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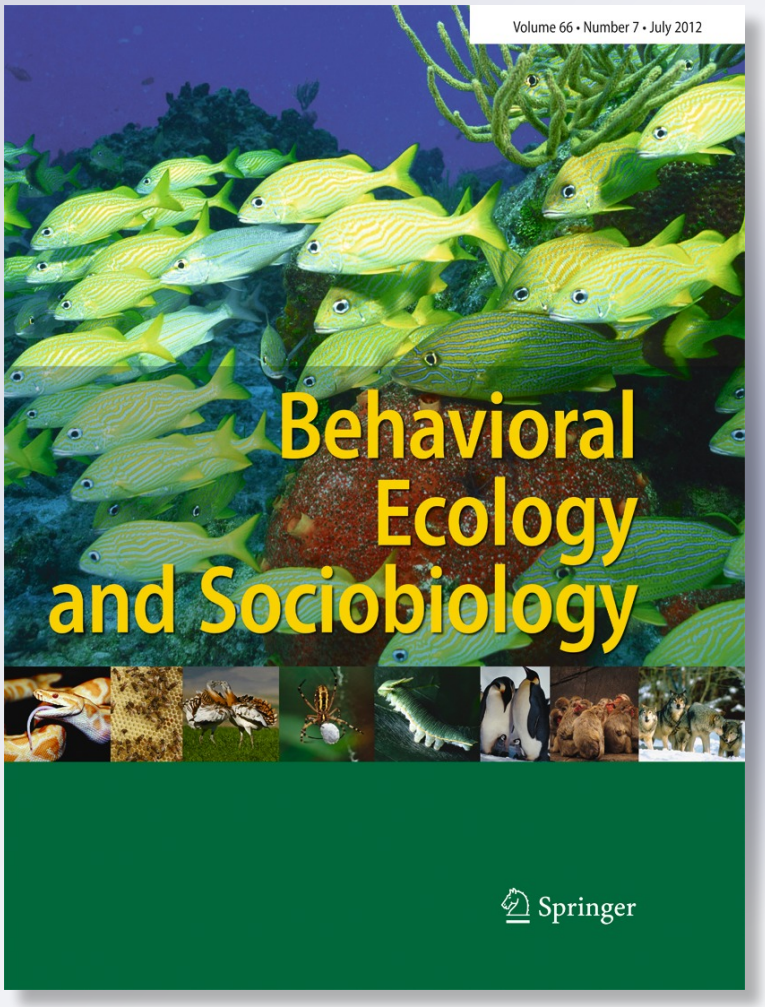

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ORIGINAL PAPER

Reproductive allocation in female house wrens is not influenced by experimentally altered male attractiveness

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Abstract The differential allocation hypothesis proposes that females mated to attractive males should invest more resources in their offspring than those mated to less-attractive males, whereas the compensation hypothesis posits that females mated to less-attractive males should invest more resources in their offspring to compensate for lower-quality young. We tested these hypotheses by manipulating attractiveness of male house wrens (*Troglodytes aedon*) prior to female arrival by adding extra nest sites to territories of some males while leaving control males with only a single nest site. Females laid their eggs sooner in the nests of attractive males, and attractive males were more likely to retain their territory over successive broods and were marginally more likely to obtain a mate for a second brood later in the season than were control males, thereby confirming the effect of our manipulation on male attractiveness. Experimentally enhanced attractiveness also led to increased hematocrit in males. However, there were no consistent differences in the number, size, or quality of eggs laid by females mated to attractive and control males, nor were there any differences in the size, health state, or immune function of nestlings produced from these eggs. There was also no effect of treatment on the number of nestlings surviving to fledging. Collectively, these results are inconsistent with both the differential allocation hypothesis and the compensation hypothesis. Future studies should consider the possibility that the criteria used by females in selecting a mate may vary temporally and be more flexible than generally thought.

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Introduction

Although females of most species are choosy when selecting a mate (Darwin 1874), they nonetheless often find themselves mated to males of differing quality and attractiveness over the course of their reproductive lives (Sheldon 2000). Variation in male attractiveness should affect how females allocate resources to their offspring because, when paired with males of different attractiveness, females can maximize their fitness by varying their reproductive effort (Mousseau and Fox 1998; Sheldon 2000; Verboven et al. 2003). Because investment in current reproductive effort is costly in terms of future survival and fecundity (Stearns 1992; Roff 2002), females paired to males of different attractiveness should adjust current investment in offspring in light of the expected fitness returns from those offspring and potential effects on their own subsequent survival and future reproduction (Trivers 1972; Sheldon 2000; Limbourg et al. 2004). It is not surprising, then, that females do respond to differences in male attractiveness and adjust their reproductive investment accordingly (e.g., Sheldon 2000; Rutstein et al. 2005; Jacot et al. 2009).

Female birds can manipulate their reproductive investment in a variety of ways during the pre-hatching and post-hatching periods of the nesting cycle. In the pre-hatch period, they can vary clutch size, egg size and content, and time spent incubating (e.g., Gil et al. 1999; Grindstaff et al. 2003; Eising et al. 2006; Giraudeau et al. 2011). Females can also manipulate nestling sex ratio and placement of the sexes in the laying sequence (Ellegren et al. 1996; Johnson et al. 2009; Bowers et al. 2011). Finally, females can differentially

invest in their offspring during the post-hatching nestling and fledgling periods by varying the rates and extent to which they brood and provision their offspring and defend their nesting sites from competitors and predators (Qvarnstrom 1997; Johnsen et al. 2005; Pryke and Griffith 2010).

Given that females often pair with males of different attractiveness and that they are able to adjust their reproductive effort in relation to environmental and social cues to maximize their fitness, the question becomes: how should females respond when mated to males of different attractiveness? Two hypotheses have been proposed to answer this question, the differential allocation hypothesis and the compensation hypothesis (Harris and Uller 2009). The differential allocation hypothesis proposes that females mated to attractive, preferred males should invest more resources in their offspring, both to maximize the quality of their offspring and to retain their attractive mates (Burley 1986; Sheldon 2000; Ratikainen and Kokko 2010). Alternatively, the compensation hypothesis predicts that females mated to less-attractive males should invest more resources in their offspring to compensate for lower-quality offspring, thereby making the best of a bad situation (Bluhm and Gowaty 2004; Navara et al. 2006).

Support for the differential allocation hypothesis comes from studies examining a wide range of taxa, including insects, mammals, amphibians, and most commonly, birds (reviewed in Sheldon 2000). Female birds mated to attractive males often lay larger clutches (Petrie and Williams 1993), larger eggs (Cunningham and Russell 2000; Uller et al. 2005; Gilbert et al. 2006; Loyau et al. 2007), and eggs containing more androgens (Loyau et al. 2007; Safran et al. 2008) than those mated to less-attractive males. Studies in which male attractiveness was experimentally manipulated support the findings from these descriptive studies, with females mated to attractive males laying earlier clutches (deLope and Møller 1993), larger clutches (Balzer and Williams 1998; Dubois et al. 2006), larger eggs (Osorno et al. 2006; Dentressangle et al. 2008), and eggs with higher levels of antioxidants (Williamson et al. 2006) and androgens (Gil et al. 1999; Kingma et al. 2009) than those mated to control males. Consistent with these studies, a recent meta-analysis found that there were small-to-moderate increases in female reproductive investment when females were paired with attractive males, with females in biparental species tending to increase the number rather than the size of their eggs (Horváthová et al. 2012). However, support for the compensation hypothesis also has been reported (Bluhm and Gowaty 2004; Navara et al. 2006).

Most tests of these two hypotheses in birds have focused on female investment in their eggs, or pre-hatch investment, but how differences in egg characteristics carry over into differences among nestlings has received comparatively little attention. Differences in characteristics of nestlings may indicate differences in pre-hatch allocation or post-hatch allocation. Several studies of differential allocation by females when paired with males of different attractiveness have examined the survival of nestlings to fledgling status, finding that females paired with attractive males generally out-reproduce those paired with less-attractive males as predicted by the differential allocation hypothesis (Burley 1986; deLope and Møller 1993; Badyaev and Hill 2002).

One difficulty involved in testing the differential allocation hypothesis and compensation hypothesis is determining the trait(s) that make a male bird attractive to a female. Many non-experimental studies have focused on male song, size, coloration, and territory quality; however, these male traits may covary with other male characteristics that influence female investment in their offspring. Females, then, might invest resources in their eggs not because of differences in mate attractiveness but because they depend on access to resources that covary with their mate's attractiveness. What is needed, then, is to separate experimentally male attractiveness from traits that covary with the attractive traits (Sheldon 2000).

We tested the differential allocation and compensation hypotheses by manipulating the attractiveness of male house wrens (*Troglodytes aedon*) as in other studies (Dubois et al. 2006; Eckerle and Thompson 2006; DeMory et al. 2010), by giving males additional nesting sites on their territories after they had settled but before they attracted females. Thus, we were able to manipulate male attractiveness independently of any other male or territory trait, and to do so without handicapping males in some way. If the differential allocation hypothesis is correct, we predicted that females mated to males perceived as attractive would invest more in their eggs and nestlings than females mated to males perceived as less-attractive [i.e., positive differential allocation *sensu* Ratikainen and Kokko (2010)]. In contrast, if the compensation hypothesis is correct, females mated to males perceived as less-attractive should invest more in their eggs and nestlings than females mated to males perceived as attractive.

Materials and methods

Study species and site

The house wren is a small (10–12 g), sexually monomorphic in size and plumage, insectivorous, migratory passerine whose breeding range encompasses much of the mid-latitudes of North America. They construct nests in preformed cavities in trees or human-made nestboxes in deciduous woodland and woodland edge (Johnson 1998). House wrens are usually socially monogamous, although facultative social polygyny also occurs (Soukup and Thompson 1997), and extra-pair mating is common (Soukup and

Thompson 1997; Forsman et al. 2008b; Johnson et al. 2009). House wrens regularly switch mates within and between breeding seasons (Drilling and Thompson 1991), making it likely that females mate with males of different attractiveness over the course of their lifetime.

When males return to the breeding grounds in late April–early May, they establish small territories around a nest cavity, into which they place large sticks, and unpaired males sing in and around their chosen nest sites to attract females (Kendeigh 1941; Johnson 1998). Females are willing to settle only with males that control territories containing at least one potential nesting site (Johnson and Searcy 1993; Johnson 1998), and females often inspect one or more males and their nest sites before choosing a social mate. There is no evidence that males reject prospective mates (Johnson 1998). After choosing a social mate, females complete the nest by lining the cup with smaller sticks and fine, soft plant materials.

House wrens in the study population typically produce two broods each breeding season. Eggs for the first brood are laid in May and for the second brood in early July (Drilling and Thompson 1991). The female alone incubates the eggs and broods the nestlings, but both the male and female provision their nestlings, which leave the nest 15–17 days after the first egg hatches (Johnson 1998).

For this study we used the East Bay (22.5 ha) study area in 2007 and the northwest corner (30.5 ha) of the Mackinaw (130 ha) study area in 2008 in McLean County, Illinois (40° 41′ N, 88°53′ W), inhabited by different groups of house wrens. The East Bay site is upland, secondary deciduous forest and the Mackinaw site is a mixture of floodplain and upland, secondary deciduous forest. Both sites are surrounded by agricultural land unsuitable for house wren breeding (Drilling and Thompson 1988, 1991). On both sites, identical, side-entrance nestboxes rest upon 48.3-cm aluminum disks that served to discourage nest predators; all nestboxes were mounted on 1.5-m metal poles. See Lambrechts et al. (2010) for details on nestbox dimensions and materials. For these experiments, we modified the standard spacing of nestboxes on the study sites (see Drilling and Thompson 1988) from 30 m apart to 60 m apart on north–south lines, which remained 60 m apart. Thus, we changed the normal density of nestboxes at the start of each experiment from 5.4 boxes/ha to 2.7/ha on the portion of each study site assigned to the experiment (see Fig. 1 in DeMory et al. 2010). We manipulated the number of nestboxes controlled by males to alter perceived male attractiveness (see below). Prior to male settlement, we removed old nest material from the available nestboxes to be able to identify when male nest-building began and to ensure that nestboxes did not differ with respect to evidence of prior use, which influences the probability that a male will choose a particular nestbox (Pacejka and Thompson 1996). Each nestbox was checked daily for evidence of male settlement. The date of settlement was the date on which the male-built stick base in the nestbox covered at least 45–50 % of the bottom and the male had been regularly singing around the nestbox (Eckerle and Thompson 2006).

Manipulating male attractiveness

To manipulate male attractiveness, we assigned one of two treatments to a male at the time of settlement but before the arrival of females: attractive with 4 nestboxes (1 original plus 3 additional) or control retaining only the original nestbox. This is the "imposed attractiveness state" described in DeMory et al. (2010). The treatment assigned to the first male to settle on the area each year was determined by the toss of a coin; thereafter, assignment of treatments alternated as other males settled. The three additional, empty nestboxes were placed 10 m directly north, south, and west of the original box on the attractive territories; the entrance of all nestboxes faced east. This design ensured that treatments were distributed evenly over the settlement period, which ended in early June, and that any correlation between male attractiveness and other male or territory traits was disrupted (Eckerle and Thompson 2006). Thus, females returning to the site were presented with a choice between attractive males (4 nestboxes) and less-attractive (1 nestbox) control males. In total, we established 14 control and 20 attractive replicates in 2007, and 17 control and 17 experimental replicates in 2008.

Female preference for males of each treatment was measured in two ways: (1) male time-to-pairing, measured as the interval from male settlement until nest-lining materials appeared in the nest (Eckerle and Thompson 2006), and (2) time-to-first-egg-laid, determined as the interval from male settlement until the first egg appeared in the nest. The former represents a female's decision to settle with a male, whereas the latter incorporates both a female's decision to settle with a male and her decision to reproduce with that male.

Field procedures

Females were captured 10–11 days after incubation began using a sliding trapdoor permanently attached to the entrance of the nestbox; banded with a numbered, aluminum U.S. Fish and Wildlife Service band; weighed to the nearest 0.1 g using a digital scale (Acculab Pocket Pro 250-B); measured for tarsus length (nearest 0.1 mm) using dial calipers, and for wing length (flattened wing chord; nearest 0.5 mm) and tail length (nearest 0.5 mm) using a stopped metal rule. The body condition index was calculated as the residual from a multiple linear regression of body mass on tarsus length and hour of capture. Males were caught during

the early part of the nestling period either when they entered the nestbox to provision nestlings or, more often, in mist nets near the nestbox when they were attracted by a tape recording of male song. Males were banded with a unique combination of 3 colored, plastic bands and 1 numbered, aluminum band (2 bands per leg). During the second brood of the season, males and females were recaptured at each nestbox to assess nestbox, territory and mate fidelity, and subsequent reproductive success.

To assess hematoserological measures of health state, we determined hematocrit (proportion of the blood volume occupied by packed red blood cells) and the ratio of albumin to gammaglobulins (Ots et al. 1998). We used a heparinized microcapillary tube to collect approximately 50 μL of blood from the left brachial vein of all adults. Blood samples were stored on ice (approximately 4 °C) until they were returned to the laboratory for further processing the same day (see below).

After females settled, lined nests were checked daily for eggs. House wrens usually lay one egg per day until the clutch is complete, with eggs increasing in size with laying order (Johnson 1998; Styrsky et al. 2002). The first egg of each clutch was marked with a single dot using a non-toxic, waterproof marker. In 2007, the second egg was collected on the morning it was laid and replaced with a dummy egg to avoid abandonment of the clutch by the female or alteration of her perception of her mate's quality (Rutstein et al. 2005). In 2008, eggs were again marked as laid and the second and fifth eggs were collected and replaced with dummy eggs. In the laboratory, eggs collected each day were weighed to the nearest 0.0001 g (Mettler AE 163) while still fresh and were subsequently frozen at −20 °C until further analysis.

Nestlings were counted and weighed on brood-days 4, 7, 9, and 12 (the first egg hatches on brood-day 0). The number of young present on brood-day 12 minus the number found dead in the nest after fledging is the number of young that survived to fledging. Nestlings also were banded with a numbered, aluminum U.S. Fish and Wildlife Service band and their tarsus measured on brood-day 12, when tarsus length is 93 % of the final adult length (Dutta et al. 1998; C. F. Thompson, unpublished data).

Immune response

We examined the immune responsiveness of house wren nestlings using two different immune challenges, tetanus toxoid immunization (Saino et al. 1997; Hanssen et al. 2005; Forsman et al. 2008a) and cutaneous immune response (Smits et al. 1999; Martin et al. 2006; Forsman et al. 2008a). On brood-day 7, we collected approximately 50 μL of blood from each nestling from the left brachial vein prior to intraperitoneal injection of 100 μg of tetanus toxoid (Iowa Vet Supply, USA, Cat. no. 202773). Tetanus toxoid is a nonpathogenic form of the neurotoxin tetanospasmin, which is produced by the bacterium *Clostridium tetani*. Injection with this antigen does not affect survival (Saino et al. 1997; Hanssen et al. 2005), and week-old nestling house wrens respond by producing antigen-specific antibodies (Forsman et al. 2008a). On brood-day 13, we took a second blood sample from the same wing to assess antibody production.

On brood-day 12, we injected nestlings with 50 μg of phytohaemagglutinin (PHA; 5 mg/mL, Sigma-Aldrich L8754), an immunostimulatory lectin protein produced by the red kidney bean (*Phaseolus vulgaris*). Prior to injection into the left wing web, we measured the thickness of the web using a digital thickness gage (Mitutoyo No. 547-500), followed by a second measurement approximately 24 h later. Three pre- and post-injection measurements were averaged to give mean final pre- and post-injection values. Post-injection wing-web thickness was measured before the post-tetanus blood sample was taken on brood-day 13.

Laboratory procedures

To measure the percentage of yolk in each egg, eggs were thawed at room temperature and the shell and albumen, which thaw more quickly than yolk (Grindstaff et al. 2005), were carefully removed, leaving the yolk intact. Whole, thawed yolks were weighed, homogenized with a sterile spatula, prepared for assays, and frozen for further analysis.

In the laboratory on the same day of collection, blood samples were centrifuged to separate plasma from the red blood cells. Hematocrit values were recorded for each blood sample. Plasma samples were microcentrifuged for several seconds (Eppendorf Centrifuge 5415) and frozen at −20 °C for future analysis of plasma proteins. We examined ratios of albumin to gammaglobulins (IgG) in adult and nestling plasma. Protein types in 3 μL of thawed plasma were separated by gel electrophoresis at 400 v for 8.5 min, following instructions by the manufacturer (Helena Laboratories, Quick-Gel System, cat. no. 3550). Stained gels were scanned with a densitometer to quantify the relative amounts of plasma proteins (prealbumin, albumin, alpha-, beta-, and gamma-globulins) present in electrophoretic bands (QuickScan 2000 WIN version 2).

IgG levels in yolk were quantified using an enzyme-linked immunosorbent assay (ELISA) modified from Grindstaff et al. (2005). Yolk samples were prepared by adding 500 mg homogenized yolk to 250 μL of PBS-Tween 20 (Fisher Scientific BP337) or PBS-T. These samples were vortexed thoroughly with glass beads and frozen until use in the ELISA procedure.

The 96-well ELISA plates (Fisher Scientific Cat. no. 07-200-39) were coated with 33 μL of anti-bird IgG capture antibody (Bethyl Laboratories A140-110A) in 5.5 mL of carbonate/bicarbonate coating buffer (0.15 M, pH 9.6) and incubated overnight at 4 °C. The plate was then washed three times for 3 min with 200 μL per well of PBS-1 % BSA-0.05 % Tween 20 buffer (50 mL 10× PBS; 450 mL distilled water; 5 g bovine serum albumin, Fisher Scientific BP1605; 250 μL Tween-20). We added 300 μL from a standard pool of samples to 1.2 mL PBS-BSA-T buffer. To create a standard curve, 200 μL from this standard solution was added to the first cells of two rows of wells at 1:10 concentration and diluted twofold across the rows to 1:20,480. Then 15 μL of yolk samples was added to another 285 μL of PBS-BSA-T buffer and pipetted to the plate in duplicate (100 μL per well). After applying samples, plates were incubated for 1 h at room temperature and washed three times. A 1:1,000 solution of buffer (10 mL) and horseradish peroxidase (HRP)-conjugated anti-bird antibody (10 μL, Bethyl Laboratories A140-110P) was added to each well (100 μL per well), and the plate incubated for another hour at room temperature. The plate was then washed twice with buffer and once with distilled water. We then added to each well 150 μL of one-step ABTS solution (Thermo Scientific 37615), a water-soluble HRP substrate that yields a green-colored end product upon reacting with peroxidase. The plate was incubated at room temperature and read at 405 nm after 60 min (Versamax tunable absorbance microplate reader).

Testosterone levels in yolk were analyzed using a competitive-binding radioimmunoassay (RIA) procedure (Bowden et al. 2001). Samples were prepared by adding 25 mg of homogenized yolk to 500 μL of distilled water. These samples were vortexed thoroughly with glass beads and frozen at −20 °C until use in the RIA procedure. We analyzed 37 samples from the second egg of each clutch in 2007. At the start of the assay, 2,000 cpm of tritiated testosterone was added to serve as a tracer for determination of recoveries. Samples were subjected to extraction using a 30:70 mixture of petroleum ether–diethyl ether and were fractionated on celite columns. The testosterone fraction was eluted with 20 % ethyl acetate–isooctane. Samples were run in a single assay with an average recovery of 68 %, and intra-assay coefficient of variation of 15.6 %. The concentration of steroids in each sample was compared to a standard curve that ranged from 1.95 to 500 pg for testosterone, and all samples were run in duplicate. We used testosterone antibody T 3003 (Wien Laboratories, Flanders, NJ), and tritiated testosterone (NET 553, Perkin-Elmer, Boston, MA).

Pre- and post-injection tetanus-specific antibody levels in nestling plasma were quantified using an ELISA (following Forsman et al. 2008a). For each nestling in a nest, pre- and post-tetanus antibody readings were compared and scored categorically, with no difference in antibody readings receiving a score of zero.

Statistical analyses

We used SAS 9.2 statistical software for all analyses (SAS Institute 2009). A Cox proportional hazards regression, as implemented in PROC PHREG, was used to examine the effect of male attractiveness on time-to-pairing and time-to-first-egg-laid. We included four covariates in the model: (1) treatment (attractive or control), (2) year (2007 or 2008), (3) settlement date (the day of the year on which the treatment was established), and (4) an interaction term involving settlement date. The interaction term was included because exploratory analyses revealed that the effect of settlement date on both time-to-pairing and time-to-first-egg-laid was not proportional over time. The two males that did not obtain mates in 2007 (both control males) were right censored at 29 days, the longest time-to-pairing observed in that year.

The number of males in each treatment that succeeded in obtaining a mate for a later, second brood; the number retaining their first-brood mate for the second brood; and the number retaining their original territory across successive broods were assessed using contingency-table analysis in PROC FREQ. Data for these comparisons were pooled across years because preliminary maximum likelihood analyses in PROC CATMOD revealed no treatment × year interaction for any of these contingencies (results not shown).

Male and female body condition indices were generated as the residuals from a multiple regression of body mass on tarsus length and hour of capture. Treatment and year effects on male and female condition indices, hematocrit, and the albumin–gammaglobulin (A/G) ratio were assessed using two-factor ANOVAs in PROC GLM.

To examine the effect of male attractiveness on the quality of eggs laid by females, we conducted two analyses in PROC GLM: (1) a two-factor ANOVA that examined the effect of treatment and year on the second egg measures (with the exception of testosterone, which was measured in the second egg in 2007 only), and (2) a repeated-measures MANOVA that examined treatment effects on the second and fifth eggs in 2008. We initially included the date on which the first egg was laid as a covariate in the models, along with associated interactions, but after finding that it had no appreciable effect on any of the egg parameters (results not shown), we omitted this variable from the final model. To examine the effect of treatment on egg testosterone in 2007, we utilized a one-factor ANOVA.

We employed mixed-model ANOVA in PROC MIXED to examine the effect of male attractiveness on nestling mass and size, health-state measures, and immune responsiveness. Nest

was included as a random effect to account for the statistical non-independence of nestlings within a brood. Treatment was included as a fixed effect, and number of nestlings on brood-day 11(brood size), brood-day 0 (time-of-season), and time-of-injection (PHA only; see effect of time of day on response to PHA injection in Forsman et al. 2010) were included as covariates. Parameter estimates were obtained using restricted maximum likelihood (REML) with a variance-components covariance structure, and degrees of freedom were estimated using the Satterthwaite approximation (Littell et al. 2006). To obtain minimal adequate models, we employed sequential backward elimination to remove non-significant terms ($P>0.15$), beginning with all two-way interactions (Crawley 1993).

Results

Female preferences

Male attractiveness did not have a significant effect on male time-to-pairing (Wald $\chi_1^2=0.68$, $P=0.41$). It did, however, have a significant effect on time-to-first-egg-laid, with females laying their eggs sooner in the nests of attractive males than in those of control males (Wald $\chi_1^2=4.39$, $P=0.0361$). The median time-to-first-egg-laid was 10 days (interquartile range=7–12 days) for control males and 8 days (6.5–12 days) for attractive males in 2007, and 10 days (6–13 days) for control males and 9 days for attractive males (7.5–12 days) in 2008. There was no significant effect of year on either measure of female preference (time-to-pairing: Wald $\chi_1^2=0.87$, $P=0.35$; time-to-first-egg-laid: Wald $\chi_1^2=1.10$, $P=0.29$). Date of male settlement had a significant effect on both measures, with males pairing sooner (Wald $\chi_1^2=18.01$, $P<0.0001$) and time-to-first-egg-laid decreasing (Wald $\chi_1^2=26.18$, $P<0.0001$), as the breeding season progressed. However, the settlement interaction term indicated that settlement date had a stronger effect on female preferences earlier in the breeding season than later in the breeding season (time-to-pairing: parameter estimate±standard error (SE)=−0.0215±0.0069, Wald $\chi_1^2=9.78$, $P=0.0018$; time-to-first-egg-laid: parameter estimate=−0.0193±0.0046, Wald $\chi_1^2=17.28$, $P<0.0001$).

Attractive males were marginally more likely to obtain a mate for a second brood in July than were control males (Likelihood Ratio $\chi_1^2=3.80$, $P=0.051$); 48.6 % of attractive males obtained a second-brood mate (18/37), whereas only 25.8 % of control males did so (8/31). Attractive males were also more likely to retain their territory over successive broods (16/18) than were control males (2/8; Likelihood Ratio $\chi_1^2=10.54$, $P=0.0012$). However, attractive males were not more likely to retain their original mate in subsequent broods (6/18) than were control males (3/8; Likelihood Ratio $\chi_1^2=0.04$, $P=0.84$).

Adult condition and health-state measures

There were no effects of treatment ($F_1=0.44$, $P<0.51$) or year ($F_1=0.48$, $P<0.49$) on male body condition index, nor was the treatment×year interaction significant ($F_1=0.19$, $P<0.66$). There were, however, significant differences between treatments and years in male hematocrit ($F_{3,\ 59}=8.51$, $P<0.0001$). Attractive males (least-squares mean± SE=49.55±0.53 %) had higher hematocrit than control males (47.03±0.55 %; $F_1=10.82$, $P<0.0017$), and male hematocrit was significantly higher in 2007 (49.60±0.55 %) than in 2008 (46.99±0.54 %; $F_1=11.60$, $P<0.0012$); the treatment×year interaction was not significant ($F_1=2.25$, $P=0.14$). In neither year was male hematocrit level and condition index correlated (2007: $r_{25}=-0.17$, $P=0.40$; 2008: $r_{28}=0.04$, $P=0.84$). There was no difference between attractive (2.61±0.25 SE) and control males (2.79±0.24) in the albumin–gammaglobulin (A/G) ratio ($F_1=0.27$, $P=0.61$), but the A/G ratio was significantly higher in 2008 (3.50±0.27) than in 2007 (1.91±0.23; $F_1=20.85$, $P<0.0001$); the treatment×year interaction was not significant ($F_1=0.80$, $P=0.38$).

There was no difference in the body condition index of females paired to control and attractive males ($F_1=0.46$, $P=0.50$), no difference in the body condition index between years ($F_1=3.15$, $P=0.081$), and no treatment×year interaction ($F_1=0.02$, $P=0.89$). There was also no difference in the hematocrit of females paired to control and attractive males ($F_1=2.40$, $P=0.13$), no difference in hematocrit between years ($F_1<0.01$, $P=0.95$), and no treatment × year interaction ($F_1=0.05$, $P=0.83$). Female condition index and hematocrit were positively correlated in 2007 ($r_{30}=0.40$, $P=0.022$), but not in 2008 ($r_{31}=-0.06$, $P=0.71$). There was no difference in the A/G ratio of females paired to attractive males (2.65±0.19) and those paired to control males (2.90±0.20; $F_1=0.82$, $P=0.37$), but, as was the case in males, the A/G ratio was significantly higher in 2008 (3.38±0.21) than in 2007 (2.17±0.18; $F_1=19.08$, $P<0.0001$); the treatment×year interaction was not significant ($F_1=0.02$, $P=0.88$).

Reproductive success

There was no effect of male attractiveness on the number of nestlings surviving to fledging (least squares mean control± SE=4.27±0.52 fledglings; attractive=5.43±0.49 fledglings; $F_1=3.04$, $P=0.11$). There was also no significant difference between years in the number of nestlings surviving to

fledging (F_1=1.51, P=0.22), nor was there a significant treatment×year interaction (F_1=1.05, P=0.31).

Egg quality

There was no difference in the clutch size of females paired with control and attractive males (least squares mean±SE: control=6.61±0.12 eggs; attractive=6.76±0.12 eggs; F_1=0.79, P=0.38), nor was there any difference in clutch size between years (F_1=0.79, P=0.38); the treatment×year interaction was also not significant (F_1=3.28, P=0.074). Two-way ANOVA of the quality of the second egg laid by females showed no effect of male attractiveness or year on egg mass ($F_{3,\ 61}$=0.41, P=0.74), percent yolk ($F_{3,\ 66}$=0.31, P=0.82), or IgG level ($F_{3,\ 66}$=1.46, P=0.23; Table 1). Male attractiveness also had no significant effects on yolk mass (F_1=3.31, P=0.0735), but there were significant differences between years in this parameter; yolks were significantly heavier in 2007 (least squares mean±SE=0.323±0.005 mg) than in 2008 (0.295±0.005 mg; F_1=15.85, P=0.0002). There was no significant difference between treatments in testosterone concentration in 2007, the only year it was measured ($F_{1,\ 35}$=0.30, P=0.59).

Repeated-measures MANOVA of the quality of the second and fifth eggs laid by females in 2008 showed no effects of male attractiveness on egg mass, yolk mass, percent yolk, and IgG level (all P>0.05; Table 1). There were significant effects of egg position on egg mass (Wilks' Lambda, $F_{1,\ 32}$=14.0, P=0.0007) and percent yolk (Wilks' Lambda, $F_{1,\ 26}$=2539.7, P<0.0001), but no effects of egg position on yolk mass and IgG level (P>0.05); fifth eggs were significantly heavier than second eggs, but the percent yolk was significantly higher in second eggs than in fifth eggs.

Nestling mass and size, health-state measures, and immune responsiveness

There was no significant effect of male attractiveness on the mass of their nestlings on brood-day 9, nor were there any effects of year, brood size, or time-of-season (all P>0.05; Table 2). There was a significant interaction between male attractiveness and time-of-season in their influence on nestling tarsus length ($F_{1,\ 54.5}$=4.31, P=0.0426). Follow-up analyses revealed that tarsus length decreased with time-of-season in nestlings of control males (parameter estimate ±SE=−0.0299±0.0107; $F_{1,\ 25.5}$=7.73, P=0.01), but was unrelated to time-of-season in nestlings of attractive males (parameter estimate=−0.0013±0.0085; $F_{1,\ 28.6}$=0.20, P=0.88). Tarsus length was unaffected by year and brood size (P>0.05).

With respect to hematoserological measures of health state, there was no significant effect of male attractiveness on nestling hematocrit or on the ratio of albumin to

Table 1 Least-squares mean (±SE) of egg-quality measures of females paired with control males and attractive males

	2007/2008 (pooled)				2008							
	Second egg		Second egg		Second egg				Fifth egg			
	Control		Attractive		Control		Attractive		Control		Attractive	
Variable	*N*	Mean±SE	*N*	Mean±SE	*N*	Mean±SE	*N*	Mean±SE	*N*	Mean±SE	*N*	Mean±SE
Egg mass (g)	32	1.31±0.04	33	1.34±0.04	19	1.35±0.02	19	1.37±0.02	18	1.40±0.03	18	1.44±0.03
Yolk mass (g)	37	0.30±0.005	36	0.32±0.005	18	0.29±0.009	17	0.30±0.008	15	0.29±0.009	17	0.30±0.008
Percent yolk	31	21.4±0.7	31	22.2±0.7	18	21.3±0.5	17	21.9±0.5	15	20.4±0.6	17	20.6±0.5
IgG (optical density units)	36	0.12±0.07	36	0.03±0.07	17	$1.08\times10^{-4}\pm.84\times10^{-4}$	18	$1.97\times10^{-4}\pm.78\times10^{-4}$	17	$2.61\times10^{-4}\pm.90\times10^{-4}$	17	$15.89\times10^{-4}\pm.84\times10^{-4}$
Testosterone (ng g^{-1}; 2007)	19	2.01±0.37	18	1.72±0.38								

Table 2 Least-squares mean (±SE) of mass, size, health-state measures, and immune responsiveness of nestlings sired by control males and attractive males

Variable (units)	Control		Attractive	
	No. of broods	Mean±SE	No. of broods	Mean±SE
Mass at brood-day 9 (g)	29	9.46±0.10	34	9.58±0.09
Tarsus length (mm)	29	18.76±0.07	34	18.81±0.07
Hematocrit at brood-day 7(%)	30	39.1±0.66	35	40.7±0.61
A/G ratio	25	3.94±0.15	31	4.13±0.14
PHA swelling (mm)	29	0.44±0.02	34	0.39±0.02
Tetanus antibody response (optical density units)	20	0.30±0.11	29	0.22±0.10

gammaglobulins ($P>0.05$). There was, however, a significant effect of time-of-season on the ratio of albumin to gammaglobulins, with the ratio decreasing as the season progressed (parameter estimate±SE=−0.0562±0.0150; $F_{1,\ 54.8}=14.14$, $P=0.0004$). There was no significant difference between years in either measure of health state, nor was there any effect of brood size (all $P>0.05$)

With respect to immune responsiveness, there was no significant effect of male attractiveness on the nestling PHA response or the tetanus toxoid response ($P>0.05$). The PHA response was influenced by hour of injection, with the response decreasing with injections done later in the day (parameter estimate±SE=$-2.9\times10^{-4}\pm1.3\times10^{-4}$; $F_{1,\ 69.2}=5.20$, $P=0.0257$). There was a significant interaction between time-of-season and year in their effect on the tetanus toxoid response ($F_{2,\ 42.7}=6.34$, $P=0.0039$). Follow-up analyses revealed that the tetanus toxoid response increased with time-of-season in 2008 (parameter estimate±SE=0.0586±0.0205; $F_{1,\ 21.7}=8.10$, $P<0.01$), but was unrelated to time-of-season in 2007 (parameter estimate=−0.0104±0.0091; $F_{1,\ 17.1}=1.31$, $P=0.27$). Brood size had no effect on either immune response ($P>0.05$).

Discussion

Success of the experimental manipulation

Females laid their eggs sooner in the nests of attractive males, and attractive males were more likely to retain their territory over successive broods and were marginally more likely to obtain a mate and produce a second brood later in the season than were control males. These results confirm the success of our treatment in rendering males more attractive by adding additional nest cavities to their territories. Two previous studies (including one on the study population) found that when males were given additional nestboxes after they had settled on a 1-nestbox territory but before females arrived, they acquired mates sooner than males left with only a single nestbox (Dubois et al. 2006; Eckerle and Thompson 2006), further underscoring the importance of male territory quality to female choice in house wrens.

Males in the attractive and control treatments did not differ in body condition index, indicating that, as in a previous experiment on the same population (DeMory et al. 2010), the experiment was successful in breaking down any correlation between attractiveness and body condition and any other male or territorial traits that covaried with the condition index. Interestingly, however, attractive males had a significantly higher hematocrit than control males. High hematocrit is associated with a superior health state, increased work load, and dehydration (Hõrak et al. 1998; Ots et al. 1998). Given the significance level associated with this difference ($P<0.0017$), it seems unlikely that we assigned a disproportionate number of males of higher hematocrit to the attractive treatment by chance alone. Instead, we suggest that the experimental enhancement of male attractiveness led to the increase in male hematocrit. This might have occurred if, upon being made more attractive, males altered their behavior by increasing nestling provisioning, maintenance behavior, or mate guarding (see Hõrak et al. 1998). Attractive males, however, are unlikely to have increased nestling provisioning, because they did not do so in another experiment using the same protocol (DeMory et al. 2010).

Testing allocation hypotheses

Pre-hatching allocation

Both the differential allocation hypothesis and compensation hypothesis predict that females mated to males of differing attractiveness will invest differentially in their offspring, with the differential allocation hypothesis predicting increased investment by females when mated to attractive males and the compensation hypothesis predicting the opposite. We found no evidence that females invested differently during the pre-hatch period when paired with attractive or control males. Females did not alter their clutch size, or the mass, percent yolk, testosterone concentration, or antibody levels of their eggs in response to male attractiveness.

Of particular interest is that females did not lay enlarged clutches with attractive-treatment males, which is consistent with Eckerle and Thompson's (2006) study of the same house wren population, but not with a study by Dubois et al. (2006) on another house wren population or with the general trend reported in the literature (Horváthová et al. 2012). Although many avian studies have found evidence of pre-hatch differential allocation in the direction predicted by the differential allocation hypothesis (see the "Introduction" section), this study and others have not. Male attractiveness did not affect female investment in clutch size (Qvarnstrom 1997; Mazuc et al. 2003; Osorno et al. 2006; Nakagawa et al. 2007), egg size (Balzer and Williams 1998; Michl et al. 2005), yolk mass (Mazuc et al. 2003), antibody transmission (Hargitai et al. 2006), or yolk testosterone concentration (Mazuc et al. 2003; Michl et al. 2005) in studies of other passerines. These and other results (Horváthová et al. 2012), as well as those of the present study, suggest that females in many species do not consistently make differential pre-hatch allocations based on the attractiveness of their mate.

Post-hatching allocation

There were no significant differences in the mass or size of nestlings of attractive and control males, and no differences between treatments in nestling health state or immunity. This is exactly what we would expect if, based on the analysis of pre-hatching allocation above, there were no differences in the pre-hatch allocations of females paired to males of the two treatments. There were significant time-of-season and year effects on measures of nestling immunity and health state, consistent with what we have found in previous studies (Forsman et al. 2010; Clairardin et al. 2011).

As with pre-hatching investment, some studies have found that post-hatching investment by females varies with mate attractiveness, including nestling body mass (Buchanan and Catchpole 2000), egg-to-independence survival (Bluhm and Gowaty 2004), and likelihood of fledging from the nest (Voltura et al. 2002). Other studies, however, including the present one, have found no differences between treatment groups in hatchling body mass (Voltura et al. 2002; Loyau et al. 2007; Nakagawa et al. 2007), nestling number (Nakagawa et al. 2007), skeletal body size (Loyau et al. 2007), or fledging success (Johnsen et al. 2005).

Females have been shown to increase nestling provisioning and defense when mated to attractive males compared with less-attractive males (Limbourg et al. 2004; Johnsen et al. 2005; Jacot et al. 2009). Naturally attractive males (Buchanan and Catchpole 2000) and males with experimentally enhanced attractiveness (Voltura et al. 2002) also have been found to expend more effort in caring for their nestlings than less-attractive males (but see Sanz 2001). In another study of our population (DeMory et al. 2010), provisioning by males, whose attractiveness had been experimentally increased using the same protocol as in the present study, did not differ. However, males with 4 nestboxes whose treatment was applied before males returned to the study areas [the "natural state" in DeMory et al. (2010)] provisioned at a lower rate than controls, leading DeMory et al. (2010) to conclude that provisioning by males is influenced more by their intrinsic quality than by their attractiveness.

Interpreting tests of the hypotheses

The lack of obvious differences between treatments raises the question as to why females did not invest differentially with respect to their mate's attractiveness. One possibility is that females did not distinguish between males in the different treatments. This seems unlikely, however, given that females laid their eggs sooner in the nests of attractive males, and attractive males were more likely to obtain a second-brood mate later in the season and to retain their territory over successive broods than were control males. In two previous studies, females also responded to an experimental alteration in male attractiveness, pairing sooner with attractive males (Dubois et al. 2006; Eckerle and Thompson 2006). Thus, it seems certain that females can distinguish between males of the two treatments, and show a clear preference for those males controlling territories containing more suitable nest sites.

A second possibility is that although territory quality influences female mating preferences, female allocation is predicated on some aspect of male phenotype not considered in the present study. Although house wrens are sexually monomorphic with respect to coloration and plumage, males produce highly complex songs that function at least partly in mate attraction (review in Johnson 1998). If female reproductive allocation decisions are based on an assessment of male song, then random assignment of males to our different nestbox treatments would have necessarily decoupled any covariance between male song structure and territory quality. Thus, although our experiment was successful in removing any correlation between attractiveness and body condition, it may have inadvertently uncoupled territory quality from a feature of male phenotype salient to female allocation decisions.

A third possibility is that all males and territories available for female assessment on the study sites we used were of varying, but generally high, quality (i.e., all had at least one suitable nest site and did not differ greatly in amount of available cover and food). Evidence for differential female investment might have been found where a greater disparity existed between or among attractiveness treatments. We cannot rule out this possibility, and future studies should

consider including one or more reduced-attractiveness treatments in their experimental design.

A final explanation for the lack of clear-cut support in the present study for either the differential allocation hypothesis or compensation hypothesis is that female pre- and post-hatching allocations are not responses to mate attractiveness, but rather are being driven by sexual conflict (Arnqvist and Rowe 2005). Another allocation hypothesis, the manipulating androgens hypothesis, proposes that females differentially invest in eggs and offspring to manipulate paternal investment (Moreno-Rueda 2007). Because androgens increase nestling begging behavior in many species (von Englehardt et al. 2006), including house wrens in our population (Barnett et al. 2011), and begging often leads to increased parental provisioning, the manipulating androgens hypothesis predicts that females deposit androgens in their eggs as a strategy to increase male provisioning rates (Moreno-Rueda 2007). However, we found no evidence of differential deposition of testosterone in the eggs that we sampled, and in another study of house wrens in our population, experimental injection of testosterone in eggs did not lead to increased male (or female) nestling provisioning, despite increased nestling begging (Barnett et al. 2011). The manipulating androgens hypothesis does not specifically include predictions about female response to mate attractiveness as the differential allocation hypothesis and compensation hypothesis do because females may be selected to manipulate contributions from both higher-quality and lower-quality males. The lack of specificity in the manipulating androgens hypothesis may be one of its strengths, as it allows for female flexibility in a way that the differential allocation hypothesis and compensation hypothesis do not. The differential allocation hypothesis and compensation hypothesis both assume that females respond to their mate's attractiveness in a specific direction over the course of the nesting cycle. Instead, female preferences may vary over time and in different environments.

Such a possibility emerges from the work of Chaine and Lyon (2008) on mate choice in lark buntings (*Calamospiza melanocorys*), which indicates that females respond to different traits associated with attractiveness in different years or under different circumstances. Thus, sexual selection on male traits may vary and, in some cases, undergo directional reversals because female preferences are subject to change over years. They further suggest that females also may experience changes in preferences even within a single breeding season. The failure to find evidence of substantial differences in female investment in the present study is consistent with the suggestion that female investment decisions may be flexible (Chaine and Lyon 2008; Harris and Uller 2009; Pariser et al. 2011; Botero and Rubenstein 2012).

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Ethical standards All research activities, including banding of birds, were performed in accordance with Illinois State University Institutional Animal Care and Use Committee (Protocol 15-2006), United State Fish and Wildlife Service (USF&WS) banding permit 09211, and USF&WS collecting permit MB692148-0).

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