



Turning a deaf ear: a test of the manipulating androgens hypothesis in house wrens

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The manipulating androgens hypothesis (MAH) proposes that female birds increase the level of testosterone (T) in their eggs to promote greater offspring begging, and thereby elicit increased provisioning by their mates. We examined the effect of a positive in ovo manipulation of T on provisioning by house wren, *Troglodytes aedon*, parents, and concomitantly examined the begging response of nestlings. We also examined the mass of nestlings throughout their growth to assess the effect of T on their development, and three measures of nest performance: hatching success, nestling survival and the proportion of nestlings that fledged. Nestlings hatching from T-injected eggs begged more than nestlings hatching from control (oil-injected) eggs early in the nestling period, but not later in the nestling period. However, treatment had no effect on the levels of parental provisioning or nestling mass gain, nor any effect on hatching success or nestling survival. There was a significant increase in parental provisioning rate, but a decline in the size of prey taken to the nest over the course of the breeding season, which was likely the result of declining environmental quality. Our results support neither the MAH, nor the expectation that nestlings should grow at different rates in relation to in ovo titres of T.

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The composition and quality of eggs in altricial bird species can vary both within and among clutches within the same species. For example, there are significant levels of variation in yolk androgens within clutches, among clutches and among populations of the same species in different geographical regions (Müller et al. 2007; Martin & Schwabl 2008). Most explanations for variation in yolk androgens focus on the differential allocation of testosterone (henceforth T) by females in eggs in response to environmental factors (Mousseau & Fox 1998a, b; Groothuis et al. 2005; Müller et al. 2007). Female manipulation of the levels of androgens in eggs might alter the developmental trajectory of embryos to better equip them to their environment (reviewed in: Groothuis et al. 2005; Navara & Mendonça 2008). Variation in levels of yolk androgens might also arise from differential sex allocation (Petrie et al. 2001; but see Gil et al. 2006; Loyau et al. 2007).

Among the factors known to influence female allocation of androgens to their eggs are intrinsic factors such as body condition (Sandell et al. 2007; Safran et al. 2008), social status (Tanvez et al. 2008) and level of immune response (Gil et al. 2006). Extrinsic

factors include social or breeding density (Groothuis & Schwabl 2002; Mazuc et al. 2003; Pilz & Smith 2004), mate quality (Gil et al. 1999; Michl et al. 2004; Loyau et al. 2007; Sandell et al. 2007) and food availability (Verboven et al. 2003). In recent years, considerable attention has focused on the effects of male quality and attractiveness on the allocation of androgens by females in eggs (reviewed in: Groothuis et al. 2005; Müller et al. 2007). However, the role that sexual conflict might play in androgen provisioning strategies of female birds has received less attention.

Sexual conflicts between parents can arise because it is advantageous for individuals to coerce their mate to provide more care to the offspring than they otherwise would. In so doing, the coercer can invest less in the current reproductive event and thereby increase future reproductive value at the expense of its manipulated mate (Trivers 1972; Arnqvist & Rowe 2005; Houston et al. 2005). This fundamental conflict has favoured coercive tactics that function to increase levels of parental provisioning by an individual's mate, as well as counteradaptations to nullify these tactics (reviewed in Arnqvist & Rowe 2005). Michl et al. (2004) suggested a candidate adaptation for coercion in birds: females might strategically adjust the level of androgens allocated to eggs to manipulate the level of paternal care. This is because elevated levels of T can increase the begging behaviour of nestlings (Schwabl 1996; Eising & Groothuis 2003; von Engelhardt et al. 2006; but see Pilz

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et al. 2004; Boncoraglio et al. 2006). Begging behaviour of nestlings signals their levels of energetic need, and increased begging rates should generally result in increased rates of parental provisioning (Godfray 1995; Kilner & Johnstone 1997). However, this response is not always evenly shared by both sexes. Playback studies have shown that, in some species, males increase their provisioning rates more than females (Ottosson et al. 1997; MacGregor & Cockburn 2002).

Recently, this conflict has been formalized as the manipulation of androgens hypothesis (MAH) (Moreno-Rueda 2007; Müller et al. 2007). The MAH does not attempt to explain female T provisioning of eggs as a response to nonsocial environmental variation, but as a female tactic to manipulate paternal care. Moreno-Rueda (2007) outlined four assumptions of the MAH: (1) females can strategically manage yolk androgen content, (2) androgens increase nestling begging (and aggression), (3) there might be costs to females or nestlings in having elevated levels of androgens, and (4) males are more responsive to increases in nestling begging than females and so increase brood provisioning at a higher rate than females. The assumptions of the MAH have not been widely verified. Recently, the MAH was tested by injecting eggs of great tits, *Cyanistes major*, with flutamide, an antiandrogen (Tschirren & Richner 2008). Flutamine binds to androgen receptors, effectively reducing the effects that yolk T is able to exert on the developing embryo. The treatment had no effect on males' levels of provisioning, but females reduced their provisioning rates (Tschirren & Richner 2008). Another study positively manipulated T in eggs also found no difference in paternal provisioning between treatments (Ruuskanen et al. 2009). Neither of these results supports the MAH; however, they did not provide data on the effect of increased T on the behaviour or growth of nestlings in response to the treatment.

The objective of our experiment was to test three assumptions of the MAH in nestlings that had either been positively manipulated with T or injected with the oil vehicle. First, we predicted that if the MAH is correct, females should provision at similar rates in control and T-treated nests, whereas males should increase their levels of provisioning in T-treated nests relative to controls. Second, we predicted that nestlings hatching in T-treated nests should beg at higher rates than oil-treated broods. The MAH assumes that increased in ovo exposure to T leads to increased nestling begging compared with controls. Finally, we examined the growth of nestlings, hatching success and nestling survival. The MAH assumes that there may be costs to elevated levels of T to nestlings. These tests provide the most comprehensive test of the MAH to date.

METHODS

Study Species and Site

The house wren is a small (10–12 g), migratory, insectivorous passerine that breeds throughout much of North America. Both sexes provision the nestlings (although males are more variable than females in their provisioning), but only the females incubate the eggs and brood the nestlings. Our study was conducted at the Mackinaw study site in McLean County, Illinois (40° 40' N, 85° 53' W), which has 700 nestboxes. Males arrive before females near the end of April, returning from their overwintering sites on the coast of the Gulf of Mexico. Males place twigs in the nestbox and sing to attract mates. Pairs typically raise two, occasionally three, broods of between four and eight nestlings each breeding season (for additional information, see Johnson 1998). The birds in this population are accustomed to high rates of exposure to human activity because each nestbox is checked a minimum of twice a week throughout the breeding season, adults are caught for banding, and nestlings

are handled for weighing and counting at least three times during the nesting cycle. Therefore, the methods described below are unlikely to cause elevated levels of nest abandonment or predation at higher rates compared with other members of this population.

Breeding Phenology

We assessed the effect of increased T on parental feeding behaviour over the course of the 2008 breeding season (May–August). We divided each breeding attempt into three parts: egg-laying, incubation and the nestling period. The egg-laying period was defined as the time from when the first egg appeared in the nest (egg-1 day) to when no further eggs appeared in the nest. Although we did not always observe the egg-1 day, we were able to extrapolate the date that the first egg appeared because females generally lay one egg per day until the clutch is complete. Once the female had stopped laying and the eggs were warm to the touch, we considered incubation to have begun.

We used the median egg-1 date (by number of nests) as the midpoint of the breeding season. Eggs laid before the median egg-1 day were classified as early-season nests and the remainder were late-season nests. For late-season nests, we did not use pairs for which we obtained data in the early season to avoid pseudoreplication. Females incubate eggs for 12–13 days before they begin to hatch. To determine the day on which the first egg hatched (brood-day 0), we began checking the nests daily after the 10th day of incubation. Upon hatching, we weighed the nestlings throughout the nestling period (described in more detail below) and assessed the provisioning behaviour of parents on brood-days 9–10.

Capture and Banding of Adults

Prior to brood-day 4, we captured females and males in their nestboxes using a permanently mounted sliding metal trapdoor over the entrance to the nestbox. We also used mist-nets to capture parents as they attempted to re-enter their nestboxes. Males were often more reluctant to return to the nest than females, necessitating the use of a conspecific song playback to lure the male into a mist-net. We weighed birds to the nearest 0.1 g (Acculab Pocket Pro 250 or PP-201), measured their tarsus (tarsometatarsus) to the nearest 0.1 mm with dial callipers, and measured wing length and tail length to the nearest 0.5 mm with a stopped rule. Captured birds were banded with a numbered U.S. Fish and Wildlife aluminium band (both sexes) and three colour bands (on males) to aid in individual identification. After processing, we released birds near the nestbox where they had been captured.

Testosterone Manipulation in Eggs

We established two treatments in which eggs from randomly selected nests were injected either with T or the oil vehicle (sham-control nests). Nests were identified for injection towards the end of the egg-laying period. When selecting nests, we attempted to identify those that had similar numbers of eggs in each treatment and avoided using nests that had fewer than three eggs (although this was not always possible). By brood-day 9, there were no differences in the number of nestlings either by treatment ($\bar{X} \pm \text{SE}$: control = 4.50 ± 0.366 , treatment = 4.60 ± 0.330 ; t test: $t_{44} = 0.211$, $P = 0.834$) or time of season ($\bar{X} \pm \text{SE}$: early = 4.48 ± 0.308 , late = 4.82 ± 0.366 ; t test: $t_{44} = 0.926$, $P = 0.360$). Once females began laying, we visited the nest 5 or 6 days after egg-1 day, but before the start of incubation, to inject each egg in the nest. We knew the female was not incubating because the nest was checked every day for egg warmth and we avoided using nests in which incubation had begun. We did this to ensure that embryos within a clutch were

at a similar, early stage of development when they were injected. We also candled the eggs to ensure that the embryo was not developing at the time of injection. After the first injection, we returned to the nest every 2 days to inject newly laid eggs until the clutch was complete and the female was incubating.

To inject eggs, we removed the freshly laid eggs and replaced them with fake, plastic eggs so that if the female returned to the nest during injection, the number of eggs was the same as when she departed. The eggs were taken from the vicinity of the nest and a betadine solution (10% providone-iodine, Purdue Pharma) was applied to the acute pole of the egg before injection. Eggs were laid on their side and the yolk visualized using a LED torch. We then made a hole at the acute pole of the egg with a sterilized 27-gauge needle through which we inserted the needle of a Hamilton syringe. We used a 100 μ l Hamilton syringe (Hamilton Company, Reno, NV, U.S.A.) to inject either 2 ng of T dissolved in 5 μ l of sterile sesame oil or 5 μ l of sterile sesame oil into the albumen of the egg to reduce the probability of damaging the yolk sac (Henry & Burke 1999; Navara et al. 2005). This amount of T was chosen because it would raise the level of T in the yolk by approximately two standard deviations above mean levels of naturally occurring T (mean \pm SD = 4.17 \pm 2.92 ng/g yolk in 2007, $N = 35$) in the yolk of house wrens in the study population (Grana 2009). After withdrawing the needle, the hole was sealed with clear wound cement, and the egg marked with a nontoxic, indelible marker and returned to the nest.

Parental Provisioning of Nestlings

We watched nests on brood-days 9 or 10 for 60 min between 0600 and 1000 hours Central Daylight Time (CDT). The start times of observations were staggered at hourly intervals so that no hourly period had a greater number of observations than would be expected by chance. Nests were watched from a distance of 25–30 m using a spotting telescope. The time and identity of the bird was recorded when it entered the nestbox with food. House wrens generally took a single prey item back to the nest with each provisioning visit. We estimated the size of each food item relative to the mean bill length of wrens (\sim 12 mm in length), which allowed us to sort the prey into three size classes: (1) small (<10 mm in length), (2) medium (10–20 mm) and (3) large (>20 mm). Ambient shade temperature was also recorded (to the nearest 0.5 $^{\circ}$ C) at the beginning of the observation period, at each parental visit, and at the end of the observation period. These measures were used to calculate the mean ambient temperature for statistical analysis. Throughout the nestling period, nests were monitored twice per week and checked daily after brood-day 12 to determine the date of fledging.

Recording Nestling Begging Behaviour

To assess the effects of experimental treatment on nestling begging behaviour, we compared the begging behaviour of nestlings at a sample of nests with T-injected eggs or vehicle-injected eggs, as described above, in the 2009 breeding season. We recorded nestling begging vocalizations using a lapel microphone (Olympus MC-15) connected to a digital Dictaphone (Olympus VN-5200PC). The microphone was placed at the top of the nestbox and the microphone wire was run down the back of the nestbox to the Dictaphone, which was tethered to the bottom of the nestbox. The recordings were made on brood-days 4–5 (1st recording) and 9–10 (2nd recording). The recordings were begun between 0630 and 1100 hours (CDT) and lasted a maximum of 120 min. After the recordings were finished, they were uploaded to a computer for later scoring. Our method of assessing nestling begging response

was novel because, unlike many previous studies, we assessed begging whilst nestlings were in situ and responding to their parents. Previous investigators have either removed nestlings from the nest for testing or attempted to elicit responses from nestlings in the nest (Schwabl 1996; Eising & Groothuis 2003; von Engelhardt et al. 2006).

We were interested in the effect of T on the begging output of the brood as a whole and so measured the proportion of time that the brood begged. We analysed each recording for 60 min after a nestling was first heard to beg after the recording had begun. From this point, we used a form of zero-one sampling whereby we recorded whether or not we heard a begging call in each second-long increment throughout the hour. This yielded a score between 0 and 3600 that we then divided by 3600 to calculate a proportion of the number of seconds in which nestlings were heard to beg. We used this measure as an approximation of the proportion of time that nestlings begged.

Hatching Success and Nestling Growth and Survival

We investigated the effect of T on nestling growth and nest performance in relation to treatment, as this would also provide insight into the potential costs of T on the development of nestlings and the success of nests. Nestlings were weighed to the nearest 0.1 g on brood-days 0–1, 4–5, 9–10 and 11. Additionally, nestlings were fitted with a single U.S. Fish and Wildlife Service numbered ring after the nest watch on brood-day 9 or 10. We also measured the nestling's tarsus to the nearest 0.1 mm on brood-day 11 using dial callipers. Nestlings can fledge anytime after brood-day 12 (typically brood-days 15–17), so nests were not disturbed beyond brief daily checks to avoid premature nest leaving. We also examined the effects of T on hatching success and nestling survival in relation to treatment and time of season. We used three measures of nest success: (1) the proportion of eggs hatching, (2) the proportion of nestlings surviving until brood-day 12 and (3) the proportion of nestlings that fledged.

Statistical Analyses

We used linear mixed models (LMMs) to analyse the provisioning data, nestling begging responses and nestling mass throughout the nestling period. For the provisioning analysis, the dependent variable was number of feeds to the nest per hour. We nested sex within the nest identifier term. In our initial model, we included all factors and first-order interactions and removed those not approaching significance (i.e. $P > 0.1$). Initially, treatment, sex and time of season were included as fixed factors in the model, as well as their first-order interactions. The mean temperature and the number of nestlings in the nest were included as covariates. The minimum appropriate model included treatment, sex and time of season as fixed factors, and the number of nestlings in the nest as a covariate. We also included a sex by time-of-season interaction in the model. All other factors and interactions were not significant and excluded from the final model. The degrees of freedom were calculated using Satherwaite's method, which accounts for within- and between-subject variance components (Littell et al. 1996).

To examine the effect of treatment on the proportion of time that nestlings begged, the initial model included treatment and nestling age (brood-days 4–5 or brood-days 9–10) as fixed factors, along with their first-order interaction. Brood size was included in the model as a covariate, which proved to be the minimum appropriate model. The proportions were all arcsine square-root transformed prior to running the model.

For the nestling mass analysis, the dependent variable was the mean nestling mass for each nest on a particular day. We used the

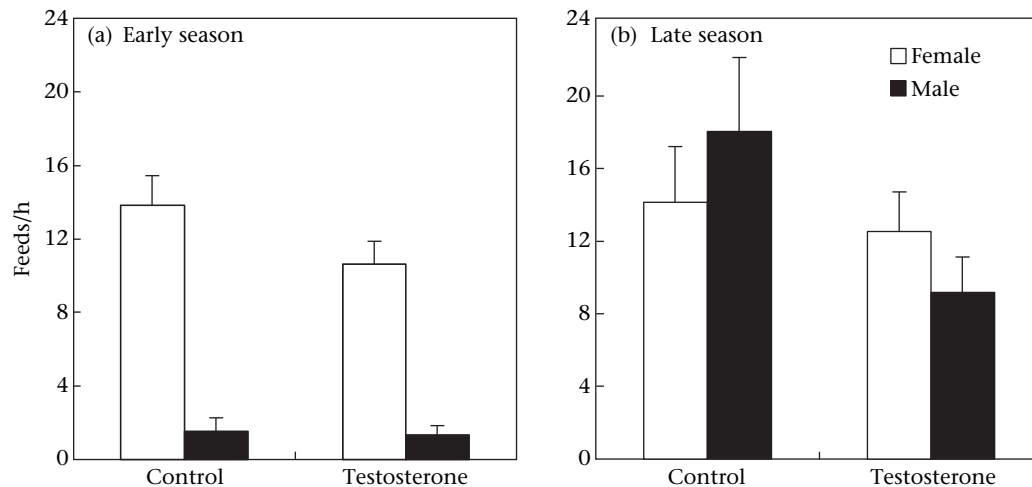


Figure 1. Mean + SE number (not REML estimates) of provisioning visits by male and female parents to (a) early-season and (b) late-season broods in each treatment.

mean because nestlings were not individually marked, so they could not be identified at different times throughout the nestling period. The initial model included treatment, time of season and brood day as fixed factors, as well as their first-order interactions. Hour of weighing and the number of nestlings were included in the model as covariates. The minimum appropriate model consisted of only brood day and time of season as fixed factors and their first-order interaction. We used a variance components covariance structure because it yielded the lowest Akaike's information criterion. We used a MANOVA to examine our three measures of nest success in relation to treatment and time of season. We included treatment and time of season as factors and an interaction between treatment and time of season.

To assess differences between treatments in the number of food items taken back to the nest, we conducted a MANCOVA analysis using a general linear model. The dependent variables were the total numbers of small, medium and large prey taken back to the nest per hour by males and females. Fixed factors in the model were treatment and time of season, and brood size was included as a covariate. The factors included in the analysis for the proportions of prey items were the same as those used in the total numbers. The proportions were all arcsine square-root transformed prior to running the model. All analyses were conducted using SPSS version 15.0 for Windows® (SPSS Inc, Chicago, IL, U.S.A.) except for the MANOVA on nest success, which was conducted using R version 2.11.1 (R Development Core Team 2010).

RESULTS

Effects of Experimental Treatment on Parental Provisioning Behaviour

We watched 46 nests on brood-day 9 or 10 (21 control, 25 treatment; 28 early-season, 18 late-season). There was no effect of experimental treatment on the number of provisioning visits to each nest (LMM: $F_{1,59.9} = 3.554$, $P = 0.064$), but there was a nonsignificant tendency for control broods to be provisioned more than T-injected broods (Fig. 1). There was a significant effect of sex, with females provisioning nestlings at higher rates than males ($F_{1,63.8} = 14.442$, $P < 0.001$; Fig. 1). Males increased their provisioning in late-season broods compared with early-season broods, which was manifest as a significant sex-by-season interaction ($F_{1,63.8} = 15.062$, $P < 0.001$; Fig. 1a versus Fig. 1b). The increased male provisioning also resulted in significantly greater

numbers of food items being delivered to late-season nests ($F_{1,63.8} = 14.756$, $P < 0.001$).

Effects of Treatment on Nestling Begging Rates

There was a significant interaction effect between treatment and nestling age in their effect on offspring begging ($F_{1,15} = 4.849$, $P = 0.044$; Fig. 2). In the early-nestling stage, nestlings in T-treated nests spent a much greater proportion of their time begging than control nestlings. At the late-nestling stage, however, there was little difference between treatments in nestling begging. In both treatments, nestling begging declined with age.

Effects of Treatment on Nest Success and Nestling Mass and Survival

There was no significant difference in the mean mass of nestlings hatching from T-injected eggs and nestlings hatching from control nests (LMM: $F_{1,80.7} = 0.665$, $P = 0.417$), nor were there significant interactions with brood day ($F_{6,118.2} = 1.412$, $P = 0.216$) or time of season ($F_{1,60.4} = 0.479$, $P = 0.491$). Hence, treatment was excluded from the final model. When treatment was excluded,

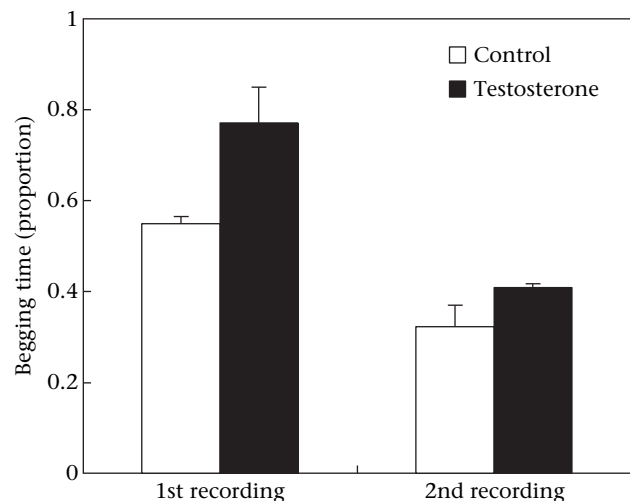


Figure 2. Mean (+SE) proportion (not REML estimates) of time that the nestlings begged in relation to treatment and nestling age (1st recording = brood-days 4–5; 2nd recording = brood-days 9–10).

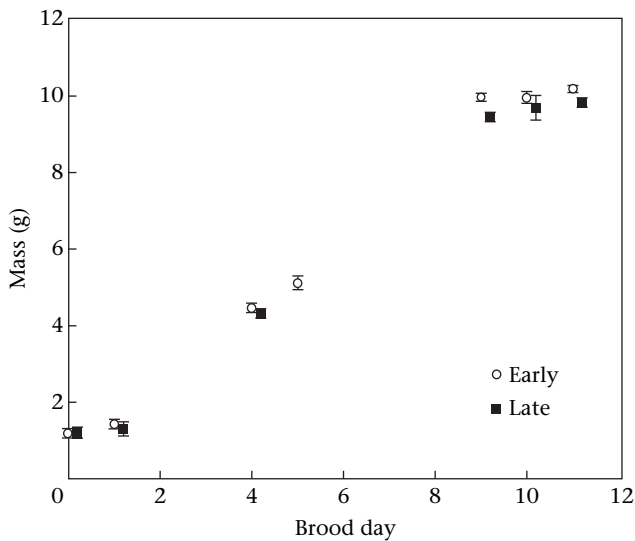


Figure 3. Mean \pm SE mass of nestlings throughout the nestling period for early- and late-season broods (not REML estimates).

there was a significant effect of time of season, with nestlings weighing less in late-season broods than in early-season broods ($F_{1,67.3} = 6.063$, $P = 0.016$; Fig. 3). Nestling mass increased with brood day until approaching asymptotic mass after brood-days 9–10 ($F_{6,130.4} = 1797.3$, $P < 0.001$; Fig. 3).

There were no differences in nest success based on treatment (Wilks' λ : $F_{3,45} = 1.444$, $P = 0.243$, $\eta^2 = 0.088$), time of season (Wilks' λ : $F_{3,45} = 0.746$, $P = 0.530$, $\eta^2 = 0.047$), or the interaction between treatment and time of season (Wilks' λ : $F_{3,45} = 1.329$, $P = 0.277$, $\eta^2 = 0.081$). Univariate tests revealed that there were no differences between treatments for either of the factors tested in relation to the dependent variables (Table 1). However, there was a nonsignificant tendency towards higher survival to brood-day 12 for nestlings whose eggs had received T injections than for nestlings whose eggs had received the oil vehicle (proportion of nestlings surviving: $\bar{X} \pm \text{SE}$: control = 0.665 ± 0.0888 , treatment = 0.855 ± 0.0602 ; $F_{1,47} = 3.212$, $P = 0.080$, $\eta^2 = 0.064$; Table 1). However, the low eta-square value from this analysis indicates that this factor explained little of the variance observed.

Size of Food Items Brought to the Nest

We compiled data on the sizes of food items provisioned to nestlings for 39 of the 46 nests (20 control, 19 T-injected). The

Table 1
Results of univariate ANOVAs for treatment and time of season, and their interaction

Source	$F_{1,47}$	P	η^2
Treatment			
Hatching success	0.557	0.459	0.012
Nestlings at BD12	3.212	0.080	0.064
Fledging success	1.163	0.286	0.024
Time of season			
Hatching success	1.482	0.230	0.031
Nestlings at BD12	0.311	0.580	0.007
Fledging success	1.903	0.174	0.039
Treatment*time of season			
Hatching success	2.098	0.154	0.043
Nestlings at BD12	0.048	0.828	0.001
Fledging success	0.070	0.792	0.001

BD = brood-day.

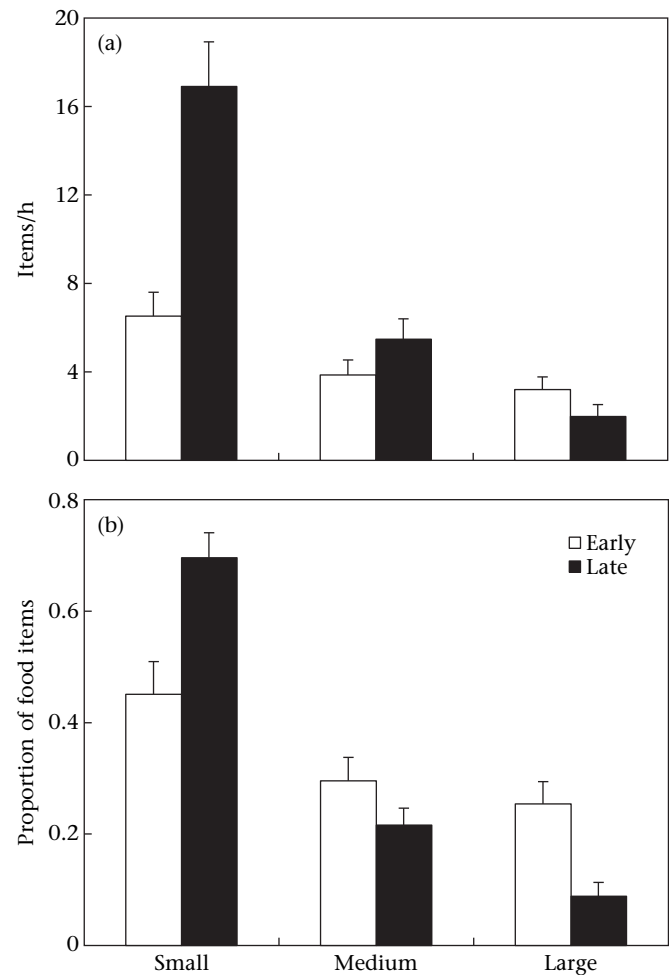


Figure 4. Mean \pm SE (a) numbers and (b) proportions of small, medium and large food items delivered to early-season and late-season nests/h (not REML estimates).

number of nestlings was included in the model as a covariate and had a significant effect on the numbers of food items taken back to the nest (Wilks' λ : $F_{3,32} = 4.459$, $P = 0.010$, $\eta^2 = 0.295$). Therefore, we controlled for the effect that brood size had on the parents' provisioning. There was no difference in the numbers of each food size taken back to the nest in relation to treatment (Wilks' λ : $F_{3,32} = 1.315$, $P = 0.287$, $\eta^2 = 0.11$). Time within the breeding season (early- or late-season) had a significant effect on the numbers of the prey of different size taken back to the nest (Wilks' λ : $F_{3,32} = 9.521$, $P < 0.001$, $\eta^2 = 0.472$; Fig. 4a). The interaction between treatment and time of season was not significant (Wilks' λ : $F_{3,32} = 0.785$, $P = 0.511$, $\eta^2 = 0.069$). Univariate tests performed within the MANCOVA for differences in the numbers of food items of each size in the early- and late-season nests revealed that there was a significant difference in the numbers of small prey per hour taken to the nest in early-season versus late-season broods (REML estimates of $\bar{X} \pm \text{SE}$: early = 6.16 ± 1.367 , late = 17.12 ± 1.658 ; $F_{1,34} = 25.692$, $P < 0.001$, $\eta^2 = 0.430$; Fig. 4a). However, there were no statistically significant differences for either the medium (REML estimates of $\bar{X} \pm \text{SE}$: early = 3.998 ± 0.716 , late = 5.462 ± 0.868 ; $F_{1,34} = 1.669$, $P = 0.205$, $\eta^2 = 0.047$) or the large prey items (REML estimates of $\bar{X} \pm \text{SE}$: early = 3.422 ± 0.516 , late = 1.98 ± 0.626 ; $F_{1,34} = 3.117$, $P = 0.086$, $\eta^2 = 0.084$; Fig. 4a).

A similar pattern emerged when we analysed the proportions of the total prey in each size class taken back to the nest. The proportion of prey in each prey class was not affected by the number

of nestlings in the nest (Wilks' λ : $F_{3,32} = 0.359$, $P = 0.761$, $\eta^2 = 0.035$). The proportion of each prey size class taken back to the nest did not differ significantly between treatments (Wilks' λ : $F_{3,32} = 0.359$, $P = 0.783$, $\eta^2 = 0.033$). However, time of season had a significant effect on the proportion of prey items of each size class taken back to the nest (Wilks' λ : $F_{3,32} = 4.216$, $P = 0.013$, $\eta^2 = 0.283$; Fig. 4b). The interaction between time of season and treatment was not significant (Wilks' λ : $F_{3,32} = 1.408$, $P = 0.258$, $\eta^2 = 0.117$). Univariate tests performed within the MANCOVA for each prey size class revealed that there was a significant increase in the proportion of small prey ($\bar{X} \pm \text{SE}$: early = 0.451 ± 0.058 , late = 0.696 ± 0.045 ; $F_{1,34} = 9.682$, $P = 0.003$, $\eta^2 = 0.225$) and a significant decrease in the proportion of large prey ($\bar{X} \pm \text{SE}$: early = 0.254 ± 0.040 , late = 0.088 ± 0.025 ; $F_{1,34} = 11.336$, $P = 0.002$, $\eta^2 = 0.250$; Fig. 4b) taken back to the nest during the late season. However, there was no difference in the proportion of medium-sized prey taken back to early- and late-season nests ($\bar{X} \pm \text{SE}$: early = 0.295 ± 0.042 , late = 0.216 ± 0.031 ; $F_{1,34} = 1.971$, $P = 0.169$, $\eta^2 = 0.055$; Fig. 4b).

DISCUSSION

In this study, we examined three assumptions of the MAH and found little support for the overall hypothesis that changes in the in ovo levels of T affect the levels of parental provisioning. First, and most importantly, we found that injecting eggs with T had no effect on the provisioning rates of either parent. This suggests that in house wrens, at least, the differences in T between clutches do not result in males changing their levels of provisioning, a result that is inconsistent with the MAH. Second, we found that nestlings from T-injected eggs begged at higher rates on brood-days 4–5, but that this difference was not evident on brood-days 9–10. This provides evidence that increased in ovo levels of T also increased nestlings' begging output, which is consistent with the MAH. Finally, we found no difference between treatments in either the growth or the survivorship of nestlings to various stages of development. This indicates that in house wrens, there are few costs or benefits of increased T on growth of nestlings or performance of broods. Overall, these results indicate that T alters the behaviour of nestlings, but that parents do not respond to the changes in nestlings' behaviour.

The failure of males to respond to the increased frequency of begging by young nestlings suggests that males were either immune to the nestlings' signals, were unable to perceive their calls, or were preoccupied with seeking additional social mates or extrapair mating opportunities. The previous two studies that have examined the effect of levels of T on parental provisioning have found that both positive and negative manipulation of T in the yolk had no effect on the levels of male provisioning (Tschirren & Richner 2008; Ruuskanen et al. 2009). Lessells (2006) suggested that manipulative behaviour in sexual conflicts might be rare because the benefits of coercion are likely to be small relative to the costs if it is ineffective in manipulating the partner's behaviour. Therefore, we might expect to find that coercion of mates over parental investment may be maladaptive and unlikely to have the power to generate significant selection pressure necessary for the evolution of coercion of parental care. Hence, the evidence from this and the previous two studies together suggests that the MAH may be inadequate in explaining within-species variation in yolk T titres among clutches.

It is possible that there was no treatment effect on the provisioning of parents because the T had no effect on the developing nestlings. This is unlikely in this study, because in ovo T-injection not only affected the begging rate of young nestlings, it also affected their immunocompetence when measured using the phytohaemagglutinin (PHA) and serum bactericidal assays (unpublished

data). Testosterone has been shown to increase the begging response of young birds towards their parents in some species (Schwabl 1996; Eising & Groothuis 2003; von Engelhardt et al. 2006), but not all, with some studies finding no effect or a negative effect (Pilz et al. 2004; Boncoraglio et al. 2006; Müller et al. 2010). Therefore, further research into the effect of T on nestling behaviour is needed.

A possible reason for the decline in nestling begging as the brood ages is that by brood-day 9, the nestlings are larger and their eyes have opened. Hence, they are able to be more discriminating with regard to the stimuli to which they respond (e.g. begging only when their parents arrive at the nest). When nestlings were younger, they begged in response to most movements in the nest and spent much more time begging in the absence of any obvious stimuli. As nestlings grew larger, their begging calls also became louder and lower in frequency. Therefore, indiscriminate begging might make nestlings easier for predators to detect and also expose broods to higher levels of predation (e.g. Briskie et al. 1999). Older nestlings should, therefore, beg only when they can be sure that there will be a benefit to them.

Our results show that neither parent responds to the increased begging response of nestlings by increasing their provisioning. This might be because T has its largest effect when the nestlings are young (≤ 5 days of age). When males feed the nestlings at this time, they rarely enter the nestbox and, instead, pass food to the females, which then feed the nestlings. At this age, females are still brooding the nestlings for substantial periods of time, which restricts the amount of time that they can spend provisioning the brood. Because males do not usually enter the nest, they are not well placed to respond to nestling signals, whereas females are unable to respond to the signals because they are brooding.

There was a significant seasonal effect on both the rates of provisioning and the mass of nestlings. Males increased their rates of provisioning of late-season broods, yet these nestlings attained a lower mass than those in early-season broods. At first glance, this seems paradoxical because the nestlings in late-season broods were fed by both parents at higher rates than those in early-season broods. However, the analysis of the size of the food items provided to nestlings revealed that the increase in feeding visits was disproportionately in the form of small food items. The increased numbers of food items were insufficient, therefore, to make up for their lower nutritional value. Hence, in late-season broods, it required the effort of both parents to provision the nestlings adequately, whereas lone females could accomplish this in early-season broods.

Earlier studies on house wrens in our study area have found modest increases in provisioning later in the breeding season. Morton (1984) found that males increased their provisioning from 7.9 trips/h in early-season broods to 12.3 trips/h in later broods. This was a significant increase, but not as extreme as the increase observed in the current study. Conversely, evidence suggests that females tend to provision broods at consistently high rates throughout the breeding season (Morton 1984; this study). A possible reason for the higher variation in male feeding rates is that the quality of available food declines later in the season, which might necessitate that males increase their provisioning of the brood later in the year to provide enough food to meet the developing nestlings' needs (Kendeigh 1979; Morton 1984; Styrsky et al. 1999).

There was no effect of experimental treatment on the mass of nestlings. It is possible that T affected only one sex in the brood (e.g. von Engelhardt et al. 2006; Pitala et al. 2009). However, if there were a treatment-by-sex interaction, we would expect that this might still manifest itself as a weak treatment effect. There was no evidence of an effect despite the significant effect of the treatment

on the nestlings' begging. Testosterone has been shown to increase the rate of development of nestlings, including mass gain (Groothuis et al. 2005; Navara & Mendonça 2008; Müller et al. 2010). The general mechanisms for T-mediated accelerated development are still poorly understood because the effects of in ovo injection of T on nestling metabolic rates are conflicting. Elevation of yolk T in zebra finch, *Taeniopygia guttata*, eggs increased resting metabolic rates of nestlings (Tobler et al. 2007), but had no effect in black-headed gull, *Chroicocephalus ridibundus*, nestlings (Eising et al. 2003). However, there is abundant evidence in adults that elevated levels of T reduce metabolic rates (reviewed in Navara & Mendonça 2008). If T does slow nestling metabolic rates, this could explain the seemingly counterintuitive result of Pilz et al. (2004), who found that European starling, *Sturnus vulgaris*, nestlings that had been treated in ovo with T, begged less, yet still gained mass more quickly than control nestlings within populations of the same species.

We also found that there were no differences in the levels of nestling success based on T treatment, which indicates that our treatment had few effects on nestling survival. Increased levels of in ovo T are thought to have a detrimental effect on embryo and nestling success (e.g. Sockman & Schwabl 2000; Navara et al. 2005), although other studies have found that T either has no effect on survival (e.g. von Engelhardt et al. 2006; Pitala et al. 2009), or increases survival (e.g. Eising et al. 2001). Therefore, further study of the effect of T on nestling survival is needed.

As with the provisioning data, we found a significant effect of time of season on the mass of nestlings throughout their development. However, contrary to expectation, the mass of nestlings was lower later in the breeding season, despite the greater number of provisioning visits from parents. Morton (1984) found that increases in parental provisioning later in the season were associated with a modest, but nonsignificant, increase in dry mass of food taken to nests, but this analysis was hampered by small sample sizes. Our analysis of the food items that parents took back to the nests was also a subsample of the total number of nests watched. However, there was a significant seasonal difference in the composition of food items taken back to the nest. Early in the season, the smaller food items were less numerous and taken to the nest in only about half of the parental visits. Later in the breeding season, smaller food items represented about 70% of all food items. These food items were the least nutritionally valuable and so their value compared with medium and large items can be discounted in relation to their numeric or proportional contribution to nestling diet. This is because the mass (and hence nutritional value) of insects does not increase linearly with length, but instead increases at about 2.79 times the rate at which length increases in most insects (Benke et al. 1999). It was recently shown that fledging masses of house sparrows, *Passer domesticus*, were positively related to the number of large food items (i.e. >2 cm in length) delivered to the nest rather than to the overall number of food items delivered to the nest (Schwagmeyer & Mock 2008). Our results revealed a nonsignificant tendency for parents to deliver fewer large food items to the nest later in the breeding season, which might explain the difference in mass between early- and late-season nestlings. Therefore, larger food items may contribute a disproportionate amount of the energy to nestlings' diets despite their numerical inferiority.

Male house wrens are socially and genetically facultatively polygynous (e.g. Forsman et al. 2008), so it is possible that early in the breeding season, when food is more plentiful, males can invest more time in seeking additional social mates and extrapair copulations without harm to their primary broods. This might be why males provision the broods less early in the breeding season than later in the breeding season. Later in the breeding season, when

food becomes limiting (Kendeigh 1979; Styrsky et al. 1999), males may be forced to provision their brood to ensure its success, and consequently, must forgo additional opportunities for attracting additional mates. Although we have no direct evidence of energetic stress in nestlings from late-season broods, nestlings from these broods were lighter. However, it is unknown whether nestlings increase their begging responses later in the breeding season to signal their energetic need to parents or whether the parents use some other signal to inform their provisioning decisions. It would also be interesting to investigate how food availability affects male behaviour. This is because the males' behaviour changed noticeably throughout the breeding season, as evidenced by the numbers of males that fed their broods at different times of the year.

In conclusion, we found little support for the MAH because males did not increase their levels of provisioning in response to in ovo injections of T even though T-treated broods spent more time begging than control broods. This also suggests that although there was a physiological effect of T on the nestlings, both parents were either unable or unwilling to respond to the increased begging behaviour of T-treated nestlings. There was also no difference in the masses of nestlings in either of the two treatments, which suggests that the elevated begging levels of nestlings did not lead to increased provisioning effort by parents or higher masses in nestlings. Finally, there were no differences in the survival of nestlings in relation to their experimental treatment. Therefore, the effects of T on developing avian embryos and nestlings are far from clear. These findings question the general applicability of the MAH and also conflict with the generally held assumption that T increases rates of development in avian species. Interestingly, we found a significant increase in provisioning rates later in the breeding season. This could have been a response to declining invertebrate size, suggesting that parents had to increase their foraging rates later in the year to provision broods adequately.

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