

Phenotypic plasticity in hormonal and behavioural responses to changes in resource conditions in a migratory songbird



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An emerging question in animal behaviour is whether and how behavioural plasticity will enable organisms to adjust to human-induced, rapid environmental changes that affect breeding conditions. Adaptive behavioural plasticity in response to changing resource conditions will depend on the sensitivity of the neuroendocrine system to food stimuli and on constraints in the mechanisms mediating the expression of reproductive behaviours. We tested the hypotheses that food availability mediates plasticity in androgen and corticosterone (CORT) responses, and that circulating concentrations of these hormones in turn mediate investment into competing mating and parental behaviours in a migratory songbird. We provided supplemental food to black-throated blue warblers, *Setophaga caerulescens*, breeding in habitats with natural low and high food abundance. The effects of supplemental feeding were most pronounced in food-poor habitat. During their social mates' fertile stage, fed males sang less near their nest sites, had lower plasma androgen and CORT levels, and had higher residual mass than control males. Fed males benefited indirectly from their mates' access to supplemental food because fed females provided more parental effort than control females. Fed males did not increase their own parental effort. Our results suggest that fed males increased mate-guarding effort over extrapair mate attraction while their social mates were fertile and invested additional resources into territorial defence or self-maintenance during the parental stage. These findings reveal plastic patterns of covariation among endocrine parameters and mating behaviours, but not parental behaviours, in response to food supplementation. These responses enabled individuals to adjust their reproductive effort rapidly to changes in resource conditions. Our findings suggest that adaptive reproductive investment in response to environmental change is unlikely to be constrained by the hormonal mechanisms underlying the mediation of competing mating and parental behaviours.

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A major question in animal behaviour is whether and how organisms will respond to human-induced, rapid environmental change (Sih, Ferrari, & Harris, 2011; Tuomainen & Candolin, 2011; Wingfield, 2003). Behavioural plasticity is one potential mechanism that can buffer individuals within a population against environmental change and facilitate adaptive evolution (Duckworth, 2009; Ghalambor, McKay, Carroll, & Reznick, 2007; Snell-Rood, 2013). Such plasticity depends on both the sensitivity to environmental stimuli and the physiological mechanisms that animals use to regulate seasonal reproduction (Dawson, 2008; Lessells, 2008). A strong candidate proximate mechanism underlying adaptive behavioural plasticity is the neuroendocrine system, and its

downstream mechanisms (i.e. hypothalamic–pituitary–gonadal and hypothalamic–pituitary–adrenal axes) (Wingfield, 2008). Steroid hormone levels and the responsiveness of target tissues are seasonally dynamic and mediate suites of reproductive behaviours that can enhance fitness (Ketterson, Nolan, Wolf, & Ziegenfus, 1992; McGlothlin, Jawor, & Ketterson, 2007; Ricklefs & Wikelski, 2002; Sinervo & Svensson, 1998). A critical gap in our knowledge, however, is how environmental stimuli are perceived and then translated by the neuroendocrine system to mediate phenotypically plastic expression of reproductive behaviours (Wingfield et al., 2011; Wingfield, Visser, & Williams, 2008). Understanding these links is especially timely given that rapid environmental change has exposed animals to modifications in habitat quality and environmental stimuli such as food availability (Both et al., 2010; Visser, Holleman, & Gienapp, 2006). These changes in breeding condition can influence reproductive decisions and reproductive success

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(Both, Bouwhuis, Lessells, & Visser, 2006; Visser, van Noordwijk, Tinbergen, & Lessells, 1998). Sensitivity of the neuroendocrine system to environmental stimuli, as well as plasticity and constraints in the signalling mechanism, will determine the extent to which animals will be able to respond and the rate at which they will do so (Hau, 2007; Ketterson, Atwell, & McGlothlin, 2009; Lessells, 2008). A mechanistic understanding of behavioural responses to changing environmental stimuli is therefore necessary to determine the potential evolutionary constraints on these responses and whether adaptive plasticity in behaviour is sufficient to compensate for rapid environmental change (Adkins-Regan, 2008; Wingfield, 2008, 2013; Wingfield et al., 2011).

The multiple effects of steroid hormones on behavioural traits has led to the question of whether this signalling mechanism constrains or permits adaptive physiological and behavioural plasticity (Adkins-Regan, 2008; Hau, 2007; Ketterson et al., 2009; Lessells, 2008; McGlothlin & Ketterson, 2008; Sinervo & Svensson, 1998). When suites of traits depend on a shared mechanism, adaptive responses may be constrained because each trait is not controlled independently and their expression may show limited plasticity (Hau, 2007; Ketterson et al., 2009). When suites of traits are independently regulated, these traits may have a greater potential to adapt rapidly to changing environmental conditions and exhibit greater plasticity (Adkins-Regan, 2008; Ketterson et al., 2009).

Studies of birds have been especially valuable in advancing our understanding of complex relationships among hormones, reproductive behaviours and changing environmental stimuli (Adkins-Regan, 2005; Ball & Ketterson, 2008; Hau, 2007; Wingfield et al., 2011). Research focused on the effects of social stimuli on short-term hormonal responses has revealed that individuals can respond rapidly and flexibly to environmental challenges (Kempnaers, Peters, & Foerster, 2008). Less empirical work has investigated neuroendocrine responses to nonsocial environmental stimuli, such as food availability (Goymann, Landys, & Wingfield, 2007). Yet, one of the major impacts of climate change on breeding birds has been the reduced availability of food (e.g. insect prey) with shifting spring phenology (Visser et al., 2006). Although food availability is known to influence the hormones regulating the onset of reproduction in birds (reviewed in: Ball & Ketterson, 2008; Schoech, Rensel, Bridge, Boughton, & Wilcoxon, 2009), we know less about the interaction between food availability and the steroid hormones that may mediate plasticity of mating and parental behaviours (Schultner, Kitaysky, Gabrielsen, Hatch, & Bech, 2013).

Steroid hormones appear to be important mediators of reproductive trade-offs between mating effort (e.g. pursuit of extrapair mating), parental effort and self-maintenance behaviours in birds (Hau, 2007; Ketterson & Nolan, 1999; Ketterson, Nolan, Cawthorn, Parker, & Ziegenfus, 1996; Magrath & Komdeur, 2003; Wingfield, Hegner, Dufty, & Ball, 1990). Testosterone (T) and corticosterone (CORT) regulate key behavioural and physiological processes and can have interactive effects on reproductive performance (Hau, 2007; Hau, Ricklefs, Wikelski, Lee, & Brawn, 2010). Androgens, such as T, generally support behaviours that increase male fecundity and, when elevated for long durations, can reduce survival (Reed et al., 2006). Hormone manipulations have demonstrated causal links between T and both sexual and aggressive behaviours (reviewed in Fusani, 2008). The suppression of male parental behaviours by T, however, has received mixed support (e.g. DeVries & Jawor, 2013; Lynn, 2008). Correlative evidence also suggests a potential role for glucocorticoids, such as CORT, in regulating parental effort in response to resource conditions (Jenni-Eiermann, Glaus, Gruebler, Schwabl, & Jenni, 2008). Baseline CORT concentrations, which regulate metabolic functions, can be indicative of energetic reserves, and thus may balance reproductive effort with self-

maintenance (i.e. the likelihood of survival) given local resource conditions (Schoech, Bowman, Bridge, & Boughton, 2007). Elevated CORT affects self-maintenance behaviours that promote adult survival (reviewed in Wingfield & Ramenofsky, 1999), at a cost to parental care of nestlings (Almasi, Roulin, Jenni-Eiermann, & Jenni, 2008; Silverin, 1998). Evidence is also accumulating that higher baseline CORT levels during the nestling stage are adaptive when positive feedback results in increased parental provisioning effort (reviewed in Bonier, Martin, Moore, & Wingfield, 2009). Alternatively, elevated baseline CORT levels may prepare individuals for investment in energetically expensive parental activities (Love, Madliger, Bourgeon, Semeniuk, & Williams, 2014). Selection on hormone-mediated trade-offs between mating and parental effort in response to food availability should occur at the level of the individual (Eikenaar, Whitham, Komdeur, van der Velde, & Moore, 2011). Therefore, it is also important to examine individual variation in plasma hormone concentrations and their relationships to mating and parental effort.

Here, we experimentally tested the hypotheses that food availability mediates plasticity in androgen and corticosterone responses, and that circulating concentrations of these hormones in turn mediate investment into competing mating and parental behaviours. To do so, we provided supplemental food to a free-living, Nearctic-Neotropical migratory songbird, the black-throated blue warbler, *Setophaga caerulescens*, breeding in habitats with low and high food abundance at the Hubbard Brook Experimental Forest (HBEF), New Hampshire, U.S.A. We predicted that (1) relative to controls, fed males would invest additional resources into mating effort and reduce parental effort and (2) increased mating effort would be associated with higher plasma androgen and lower baseline CORT levels. We also expected that male reproductive effort and hormone levels would be positively associated with energetic reserves during their social mate's fertile stage (i.e. egg laying) and during periods of biparental care (i.e. provisioning nestlings). In the context of habitat quality, we predicted that supplemental feeding would significantly mediate hormonal and concomitant behavioural responses in low-quality habitat, but have a reduced or negligible effect in high-quality habitat. Finally, our hypothesis predicts that hormonal responses should covary with an individual's behavioural responses and energetic reserves. Accordingly, we examined whether circulating steroid hormones mediate male reproductive trade-offs between mating and parental effort for all sampled males (fed and control combined). We predicted that androgen concentrations would be (1) higher in males during their social mate's fertile stage than during the parental stage, (2) positively correlated with mating effort and (3) negatively correlated with parental effort. We predicted that elevated baseline CORT concentrations in males would be associated with lower energetic reserves, but higher parental effort.

METHODS

Study Population

We studied a marked population of black-throated blue warblers breeding in mature northern hardwood forest in the 3160 ha HBEF, Woodstock, New Hampshire, U.S.A. (43°56'N, 71°45'W). We collected data over a 4-year period (May–August, 2009–2012) on three, gridded study plots established at low (250–350 m; 85 ha), mid (450–600 m; 65 ha) and high (750–850 m; 35 ha) elevations (Rodenhouse, Sillett, Doran, & Holmes, 2003). The abundance of Lepidoptera larvae, the primary food source and important predictor of territory quality for black-throated blue warblers (Holmes, Rodenhouse, & Sillett, 2005), is positively correlated with elevation at the HBEF (Cline, Strong, Sillett, Rodenhouse, & Holmes, 2013;

Rodenhouse et al., 2003). Natural variation in food availability, as well as reproductive output at the mid-elevation plot are, on average, similar to that on the high-elevation plot, but differ substantially from the low-elevation plot (Cline et al., 2013; Rodenhouse et al., 2003). Therefore, we grouped data by habitat quality (low quality: low-elevation plot; high quality: mid- and high-elevation plots). All individuals were captured and marked with a unique combination of three coloured leg bands and one aluminium U.S. Geological Survey leg band. The black-throated blue warbler is socially monogamous and genetically polygamous, with relatively high rates of extrapair paternity at the HBEF (34%, 1995–1998) (Webster, Chuang-Dobbs, & Holmes, 2001). Males guard fertile females during the nest-building and egg-laying stages, following females closely. Females incubate (mean clutch size = 3.6, range 2–5 eggs) without assistance from males for about 12 days. Both sexes feed nestlings for approximately 9 days until fledging (see Holmes et al., 2005 for more information on breeding biology). Adults are insectivorous and deliver mostly Lepidoptera larvae (~90% of nestling diet) to nestlings (Goodbred & Holmes, 1996; Rodenhouse & Holmes, 1992). Pairs will often attempt second broods, depending on food availability (Nagy & Holmes, 2005a).

Field Methods

During each breeding season, we mapped male territories and monitored all nest attempts. Following territory establishment, we randomly assigned six to eight territories per study plot to the food-supplemented (hereafter, 'fed') treatment and designated 15–20 territories per study plot as controls. Fed and control territories were separated by at least one territory to reduce the probability of control males finding and using feeding trays. We typically found nests during building. Supplemental feeding started on first nest attempts 2–3 days after the onset of incubation to avoid affecting lay dates or causing nest abandonment.

We initially placed feeding trays 1 m from nests at nest height, and then moved them further from the nest (~5 m) once we visually confirmed that the female and/or male were regularly feeding from the tray. Details on the design of the feeding tray are described in Nagy and Holmes (2005b). We conducted daily observations at the feeding trays to determine whether other species were taking food from the trays (e.g. small mammals and other songbirds). If other species were detected feeding from a tray (this occurred rarely), we moved it to a new location near the nest. If a nest failed, we paused food delivery until the new nest was found and began feeding at the new nest once the female began incubating her new clutch. When a brood fledged, we continued to provide food at the tray until it was no longer being taken (i.e. fledglings dispersed), or we moved the tray to the new nest if the female initiated a second clutch.

We adjusted the amount of food provided based on the nest stage. We initially provided 5 g of waxmoth larvae (Lepidoptera: *Galleria mellonella*). Once females were documented feeding, we increased the amount of food delivered to 7 g (52 kJ), which included 2 g of waxmoth larvae and 5 g of mealworms (Coleoptera: *Tenebrio monitor*) that we had gut-loaded with cricket meal (Zilla Gut Load Cricket & Insect Food) to increase protein and calcium content. After nestlings hatched, we increased the amount of food delivered to 14 g (104 kJ), 10 g of mealworms and 4 g of waxmoth larvae, because adults regularly fed larvae to their young. We delivered new food daily and removed uneaten larvae from trays. We varied the time of day that food was delivered so that birds did not learn to anticipate food, which could affect hormone levels (Reneerkens, Piersma, & Ramenofsky, 2002).

We successfully fed 11 pairs in 2009, 21 pairs in 2010, 18 pairs in 2011 and 18 pairs in 2012, for which we collected data from

multiple nests on individual territories. In analyses examining the effects of food supplementation during the fertile (egg laying) and parental stages (provisioning nestlings), we only included nests where we had confirmed that adults were feeding from trays for at least one complete nest stage (e.g. fed during incubation stage prior to parental stage, or fed during nestling stage prior to subsequent fertile stage) before blood or behavioural data collection. The sample sizes for nests provided supplemental food differed between the fertile and parental stages because (1) supplemental feeding of first nests was initiated a few days after the onset of incubation, limiting sample sizes during the fertile stage (i.e. to renesting attempts and second broods), (2) adults differed in when they began taking food following the placement of trays and (3) nests were found and failed at different stages of the nesting cycle.

We captured males during their social mates' fertile stage and attempted to recapture them during days 3–7 of the nestling stage. Males were lured into mist nets with song playback and a male decoy ('simulated territorial intrusion') between 0600 and 1400 hours. We recorded the time elapsed between the initiation of playback and capture ('response latency'), where males were not always observed when playback began (mean \pm SE = 18.8 \pm 1.4 min), and the time elapsed between capture and blood sampling ('bleed time'), rounded to the nearest minute. We obtained blood samples within 1–3 min of capture (mean \pm SE = 2.2 \pm 0.1 min), by puncturing the brachial vein with a small (26-gauge) needle, and collected approximately 80 μ l of whole blood from the ~10 g males into heparinized microcapillary tubes. Total handling time was limited to 20 min. We released birds immediately after processing and recorded the health status and disposition of birds post-release. Blood samples were stored on ice in the field, centrifuged within 6 h of collection and stored at -20°C until we performed hormone assays.

We collected morphometric data from captured warblers. We aged individuals as second-year (SY) or after second-year (ASY) using plumage characteristics (Holmes et al., 2005) and measured the length of the right tarsus to the nearest 0.01 mm and mass to the nearest 0.1 g. We calculated size-corrected mass from the residuals of a linear regression of mass on tarsus length ($F_{1,433} = 13.8$, $P < 0.001$). We use 'residual mass' as an index of energetic reserves, but recognize that differences in mass can indicate lean muscle, organ or fat mass, and reflects different energetic needs, depending on life history stage (Boyle, Winkler, & Guglielmo, 2012; Labocha & Hayes, 2011; Schoech, 2009).

Male birds spend considerable time and energy singing to attract and guard mates, and to defend their territories (Kroodsma & Byers, 1991; Nowicki & Searcy, 2005). We used song rate, which is correlated with body mass and can reflect resource limitation (Grava, Grava, & Otter, 2009), as our index of male mating effort. Higher song rate would indicate greater allocation to mating effort. To measure song rate, we placed digital voice recorders (Olympus DS-30) within 10 m of nests, 2010–2012. The recorders were capable of detecting songs within a radius of 60 m from the stereo microphone. Recordings were automated to record songs of focal males for three 10 min periods at 0530, 0600 and 0630 hours on each of four consecutive mornings during their social mate's fertile stage and parental stage. We generated song rates from recordings using the band-limited energy detector in Raven 1.4 (Bioacoustics Research Program, 2014). See Supplementary material for validation of methods to detect songs of focal males from automated recordings. Male song rate was calculated by averaging the number of songs detected per minute across the three morning sampling periods (30 min total) on each of the four sampling days for the fertile (3–4 egg-laying days) and parental stages (4 days, nestling days 3–7) separately.

We extracted three measures of female and male parental effort (nestling provisioning rate, amount of prey biomass delivered, time spent at the nest) from 2 h video recordings of nests collected after dawn on day 7 of the nestling stage. Video cameras (JVC Everio GZ-MG155U) were mounted on tripods, set at the height of the nest rim, and placed approximately 10 m from nests. For each nest visit, we confirmed the colour band combination of the feeding parent, its arrival time, the number and type of prey items (Lepidoptera larvae versus adult insects) brought to the nest and their size relative to the length of the exposed bill (~7 mm) and departure time. Prey were grouped into four length classes: <7 mm, 7–14 mm, 14–21 mm and >21 mm. We converted the number and length (l) of the prey items into estimated prey biomass (b) in units of mg of prey biomass for each nest visit using the formula: $b = 0.004 \times l^{2.64}$ (Rogers, Buschbom, & Watson, 1977). We standardized each hourly measure of parental behaviour to per-nestling rates. Results did not differ when we did not standardize data for brood size (results not shown).

Hormone Assays

We obtained 10–50 μ l of plasma from males during their social mate's fertile stage and during the parental stage; these collections were separated by the incubation stage, and so were more than 12 days apart. We validated and used a commercially available testosterone (T) enzyme immunoassay kit (catalogue number 1-2402, Salimetrics LLC, State College, PA, U.S.A.) to measure total androgen concentrations of plasma samples. The testosterone antibody cross-reacts 100% with testosterone, 36.4% with dihydrotestosterone, 21.0% with 19-nortestosterone, 1.9% with 11-hydroxytestosterone, 1.2% with androstenedione and less than 1% for all other steroids (values provided by manufacturer). We followed the manufacturer's protocol except that we diluted 7 μ l of plasma samples at 1:10 with steroid dilutant according to Washburn, Millsbaugh, Morris, Schulz, and Faaborg (2007). We reran samples >600 pg/ml at a 1:40 dilution. The validation included parallelism and recovery of exogenous androgens on five pooled plasma samples. Each pool contained plasma from individuals expected to have high (territory establishment) and low (postbreeding) endogenous concentrations. We conducted assays to determine the recovery of exogenous testosterone by spiking pooled samples containing different levels of endogenous concentrations with known quantities of testosterone. Serial dilutions (1:10, 1:20, 1:40, 1:80) of pooled plasma samples yielded a displacement curve that was parallel in slope to the standard T curve. Mean recovery of exogenous T added to the high- and low-pooled plasma samples was 102.6% and 111.9%, respectively. We randomly distributed samples from different nest stages across the assays. The intra-assay coefficient of variation (CV) was less than 2% and the interassay CV ($N = 12$ assays) was less than 10%. The sensitivity of the assay was 15 pg/ml (Washburn et al., 2007).

We validated and used a commercially available radioimmunoassay kit (catalogue number 07120102, MP Biomedicals, Orangeburg, NY, U.S.A.) to measure CORT concentrations of plasma samples. We followed the manufacturer's protocol except that we miniaturized the assay by halving the volumes of reagents and by diluting 3 μ l of plasma samples at 1:50 with the assay dilutant according to Washburn, Morris, Millsbaugh, Faaborg, and Schulz (2002). The validation included parallelism and recovery of exogenous CORT that was added to a low plasma pool. The low pool was derived from five birds that were bled within 3 min of capture. The high pool contained plasma from birds evaluated in a previous study that involved an injection of gonadotropin-releasing hormone and then confinement in a cloth bag for 20 min. We conducted assays to determine the recovery of exogenous CORT by

spiking pooled samples containing different levels of low endogenous concentrations with known quantities of CORT. Serial dilutions (1:25, 1:50, 1:100, 1:200) of the high-pool plasma sample yielded a displacement curve that was parallel in slope to the standard CORT curve. Mean recovery of exogenous CORT added to the low-pool plasma sample was 99.5%. The intra-assay coefficient of variation (CV) was less than 7% and the interassay CV ($N = 6$ assays) was less than 9%. The sensitivity of the assay was 3.1 ng/ml (Washburn et al., 2002), which is less sensitive than the standard radioimmunoassay (0.15 ng/ml; Wingfield & Farner, 1975). Our samples were above 3.1 ng/ml, such that high mean CORT values were not attributed to the lower sensitivity of the assay. However, we describe CORT concentrations as 'initial CORT' because our analyses indicated that CORT levels increased linearly with time to bleed for samples collected within 3 min of capture (linear regression: $\beta = 6.15 \pm 2.24$ ng per ml/s, $F_{1,174} = 7.6$, $P < 0.01$). These results suggest that the initial CORT levels we measured did not reflect true baseline concentrations, but most likely reflected near-baseline levels. Some studies have shown that CORT concentrations can rise within 1–2 min of capture (Dawson & Howe, 1983; Romero & Reed, 2005; Schoech, Ketterson, & Nolan, 1999).

Statistical Analyses

Prior to analyses, we examined whether model assumptions were met, and grouped data when sample sizes were limited. We examined residual diagnostic plots to assess normality of model residuals (Fox, 2008). We used nonparametric tests when we could not meet the normality assumption. We grouped data by habitat quality (low quality = low-elevation plot; high quality = mid- and high-elevation plots) as justified above (see Study Population). All analyses were performed using R v.2.15.3 (R Development Core Team, 2013).

We tested for effects of food supplementation and habitat quality on warbler song rate, parental effort, concentrations of plasma androgens and CORT, and residual mass. We constructed four linear mixed models (LMM) using the R package 'lme4', where the response variable was song rate, plasma androgens, initial CORT concentrations or residual mass. Each of these models (except for parental effort) included male identity as a random effect because males in these data sets were represented more than once during the study and treatment (fed, control), nest stage (fertile, parental), habitat (low quality, high quality), and year as fixed effects, and the treatment*nest stage and treatment*habitat interaction terms, which were the factors of interest in the experimental design. Low sample sizes of fed males in low-quality habitat precluded our evaluation of all possible covariates in the hormone models. In the fully parameterized hormone models, we also included response latency, bleed time and time of capture as fixed effects. However, response latency, but not bleed time, was confounded with treatment in the androgen model: fed males responded less rapidly to simulated territorial intrusions (Supplementary Table S1). Bleed time (restricting samples to ≤ 3 min) and time of capture were not significant in the fully parameterized CORT model (Supplementary Table S1). We used the natural log of androgen and initial CORT levels to improve normality of model residuals. We used a White–Huber corrected covariance matrix in the ANOVA to account for heteroscedasticity (White, 1980) and partial sums of squares to account for unbalanced data (Fox, 2008). We fitted models using the restricted maximum likelihood (REML) method and used Wald chi-square values to examine the significance of variables and interaction terms (Zuur et al., 2009). We excluded year from the final models for song rate, plasma androgens and residual mass because we found no significant year effects. We tested habitat-specific differences in parental behaviour between

fed and control birds with a Wilcoxon two-sample test; no individuals in the parental data set were sampled more than once during the study.

We tested whether individual variation in plasma hormones in males (both fed and control males combined) was associated with male song rate, provisioning rate and residual mass with separate LMMs, including individual as a random effect when appropriate. First, we examined the association between plasma androgens and initial CORT concentrations and both male song rate and male residual mass across nest stages. Second, we examined the relationships between plasma androgen and initial CORT concentrations measured during the parental stage and male provisioning rate. Next, we conducted paired-sample *t* tests to test differences between the fertile and parental stages in both plasma androgen and initial CORT concentrations (log transformed) for 12 control males with hormone data from both nest stages. Lastly, we examined the relationship between plasma androgens and initial CORT concentrations, although we made no a priori prediction about the direction of this relationship.

Ethical Note

We followed protocols approved by our Institutional Animal Care and Use Committees to capture, handle, mark and sample black-throated blue warblers (Cornell University, 2009-0133; Smithsonian National Zoological Park, 08-11, 12-12; Wellesley College, 1304). All work was performed under scientific permits from the U.S. Geological Survey Bird Banding Lab (22665) and the New Hampshire Department of Fish and Game (MB207492-1). All procedures performed in this study were in accordance with the ASAB/ABS Guidelines for the treatment of animals in behavioural research. Sampling and processing had no discernable negative impacts on individuals. Behavioural observations did not appear to disrupt the normal activities of individuals.

RESULTS

Behavioural and Hormonal Responses to Food Supplementation

Male song rate was correlated with supplemental feeding, but in the direction opposite that predicted: control males had significantly higher recorded song rates than fed males in low-quality habitat, (LMM: treatment*habitat, Wald, $P = 0.032$; Fig. 1). Song rates did not differ between treatments in high-quality habitat. Results were similar when we excluded individuals where no songs were detected over all sampling periods (not shown). Song rate was significantly higher during the fertile stage than during the parental stage, as expected (fertile: 0.58 ± 0.04 songs/min, $N = 209$; parental: 0.28 ± 0.02 songs/min, $N = 172$; LMM: Wald $\chi^2_1 = 37.69$, $P < 0.001$).

Food supplementation did not directly affect parental behaviour of males, contrary to our prediction, but could have had indirect effects via changes in female parental behaviour (Fig. 2, Table S2, model results). Fed males did not reduce or increase their nestling provisioning efforts compared with control males (Fig. 2b, d, f). In low-quality habitat, fed females provisioned at a significantly higher rate and delivered significantly more prey biomass than control females (Fig. 2a, c). Fed females also spent significantly more time at the nest than control females in both low- and high-quality habitats (Fig. 2e), suggesting greater parental effort.

Plasma androgen concentrations in males were correlated with the availability of supplemental food, but in the opposite direction than predicted. Fed males had significantly lower androgen concentrations than control males during the fertile stage (Fig. 3a), but only in low-quality habitat (Table 1). Males (fed and control) in

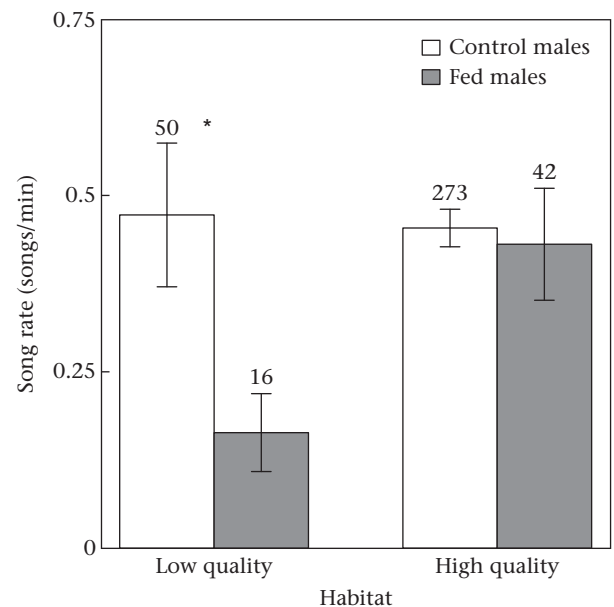


Figure 1. Effect of food supplementation on male song rate in low- and high-quality habitat. Data are pooled across nest stages and shown as means \pm SE. Sample sizes are given above the error bars. Significance levels are from a linear mixed model controlling for fixed and random effects ($*P = 0.032$).

high-quality habitat had significantly higher androgen concentrations than males in low-quality habitat, as predicted (low quality: 2.68 ± 0.43 ng/ml, $N = 63$; high quality: 3.32 ± 0.28 ng/ml, $N = 173$; Supplementary Table S1).

Food supplementation affected initial CORT concentrations and male residual body mass in the predicted direction during the fertile stage, but not during the parental stage (Fig. 3b, c). Fed males with fertile mates had significantly lower initial CORT concentrations (Table 1, Fig. 3b) and higher residual mass (LMM: treatment*nest stage: Wald $\chi^2_1 = 4.27$, $P = 0.039$; Fig. 3c), relative to controls. Fed and control males had similar initial CORT concentrations and residual mass during the parental stage (Fig. 3b, c). We also found no significant differences between males in low- and high-quality habitat in initial CORT concentrations (low quality: 40.36 ± 3.32 ng/ml, $N = 48$; high quality: 37.93 ± 1.83 ng/ml, $N = 131$; Supplementary Table S1) or in residual mass (low quality: 0.04 ± 0.05 residual mass/tarsus, $N = 63$; high quality: -0.004 ± 0.03 residual mass/tarsus, $N = 173$; LMM: Wald $\chi^2_1 = 0.03$, $P = 0.85$).

Hormones and the Mediation of Male Reproductive Effort

Circulating androgen concentration in males was associated with mating effort and residual body mass, but not with parental effort. Males with high androgen concentrations had higher song rates (LMM: $\beta = 0.14 \pm 0.05$, Wald $\chi^2_1 = 7.38$, $P = 0.007$) and lower residual mass than males with low androgen concentrations (LMM: $\beta = -0.85 \pm 0.20$, Wald $\chi^2_1 = 18.13$, $P < 0.001$). Androgen concentrations during the parental stage were not associated with male provisioning rate (LMM: $\beta = -0.04 \pm 0.13$, Wald $\chi^2_1 = 0.10$, $P = 0.75$).

We found indirect support for the role of plasma androgens in mediating mating effort and parental effort. Mean androgen concentrations were significantly higher in the fertile stage than in the parental stage, as predicted (fertile: 4.52 ± 0.33 ng/ml, $N = 142$; parental: 1.17 ± 0.15 ng/ml, $N = 94$; Supplementary Table S1). Similarly, males that were sampled during both the fertile and parental stages had significantly higher androgen concentrations during the fertile stage (paired *t* test: $t_{11} = -2.75$, $P = 0.018$).

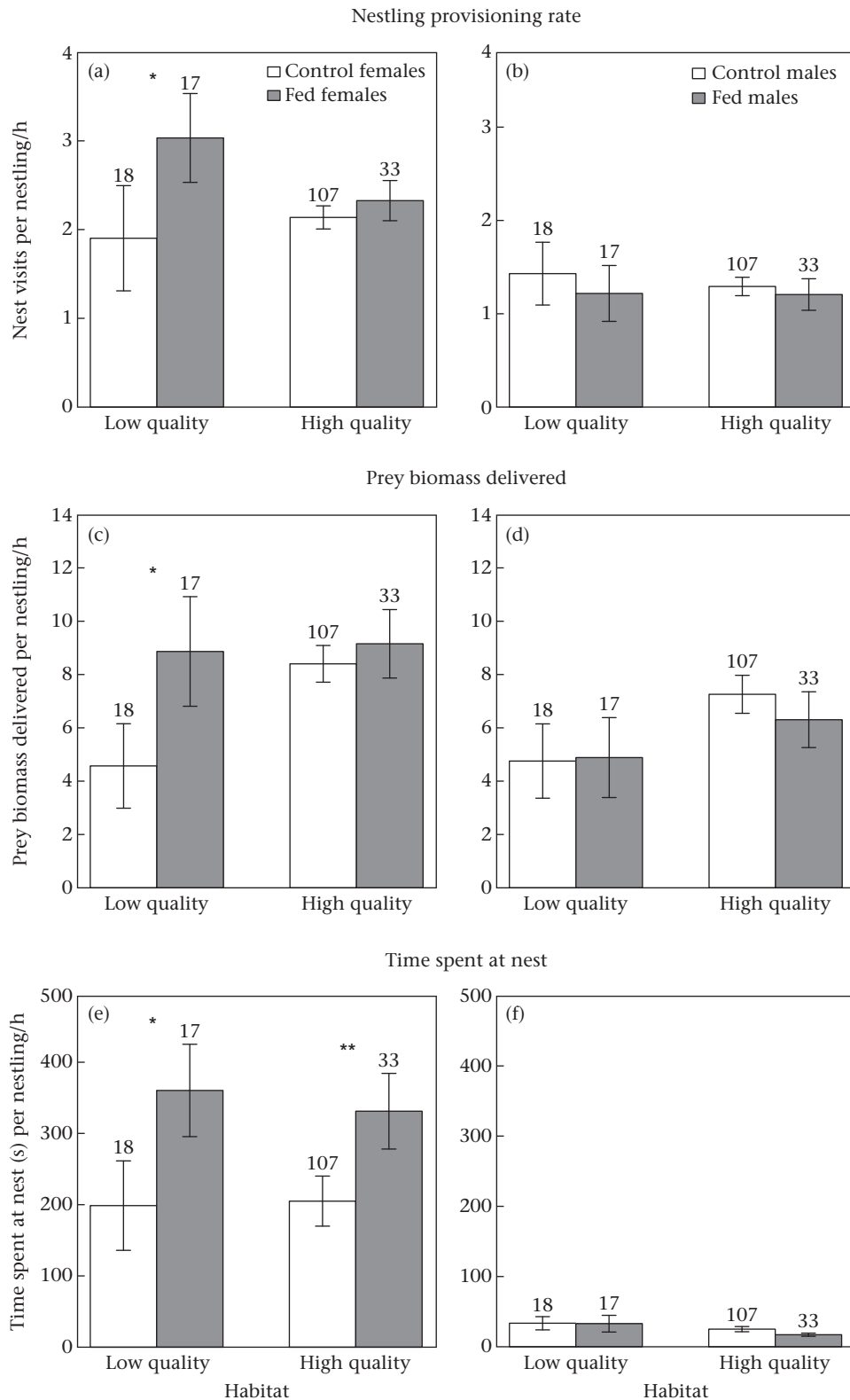
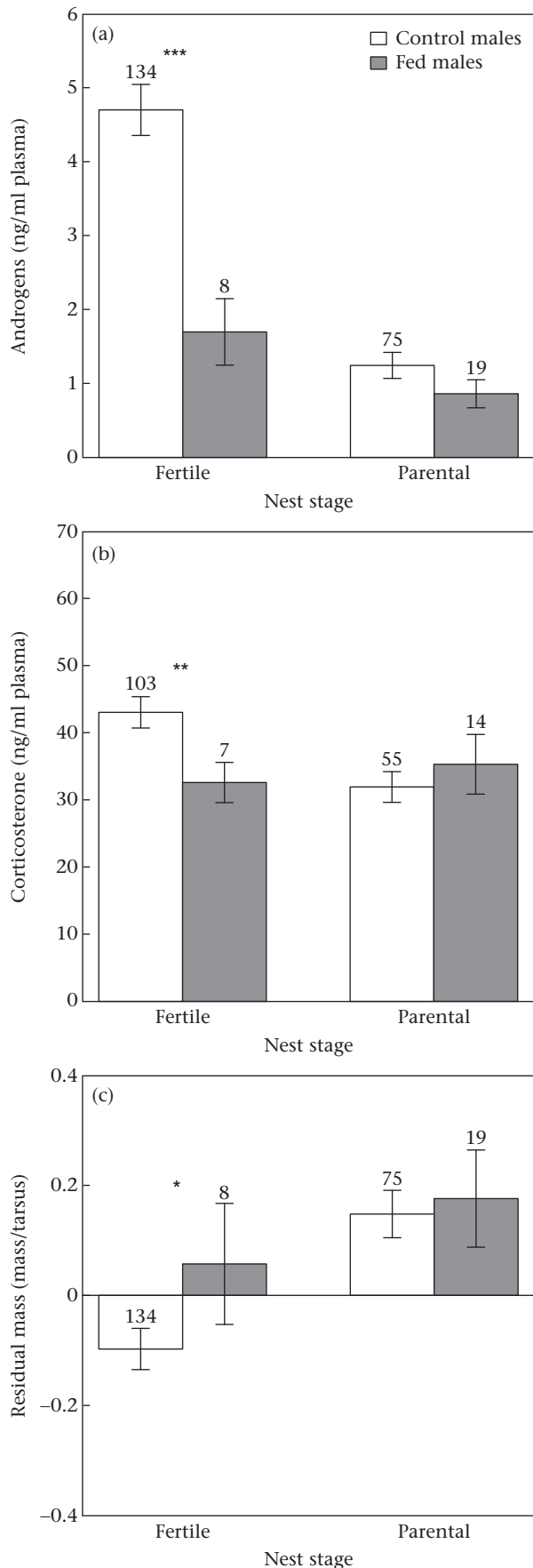


Figure 2. Effect of food supplementation on (a, b) nestling provisioning rate, (c, d) prey biomass delivered to nestlings and (e, f) time spent at the nest by female (left panel) and male (right panel) black-throated blue warblers breeding in low- and high-quality habitat. Data are shown as means \pm SE and sample sizes are given above the error bars. * $P < 0.05$; ** $P < 0.01$ (see [Supplementary Table S2](#)).

However, we found substantial within-male variation in androgen concentrations, and two of the 12 males sampled during both the fertile and parental stages had higher androgen concentrations during the parental stage (Fig. 4).

Initial CORT concentration in males was associated with residual body mass, but not with mating effort or parental effort. Males with high initial CORT concentrations had lower residual mass than males with low initial CORT levels (LMM: $\beta = -0.28 \pm 0.10$, Wald



$\chi^2_1 = 8.11, P = 0.004$). We found no relationship between initial CORT concentrations and song rate (LM: $\beta = 0.18 \pm 0.12, F_{1,62} = 2.14, P = 0.15$) or male provisioning rate (LM: $\beta = -0.25 \pm 0.38, F_{1,32} = 0.44, P = 0.51$).

Indirect evidence supports the association between initial CORT levels, residual body mass and mating effort. Mean initial CORT levels were significantly higher in the fertile stage (when song rate was higher and residual mass lower) than in the parental stage (fertile: 42.35 ± 2.21 ng/ml, $N = 110$; parental: 32.56 ± 2.03 ng/ml, $N = 69$; [Supplementary Table S1](#)). However, males sampled during both the fertile and parental stages did not show a significant change in initial CORT concentrations across these stages (paired t test: $t_{10} = -0.82, P = 0.43$). Lastly, plasma androgen and initial CORT concentrations were positively associated within males (LMM: $\beta = 0.44 \pm 0.18, \text{Wald } \chi^2_1 = 6.39, P = 0.012$), suggesting that the increased energetic demand of testosterone-regulated behaviours was met with increased initial CORT concentrations.

DISCUSSION

Black-throated blue warblers breeding in northern hardwood forest showed plastic patterns of covariation among endocrine parameters and mating behaviours, but not parental behaviours, in response to food supplementation, particularly in habitats with naturally low levels of food abundance. These responses enabled individuals to adjust their reproductive effort rapidly to changes in resource conditions that limit reproduction, although these adjustments were not necessarily in the directions we predicted. In low-quality habitat, fed males showed decreased singing rates, probably because they increased their mate-guarding effort over pursuing extrapair mating while their social mate was fertile (see below). Our results suggest that increased mate-guarding effort by fed males was facilitated by increased energetic reserves and was reflected in plasma androgen and CORT levels. Fed males did not adjust their parental effort, probably because their social mates increased parental activity at the nest. Increased female parental effort possibly allowed fed males to invest supplemental resources into other activities, such as territorial defence and self-maintenance during the parental stage. Furthermore, because the neuroendocrine system may mediate reproductive trade-offs, we examined individual variation in plasma hormones and their relationships to mating and parental behaviours to understand how responses to environmental factors may be mechanistically linked. Elevated plasma androgens and initial CORT levels were associated with lower residual mass, and high peripheral concentrations of androgens were correlated with higher song rate, but did not appear to interfere with male parental care. Our findings link environmental stimuli affected by changes in resource conditions to the endocrine mechanisms that mediate reproductive effort in a migratory songbird.

Behavioural and Hormonal Responses to Food Supplementation

Song rate, which differed by treatment and habitat, was associated with higher androgen concentrations and may have reflected increased investment in extrapair mate attraction. Supplemental feeding only affected male song rates and androgen concentrations in low-quality habitat. However, our recorders detected a lower,

Figure 3. Effect of food supplementation on male (a) plasma androgen concentrations, (b) initial corticosterone concentrations and (c) residual mass by nest stage for black-throated blue warblers. Data are shown as untransformed means \pm SE and sample sizes are given above the error bars. Significance levels are from linear mixed models ([Table 1](#)) controlling for fixed and random effects: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 1

Results from linear mixed models testing the effects of treatment and habitat quality on plasma androgen and corticosterone concentrations of breeding male black-throated blue warblers, Hubbard Brook Experimental Forest, NH, U.S.A.

Model term ^a	Coefficient	Wald χ^2	df	P
Model (androgen) N=225				
Intercept	1.12±0.11			
Treatment (fed)	-0.75±0.47	15.04	1	<0.001
Treatment*nest stage		83.64	2	<0.001
Control*parental stage	-1.40±0.16			
Fed*parental stage	-0.80±0.45			
Treatment*habitat		6.77	2	0.034
Control*low quality	-0.45±0.18			
Fed*low quality	-0.28±0.41			
Model (corticosterone) N=174				
Intercept	3.56±0.12			
Treatment (fed)	0.03±0.25	0.01	1	0.96
Treatment*nest stage		8.00	2	0.018
Control*parental stage	-0.26±0.09			
Fed*parental stage	-0.02±0.25			
Treatment*habitat		1.83	2	0.40
Control*low quality	0.11±0.10			
Fed*low quality	-0.19±0.23			
Year (2009)		17.54	3	<0.001
2010	-0.20±0.12			
2011	0.24±0.13			
2012	0.08±0.14			

Significant values are shown in bold.

^a Each model included individual identity as a random effect.

rather than higher, song rate for fed males in low-quality habitat. Moreover, these males had lower plasma androgen levels during their mates' fertile periods than did control males.

Several lines of evidence suggest that the reduced song rates of fed males in low-quality habitat were due to these males increasing their investment in mate guarding, at the expense of extrapair mating effort, during their social mate's fertile stage. First, females spend their time and energy foraging during the fertile stage to support egg production, and therefore only visit nest sites to lay

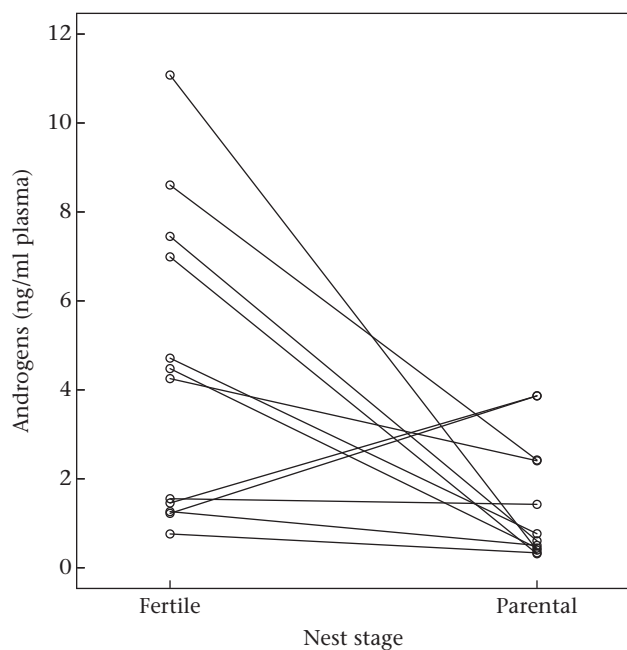


Figure 4. Within-male variation in endogenous androgen concentrations for 12 male black-throated blue warblers from the control group sampled during their social mate's fertile stage and the parental stage. Paired androgen concentrations of each male are connected by a solid line.

eggs (Holmes et al., 2005). Second, males typically guard their social mates closely during this time to prevent loss of paternity (Chuang-Dobbs, Webster, & Holmes, 2001) and, accordingly, guarding males also spend little time near the nest. Third, territories in low-quality habitat were, on average, 30% larger than territories in high-quality habitat (Cline et al., 2013). Black-throated blue warbler males typically sing multiple times per hour when their mates are fertile (Sillett, Rodenhouse, & Holmes, 2004). Nevertheless, our song recordings from low-quality habitat included sampling periods when no songs were detected, suggesting that these males were outside the 60 m detection radius of our digital recorders. Finally, fed males in low-quality habitat were both less likely to lose paternity and less likely to sire extrapair young than were control males (Kaiser, 2013).

Fed males were better able to meet their energetic needs during the fertile stage relative to control males, as indicated by their lower initial CORT concentrations and higher residual body mass. These results suggest that fed males were also able to spend additional resources on self-maintenance behaviours, such as foraging, possibly while mate guarding. Considerable evidence has linked reduced energetic condition and reduced food availability to elevated baseline CORT level, but less is known about the role of reproductive hormones in this context (reviewed in Schoech et al., 2009). CORT mobilizes glucose stores to meet energetic challenges, and baseline levels in breeding males are typically elevated to meet the energetic demands of testosterone-regulated reproductive behaviour (Romero, 2002). For this reason, CORT and T can be positively correlated (e.g. Van Hout, Eens, Darras, & Pinxten, 2010), the relationship we observed. Elevated CORT may be adaptive to increase immediate availability of energy, but prolonged or highly elevated CORT due to adverse conditions and low resource availability may be maladaptive and interfere with reproduction (Lynn, Breuner, & Wingfield, 2003; Schoech et al., 2007; Wingfield, 1985). Hence, individuals that maintain lower baseline CORT concentrations and more energetic reserves during the fertile stage may be better able to guard their social mates or defend their territories.

In addition to their low initial CORT concentrations and high residual body mass prior to the parental stage, fed males with nestlings may have been able to spend more time and energy in nonparental activities, such as territorial defence or their own self-maintenance, because their social mates invested more into parental effort relative to controls. Lozano and Lemon (1995) found a similar pattern with the congeneric yellow warbler, *Setophaga petechia*: females with supplemental food visited their nests more frequently than control females, but the amount of parental care provided by males was unaffected by access to additional food. Predicted higher baseline CORT levels during the parental stage for food-supplemented black-throated blue warblers would have reflected greater parental investment by males with increased resources (Bonier et al., 2009). For example, male house sparrows, *Passer domesticus*, that raise more offspring (higher reproductive effort) in a season have higher baseline CORT concentrations (Ouyang, Sharp, Dawson, Quetting, & Hau, 2011). Our results do not support this alternate parental investment hypothesis because we did not detect any effects of supplemental feeding on male parental effort or initial CORT levels during the parental stage.

Overall, our findings show that both sexes adjusted their reproductive behaviour rapidly in response to food supplementation in habitat with low food abundance, and male behavioural responses covaried with changes in peripheral hormone concentrations. Fed females increased parental effort, whereas fed males appeared to increase mating effort via mate guarding during the fertile stage and possibly defending their territories or investing in their own self-maintenance during the parental stage.

Hormones and the Mediation of Male Reproductive Effort

We found only indirect support for the role of plasma androgens in mediating male mating effort and parental effort. Most males had higher androgen concentrations during their social mate's fertile stage compared to the parental stage. Male provisioning rate, but not song rate, was independent of measured androgen concentrations. Plasma androgens, therefore, did not appear to mediate the potential trade-off males face between extrapair mate attraction and male parental effort in the black-throated blue warbler. Several songbird studies have also shown that endogenous androgens are not correlated with male feeding rates and that androgen levels can be highest in the parental stage (Eikenaar et al., 2011; McGlothlin et al., 2007; Pinxten, de Ridder, Arckens, Darras, & Eens, 2007). The substantial interindividual variation in hormone profiles observed in these studies and ours indicate that different males may have different reproductive strategies, such as increased T-regulated song output, by some males but not others, to attract extrapair mates during the parental stage. These findings demonstrate the importance of examining individual variation in plasma hormones and their relationships to mating and parental behaviours to understand the proximate role of hormones in reproductive trade-offs (Eikenaar et al., 2011; Horton, Yoon, Ghalambor, Moore, & Scott Sillett, 2010; McGlothlin et al., 2007).

Initial CORT concentrations did not appear to regulate parental effort by male black-throated blue warblers. Male provisioning rates were independent of initial CORT concentrations, although CORT levels were lower in the parental stage, when energetic demands on males are likely to be high relative to the fertile stage. It is possible that we found no relationship between initial CORT levels and provisioning rate because our initial CORT levels did not reflect true baseline levels (see *Methods*). To obtain these baseline levels might require noninvasive techniques for sampling CORT. However, other CORT measures, such as acute stress-induced CORT concentrations, or baseline measures just prior to the parental stage may better predict parental effort (Angelier & Chastel, 2009; Love et al., 2014; Ouyang et al., 2011). Males that mount a stronger response to an acute stressor, as indicated by a greater change between baseline and stress-induced CORT concentrations, may be more likely to reduce their parental effort to invest more energy into regaining homeostasis (Lendvai & Chastel, 2010; Miller, Vleck, & Otis, 2009).

Altogether, we found no antagonistic effects of circulating steroid hormones on mating and parental behaviours with our correlative analyses. If these effects had been observed, the increased expression of mating behaviour via higher circulating hormone levels would have suppressed linked parental behaviours. Hormone mediation could be adaptive if it allows individuals to allocate limited resources to competing behaviours appropriate to the environment (Ketterson et al., 2009; McGlothlin & Ketterson, 2008). However, this type of constraint on the hormone signalling mechanism could also prevent adaptive plasticity in reproductive investment in response to environmental change if selection favours novel relationships among hormone-mediated behaviours (Hau, 2007; Wingfield, 2008). Instead, our results are more consistent with an alternative view, that the links between hormones, mating and parental behaviours are plastic. Parental behaviours appear to be independently regulated by other hormones or nonhormonal factors not measured in this study (e.g. stress-induced CORT, oestradiol, prolactin, steroid receptor cofactors, enzymes, or downstream mechanisms that affect tissue responsiveness) (DeVries & Jawor, 2013; Hau, 2007; Ketterson et al., 2009). This could explain why parental behaviour varied with little to no change in peripheral hormone concentrations. However, understanding the relationship between plasma hormones and parental behaviour will require hormone manipulation. If selection acts on different components of

the hormone-signalling cascade from hormone synthesis to tissue responsiveness, this should permit rapid, adaptive behavioural response to changing environmental pressures (Adkins-Regan, 2008; Ketterson et al., 2009; Wingfield, 2008). Future studies that focus on how these downstream mechanisms respond to environmental stimuli will be especially valuable in understanding the evolutionary potential of the neuroendocrine system underlying adaptive behavioural responses.

Conclusions

Experimentally reducing food limitation increased male mating effort and female parental effort for black-throated blue warblers breeding under poor resource conditions and also revealed that male behavioural responses to food availability covaried with endocrine responses. Peripheral concentrations of androgens appear to be involved in regulating the expression of male mating behaviour, but not parental behaviour. Thus, our findings suggest that adaptive reproductive investment in response to changes in resource conditions are unlikely to be constrained by peripheral concentrations of hormones that mediate competing reproductive behaviours. Manipulations of both food availability and hormone concentrations will be an important next step to test how interactions between the environment and the neuroendocrine system shape reproductive effort. Furthermore, studies of natural systems that can be manipulated to reduce (rather than increase) food availability would provide insight into the responsiveness and resilience of breeding individuals to the effects of environmental change that degrade habitats (Wingfield, 2013). Integrating studies of environmental endocrinology and ecology remains an important way forward to determine how organisms will adjust their behaviour and physiology to cope with environmental change.

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Supplementary Material

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