* 2006). We incubated 5  $\mu\text{L}$  of fresh plasma (collected on brood-day 13) with approximately 200 colony-forming units of *Escherichia coli* (American Type Culture Collection, strain 8739) at 41 °C for 45 min following Matson *et al.* (2006). Control samples of bacteria were plated without the addition of plasma. Samples were plated in duplicate on tryptic soy agar plates and stored overnight in an incubator at 37 °C to allow colony formation by surviving bacteria. The following day we counted visible *E. coli* colonies and calculated the mean number of surviving colonies for the duplicates. We used numbers of colonies from control samples to calculate percentage of total bacteria killed for each nestling sample.

#### *DNA extraction and paternity analyses*

We isolated DNA from blood samples using a high-salt extraction protocol following Bruford *et al.* (1992). Polymerase chain reaction (PCR) amplifications were carried out in 15- $\mu\text{L}$  volumes containing 200  $\mu\text{M}$  dNTPs, 2.5 mM  $\text{MgCl}_2$ , 1 $\times$  PCR Buffer II (Applied Biosystems), and 0.133  $\mu\text{M}$  forward and reverse primers. We used a thermal profile that followed the touchdown protocol described in Johnson *et al.* (2002). Forward primers were fluorescently labeled, and PCR products were analysed using a Beckman Coulter CEQ 8000 Genetic Analysis System. We typed all samples at three loci: TA-C3 (B)2 (Cabe & Marshall 2001),  $\text{Mcy}\mu 4$  (Double *et al.* 1997), and LTMR6 (McDonald & Potts 1994). When more resolution was needed, two additional loci, TA-A5-15 and TA-B4-2 (Cabe & Marshall 2001), were used. We analysed allele data using CERVUS 2.0. No locus deviated significantly from Hardy–Weinberg equilibrium, with the exception of TA-A5–15, whose null allele frequency was estimated at 0.094.

Attendant females matched young at all loci with rare exceptions (i.e. six cases in which there was a mismatch at a single locus, attributable to mutation) and were assumed to be the genetic parent in all cases. Young that matched attendant males at all loci were assigned within-pair paternity (i.e. WPY), and those that failed to match attendant males at two or more loci were designated as extra-pair (i.e. EPY). A few young failed to match attendant males at one locus, so we retyped at the anomalous locus to prevent typing error. If they still failed to match the attendant male, we typed them at additional loci to attempt to resolve the anomaly. In some cases, they failed to match the attendant male at one or more of these additional loci and were therefore designated as EPY. If the probability of false assignment based on matching loci was lower than 0.005, then young were designated as WPY (Masters *et al.* 2003). We excluded from further analyses those nestlings whose paternity was not resolved as a result of loss of sample (usually only one sample/unresolved brood) or when repeated attempts to genotype failed to produce reliable allele data for one or

more loci because of the poor quality of the DNA sample. Exclusion probabilities for each locus were Mcy $\mu$ 4, 0.811; LTMR6, 0.691; TA-C3(B)2, 0.841; TA-B4-2, 0.641; and TA-A5-15, 0.464. For the three-locus set and five-locus set, exclusion probabilities were 0.991 and 0.998, respectively. Overall, the probability of false assignment for young designated as WPY was  $< 0.008$ .

### Statistical analyses

We compared EPY and WPY broodmates in each of our measures of quality (i.e. size, condition, immunocompetence measures) using randomized complete block analyses of variance (ANOVA). Only broods with at least one EPY and one WPY (i.e. mixed-paternity broods) were used for this analysis ( $N = 117$ ), including 18 broods in which the paternity of all young present was not resolved. Condition and immune response data for individual nestlings were not used in analyses as broodmates do not represent independent samples; hence, mean condition and immune response was calculated for EPY or WPY for each brood. Means for EPY and WPY in each brood were used as opposed to arbitrarily selecting one EPY and one WPY from each brood. Sample sizes vary among the individual ANOVAs because all broods were not necessarily assessed for the same measures of quality, particularly immune responsiveness (e.g. PHA-treated young did not also receive tetanus immunization). In a previous study we found no correlations between these immune responses among individual nestlings (Forsman *et al.* 2008). In the three years of data used in our analyses, there was only one instance of simultaneous social polygyny. In only three cases did the same pair produce two broods. In two of these, we assessed different components of immunity (PHA vs. tetanus), which means that nestlings from broods one and two were not included in the same analyses of immunity.

Data were transformed as needed to meet requirements of the ANOVA. We treated paternity as a fixed factor with two levels, within-pair and extra-pair. Brood-within-year was considered the random, block factor, allowing for a direct comparison of maternal half-siblings. We included year as a fixed effect for the dependent variables that were measured in multiple years (i.e. mass, tarsus length, haematocrit, plasma albumin/ $\gamma$ -globulin ratio, and PHA response). We did not include mass as an explanatory variable in analyses of PHA response as the former contributed minimally to PHA response variation among nestlings ( $N = 521$ ,  $P = 0.003$ ,  $R^2 = 0.017$ ). We considered differences between EPY and WPY to be significant only after controlling for the false discovery rate with  $\alpha = 0.05$  (Benjamini & Hochberg 1995), an approach that conserves statistical power while maintaining some control over the number of type I errors (Nakagawa 2004; Narum 2006; Waite & Campbell 2006). For each ANOVA, a  $P$  value of 0.05 was used to

determine significant effects. In cases with significant interactions, planned contrasts were conducted and interpreted with Bonferroni-adjusted  $P$  values. Bonferroni-adjusted  $\alpha$  for contrasts was set at 0.033 for an experiment-wise  $\alpha$  of 0.1. Where appropriate, we report least-squares means, controlling for effects of year. We report all means  $\pm 1$  SE. All analyses were done using SAS statistical software (SAS 2004).

## Results

### *Incidence of extra-pair paternity*

We assessed paternity of young in 362 broods. We were able to assign paternity to all young that survived to time of blood collection (brood-day 11 or 13) in 283 broods (total of 1466 young). Overall, 99 (35.0%) of these broods contained  $\geq 1$  EPY, and 244 young (16.6%) were sired by extra-pair males. In 13 of these broods (4.6%), all young were EPY. On average, 1.8 young in broods with mixed paternity were sired by extra-pair males. Average brood size on brood-day 11 was 5.1 young.

### *Indices of physiological health and condition of young*

Mean mass of young differed significantly among years ( $F_{2,114} = 3.21$ ,  $P = 0.04$ ), with mass in 2004 (10.0 g) exceeding that in 2005 (9.6 g;  $P < 0.0001$ ) and 2006 (9.7 g;  $P = 0.004$ ). Mass of young was also greater in 2006 than in 2005 ( $P = 0.02$ ). EPY and WPY half-siblings did not differ significantly in mass (Table 1).

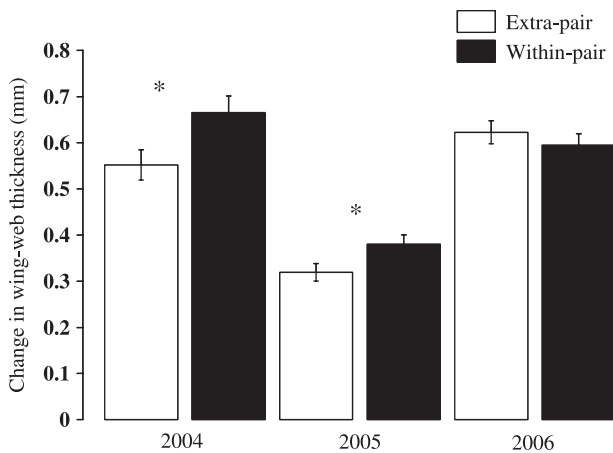
Tarsus length of young did not differ significantly among years ( $F_{2,70} = 0.22$ ,  $P = 0.81$ ). Paternity had a significant effect on tarsus length, with EPY having longer tarsi, on average, than WPY (Table 1). This effect occurred in all three years of study (no paternity-by-year interaction:  $F_{2,70} = 2.08$ ,  $P = 0.13$ ).

Haematocrit levels did not differ among years ( $F_{2,104} = 1.52$ ,  $P = 0.22$ ). EPY had higher haematocrit levels than their WPY half-siblings; however, the difference was just short of significance (Table 1). The relationship between paternity and haematocrit was consistent among years (no paternity-by-year interaction:  $F_{2,103} = 0.79$ ,  $P = 0.46$ ).

Mean albumin/ $\gamma$ -globulin ratios of young did not differ significantly among years ( $F_{2,87} = 0.79$ ,  $P = 0.46$ ). In contrast to haematocrit, EPY had significantly lower plasma albumin/ $\gamma$ -globulin ratios than their WP half-siblings (Table 1). This result was consistent across years (no paternity-by-year interaction:  $F_{2,87} = 2.25$ ,  $P = 0.11$ ).

### *Immune responsiveness of young*

Phytohaemagglutinin response of young differed significantly among years ( $F_{2,99} = 11.86$ ,  $P < 0.0001$ ). Mean PHA response in 2005 (0.35 mm) was significantly lower than in



**Fig. 1** Phytohaemagglutinin (PHA) responses of extra-pair and within-pair half-siblings within each year. Shown are least-squares means ( $\pm 1$  standard error). Within-pair young mounted stronger PHA responses than their maternal half-siblings of extra-pair origin in 2004 ( $N$  of broods = 22,  $P = 0.02$ ) and 2005 ( $N = 38$ ,  $P = 0.03$ ) but not in 2006 ( $N = 42$ ,  $P = 0.43$ ). Significant differences are denoted with asterisks (i.e.  $P \leq 0.05$ ). Bonferroni-adjusted  $\alpha$  for contrasts was set at 0.033 for experiment-wise  $\alpha$  of 0.1.

2004 (0.61 mm;  $P < 0.0001$ ) and 2006 (0.61 mm;  $P < 0.0001$ ), but did not differ in 2004 and 2006 ( $P = 0.959$ ). Paternity had a significant effect on nestling PHA response (Table 1); however, this effect differed among years (paternity-by-year interaction:  $F_{2,99} = 3.57$ ,  $P = 0.032$ ). Follow-up contrasts indicated that, contrary to prediction, EPY had a lower PHA response than their WPY half-siblings in 2004 and 2005 but not in 2006 (Fig. 1).

There were no significant differences between EPY and WPY in tetanus-specific antibody response or bactericidal activity in the single years that we examined these measures of immunity (2005 and 2006, respectively; Table 1).

## Discussion

Female birds have been hypothesized to engage in EPM to obtain alleles that enhance the quality of their offspring, including immunocompetence (Jennions & Petrie 2000). Our study provides the most comprehensive test of this hypothesis to date as we examined not only cutaneous immune activity (PHA response) but also antibody response and blood bactericidal activity (i.e. both adaptive and innate immune responses). Despite the large sample size, in none of these three measures of immunocompetence did EPY mount a stronger response than their WPY half-siblings. In fact, EPY had significantly lower cutaneous immune activity than their WPY half-siblings in two of the three years. This finding matches that of a recent study on a passerine bird (red bishop, *Euplectes orix*, Edler & Friedl 2008). However, it contrasts with results obtained for four

other passerines species in which EPY were found to have had either enhanced cutaneous immune activity (bluethroats, *Luscinia svecica*, Johnsen *et al.* 2000; Fossøy *et al.* 2008; common yellowthroats, *Geothlypis trichas*, Garvin *et al.* 2006) or equivalent activity (reed buntings, *Emberiza schoeniclus*, Kleven & Lifjeld 2004; North American barn swallows, *Hirundo rustica*, Kleven *et al.* 2006) when compared with their WPY broodmates. Although, in one of the latter species (the reed bunting), WPY tend to have greater cutaneous immune activity than EPY in a majority of broods, which is consistent with both what we found with house wrens and what occurs in red bishops (Edler & Friedl 2008). This raises the possibility that, in some species, EPM may actually decrease female fitness. This might occur, for example, if females are routinely forced or coerced into accepting copulations from extra-pair males that are of lower quality than their social mates. However, forced or coerced EPCs are rare in passerines and have not been reported in house wrens, despite extensive observational studies on this species (Kendeigh 1941; Johnson & Kermott 1989; Johnson 1998; Brylawski & Whittingham 2004). Nevertheless, researchers will want to be cognizant of this possibility in future studies. At the very least, the variety of results obtained in studies done to date suggest that there is no consistent benefit of EPM to females in the form of offspring cutaneous immune activity across passerine species. The lack of consistency may also be attributable, in part, to differences in immune investment among species or geographical areas (Ardia & Rice 2006), or differences in immunological and analytical methodology.

We also found that EPY had lower plasma albumin/ $\gamma$ -globulin ratios than their WPY half-siblings. Thus, WPY were not only able to mount stronger PHA responses than EPY, but they were also in better condition based on albumin/ $\gamma$ -globulin ratios. Other studies have also found a positive relationship between other measures of condition and PHA response (mass relative to body size, e.g. Saino *et al.* 1997b; Christe *et al.* 1998). However, the conclusion that WPY were in better condition than EPY is not supported by measures of haematocrit, tarsus length, and body mass. Indeed, EPY tended to have a higher haematocrit and longer tarsi than their WPY broodmates. It is noteworthy that we did not find a difference in mean body mass between EPY and WPY given that probability of recruitment is correlated with mass at fledging in this population (C. F. Thompson, unpublished data). If females derive indirect genetic benefits from extra-pair males, we would expect higher values in EPY for recruitment-related measures, such as mass.

Extra-pair young were significantly larger than their WPY half-siblings, as measured by tarsus length, in all three years of the study. This also occurs in reed buntings (Bouwman *et al.* 2007). Tarsus growth is essentially complete

in house wrens by brood-day 11 (Lago *et al.* 2000), the day on which we measured tarsus length, and thus tarsus length can provide a reliable indicator of structural size as an adult (Rising & Somers 1989). Tarsus length is also a heritable trait (Smith & Dhondt 1980; Dhondt 1982; Alatalo & Lundberg 1986) and is positively correlated with probability of survival and reproductive advantage in many species. For example, Christie *et al.* (1998) found that house martin (*Delichon urbica*) young that did not survive to leave the nest had shorter tarsi than the mean tarsus length of their broodmates. Similarly, Alatalo & Lundberg (1986) found that pied flycatchers (*Ficedula hypoleuca*) with the shortest tarsi had reduced survival from fledging to their first breeding season. There is also evidence that female birds with longer tarsi produce both heavier young (Potti & Merino 1994) and larger clutches than those with shorter tarsi (Alatalo & Lundberg 1986), and that cuckolded males have shorter tarsi (Kempnaers *et al.* 1992, 1997; Edler & Friedl 2008) and lower survival (Kempnaers *et al.* 1997) than noncuckolded males.

We do not know what effect tarsus length has on fitness in house wrens. However, one aspect of the natural history of this species suggests that larger males may have an advantage in competition for nest sites and hence mates. In this species, resident males are frequently challenged by nonresident 'floater' males for nest sites (Johnson & Kermott 1990). During challenges, which often last for several hours, the resident male vigorously chases the intruder, sometimes engaging him in direct physical fights. In one study, about 50% of observed nest takeover attempts were successful, and successful challengers almost always killed offspring of former residents, if present (Kermott *et al.* 1991).

Structural size differences between EPY and WPY could also arise from differential maternal effects or allocation of parental care as opposed to genetic effects. There is currently no evidence that female birds preferentially allocate resources to individual eggs within a clutch based on paternity, or that either sex discriminates between EPY and WPY when provisioning young with food (Whittingham & Dunn 2001; Whittingham *et al.* 2003; Krist *et al.* 2005). However, in house sparrows (*Passer domesticus*) and collared flycatchers (*Ficedula albicollis*), EPY were significantly more likely than WPY to come from the earlier-hatching eggs in the clutch (Cordero *et al.* 1999; Krist *et al.* 2005, respectively). In an asynchronously hatching species, such as the house wren (Harper *et al.* 1992), this could put EPY in a strong competitive position for access to food relative to their WPY nest mates. Because we have no data on the relationship between paternity and hatching order in mixed-paternity broods in our house wren population, we cannot dismiss the possibility that this type of maternal effect could have contributed to our finding that EPY have longer tarsi than WPY. However, our data do not suggest that EPY are

competitively superior to WPY during the nestling period, as we found no significant difference in body mass near the time of fledging.

That EPY had longer tarsi but lower PHA responses than their WPY half-siblings is, at first glance, a disconcerting inconsistency. However, evidence is mounting that immune system development and response are energetically costly (Lochmiller & Deerenberg 2000; Norris & Evans 2000; Alonso-Alvarez & Tella 2001; Bonneaud *et al.* 2003; Martin *et al.* 2003) and that both may be suppressed in favor of other physiological functions when resources are limited (Sheldon & Verhulst 1996; Svensson *et al.* 1998; Chin *et al.* 2005). Studies have shown that nestlings with experimentally elevated immune investment have lower growth rates than control young (magpies, *Pica pica*, Soler *et al.* 2003; blue tits, *Parus caeruleus*, Brommer 2004), which provides direct evidence for such a trade-off. Whether growth or immune system development will be favoured in a population should depend on the relative fitness benefits of enhanced immunocompetence vs. structural size, which has yet to be determined in our study population.

Our results suggest that through EPM, females increase structural size of offspring, but not offspring immune responsiveness or physiological condition. We assume that females acquire alleles from extra-pair sires that enhance offspring size. This could result from active choice of certain males by females, or variation among males in success at gaining sexual access to females, or both. However, we suspect that the latter may play a larger role than the former. Previous observational studies suggest that, in house wrens, EPMs occur when neighbouring males intrude into the territories of other males (e.g. Johnson & Kermott 1989; Johnson *et al.* 2002). If structurally larger males are able to dominate physically smaller males to gain access to neighbouring females, this may explain why EPY are larger, on average, than their WPY counterparts. However, all available evidence suggests that resident males always evict males intruding from other territories, if detected, regardless of any differences in size (Johnson & Kermott 1989). Alternatively, larger males may be generally more fit and, as a result, may return earlier from the wintering grounds than smaller males, thereby putting themselves in good position to sire EPY. In house wrens, earlier-settling males are significantly more likely to sire EPY in nests of late-settling neighbours rather than the reverse (Johnson *et al.* 2002). This probably results from the fact that often by the time that mates of late-settling males are fertile, mates of earlier-settling males have finished laying and are no longer fertile, thus freeing earlier-settling males to pursue EPMs (Johnson *et al.* 2002).

In summary, we found no evidence that females engaging in EPMs gain indirect genetic benefits enhancing condition or immunocompetence for their EPY. We did, however, find that EPY were structurally larger than WPY, suggesting

either that females preferentially seek extra-pair copulations with larger males or that larger, more aggressive males are more likely to gain EPMS. We think that it is unlikely that size differences between EPY and WPY arise from differential maternal effects or allocation of parental care.

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Anna M. Forsman conducted this research as part of her M.S. thesis and is currently a Ph.D. student at Cornell University studying eco-immunology and life-history trade-offs in altricial birds. Laura Vogel, who was trained in medical immunology, has broad interests in immunology, including eco-immunology, cellular immunology, and immunity during aging. Scott Sakaluk normally studies the sex lives of insects. He was originally slated to become a bit player in this research, but got drawn into full-time field work over three consecutive summers by the sheer joy of working on something new. Scott and Bonnie Johnson have broad interests in the biology and reproductive ecology of songbirds. Scott Johnson has done research on the model species in this project, the house wren, for almost two decades. Brian Masters' interest is in the area of molecular ecology. His current research involves the application of molecular techniques to the study of the evolution and ecology of avian mating behaviour. Charles F. Thompson, who has worked on this house wren population for 29 field seasons, studies the ecology and behaviour of birds.

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