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ORIGINAL ARTICLE

No evidence of sex ratio manipulation by black-throated blue warblers in response to food availability

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Abstract
Sex allocation theory predicts that females should bias their offspring sex ratios when the fitness benefits of producing sons or daughters differ depending on rearing environment. The Trivers-Willard hypothesis proposes that whether females produce more sons or daughters depends on food availability via both intrinsic maternal condition and differing reproductive potential (typically from mating system structure) for sons versus daughters. However, tests of its key predictions are often based on untested, implicit assumptions that are difficult to quantify, especially in migratory animals. In a 5-year study, we manipulated food availability in low- and high-elevation forest to test the Trivers-Willard hypothesis in the migratory black-throated blue warbler (*Setophaga caerulescens*). We found that the population-wide offspring sex ratio was significantly male-biased (population mean: 0.58), which was driven by an overproduction of sons in high-elevation forest (high-quality habitat mean: 0.59). Yet, we found no effect of food availability on offspring sex ratio from either natural variation or supplemental feeding. Sex-specific developmental costs did not differ for sons and daughters reared under low and high food availability. These results suggest that female black-throated blue warblers do not manipulate offspring sex ratios in response to food availability and are not consistent with the predictions of the Trivers-Willard hypothesis. This study highlights challenges of examining mechanisms driving patterns in offspring sex allocation in migratory species for which both the costs of rearing and relative fitness benefits of sons and daughters cannot be tracked into adulthood.

Significance
Birds can optimize their fitness return on parental investment by biasing offspring sex ratios. When the costs and benefits of raising sons or daughters differ under low and high food availability, females could produce either more sons or daughters depending on those conditions. Black-throated blue warbler offspring sex ratios were male biased in high quality habitat. However, we found no evidence that food-supplemented females or females on territories with higher caterpillar abundance produced more sons than daughters as predicted by the Trivers-Willard hypothesis. The relative costs of producing sons or daughters did not differ under low and high food availability, but we could not directly examine differences in relative benefits. Findings indicate that this migratory bird does not adjust sex ratios in response to food availability and highlights the need for evaluating future fitness benefits of sons and daughters in migratory species.

Keywords Food supplementation · Offspring sex allocation · *Setophaga caerulescens* · Sex ratio bias · Trivers-Willard hypothesis

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Introduction

Sex allocation theory predicts that females should bias their offspring sex ratios to maximize their own fitness when rearing sons and daughters yields different net fitness benefits (Fisher 1930; Trivers and Willard 1973; Charnov 1982; Frank 1990). From the female's perspective, the return on their reproductive investment for raising sons versus daughters is expected to differ if sons and daughters (1) cost different resources (i.e., amount and quality of food) to successfully rear and (2) provide differential benefits to the female through both direct and indirect benefits. These trade-offs of raising sons versus daughters can be driven by intrinsic (e.g., maternal condition) and extrinsic (e.g., food availability) factors that influence sex ratio bias through their effects on parental investment (i.e., foraging success of female, provisioning effort) and the rearing environment (i.e., food availability and diet quality) (Frank 1990; West 2009; Navara 2018). Studies have found support for the effect of maternal condition and parental provisioning on offspring sex allocation (Love et al. 2005; Nooker et al. 2005; Baeta et al. 2012; Merkling et al. 2012), often as an indirect proxy for food availability (Nager et al. 1999; Hasselquist and Kempenaers 2002). Because maternal condition and parental provisioning are associated with food availability and diet quality, whether females are responding to their own physiological condition or, alternatively, to the quality of the rearing environment for their offspring is unclear (Komdeur et al. 1997; Nager et al. 1999; Cockburn et al. 2002; Ewen et al. 2004; Pryke and Rollins 2012).

The Trivers-Willard hypothesis (Trivers and Willard 1973) has been proposed, and extensively tested, as a framework for how food availability might influence offspring sex ratio bias in sexually dimorphic species (Table 1). Fisher's classic sex allocation work theorized that the evolutionary stable strategy is to invest equally in the production of sons and daughters, which would maintain a 1:1 sex ratio if sons and daughters were equally costly to raise to independence (Fisher 1930). Thus, Trivers and Willard hypothesized that females in better condition (e.g., from areas with abundant food or high-quality diets) should produce more of the sex that yields the greatest future fitness return from concomitant increases in parental investment, which increases females' indirect fitness benefits (i.e., more grandoffspring).

However, the Trivers-Willard hypothesis hinges on several implicit, often untested, assumptions (Table 1). Briefly, the assumptions are: (1) high food availability in the environment translates to high quality females on those territories, (2) females in good condition and/or with access to high food availability increase their parental investment, (3) increased female parental investment yields offspring in good condition, (4) an individuals' condition during

Table 1 An experimental framework for testing the Trivers-Willard hypothesis and associated predictions for this study and similar species

Hypothesis	Assumptions	Predictions	Variables Measured (this study)
Trivers-Willard Hypothesis: (1973) Females in better condition will produce more of the sex that yields the greatest fitness benefit under different resource conditions	1. High food availability is associated with females in good condition 2. Females in good condition and/or with high food availability increase their parental investment 3. Increased female parental investment results in offspring in good condition 4. Offspring condition is correlated with later condition as adults 5. Parental investment affects the future reproductive performance of sons and daughters differently	1. Females produce male-biased sex ratios under abundant resource conditions 2. Females produce female-biased sex ratios under poor resource conditions	1. Habitat quality (low- and high-quality habitat at low and high elevations) 2. Natural variation in food availability (caterpillar density) 3. Experimental food supplementation 4. Maternal condition (scaled mass index)

development is correlated with their condition as an adult, and (5) increased parental investment in offspring differentially impacts the future reproductive benefits for sons versus daughters in mating systems with sex-specific differences in reproductive potential (e.g., polygynous systems). These assumptions broadly translate to the expectation that differences in offspring condition from increased parental investment carry-over into adulthood. In socially and genetically polygynous species, sons in good condition from increased parental investment are expected to have higher survival and future reproductive performance as adults compared to sisters in similar condition (Lindström 1999; Shuster and Wade 2003). For example, adult males in good condition often acquire more extra-pair fertilizations than males in poor condition (McElligott et al. 2001; Hill 2002; Holzer 2003; McGraw and Ardia 2003). This condition-dependent difference can lead to high variance in male reproductive success, whereas female reproductive success is generally less variable (Clutton-Brock 1988; Lukas and Clutton-Brock 2014). Thus, for polygynous systems, increases in offspring condition from greater parental investment is expected to disproportionately affect the future reproductive success of sons relative to daughters under high food availability. Conversely, because female reproductive success is generally less variable than that of males, daughters are expected to have higher fitness than their brothers when reared under low food availability. Accordingly, the Trivers-Willard hypothesis predicts females should produce more sons under high food availability and more daughters under low food availability (Trivers and Willard 1973; Cameron and Linklater 2002). However, few studies exploring the Trivers-Willard hypothesis as a mechanism driving patterns of sex ratio manipulation have robustly examined the implicit assumptions of the Trivers-Willard hypothesis before testing the hypothesis.

In this study, we use a combination of correlational and experimental approaches to test the key predictions of the Trivers-Willard hypothesis in a wild population of migratory black-throated blue warblers (*Setophaga caerulescens*). We assessed the effects of food availability, maternal condition, parental provisioning, and their potential relationships on offspring sex ratio. Birds are an ideal system to test the Trivers-Willard hypothesis because females, the heterogametic sex, could bias sex ratios by controlling sex chromosomes in the ovum (Pike and Petrie 2003). In *ZW* sex-determination systems, such as birds, the ovum, not the sperm, determines the sex of the offspring. While a precise mechanism for how females adjust offspring sex ratios is uncertain (Komdeur and Pen 2002; Pike and Petrie 2003; Cameron 2004; Alonso-Alvarez 2006; Rutkowska and Badyaev 2008), a few compelling studies have demonstrated adaptive offspring sex ratio adjustment in *ZW* systems in response to aspects of the rearing environment (e.g., snakes: Madsen and Shine 1992, amphibians: Sakisaka et al. 2000, birds: Heinsohn et al. 1997; Komdeur et al. 1997; Bradbury and Blakey 1998; Hasselquist and Kempenaers 2002). Additionally, black-throated blue warbler males have high reproductive variance from extra-pair mating and double brooding (Kaiser et al. 2015, 2017; Germain et al. 2021), making them a suitable system to explore the Trivers-Willard hypothesis. Furthermore, our marked breeding population and robust measures of natural food availability enable us to experimentally disentangle the effects of food availability from proxies of food availability (e.g., maternal condition, parental provisioning) in a wild system.

Testing hypotheses on biased offspring sex ratios has been challenging because their key predictions are often based on untested assumptions that are difficult to quantify (Navara 2018). We first explored as many of the implicit assumptions of the Trivers-Willard hypothesis as feasible in our system (assumptions 1–3 described above; Table 1), to evaluate whether they were met. We previously showed that food-supplemented females increase their parental investment (assumption 2 was met; Kaiser et al. 2014). However, we could not also evaluate whether females in good condition increase their parental investment because females were not reliably captured at the nest during the nestling period to measure condition while they provisioned offspring. We could not evaluate assumptions 4 and 5 because black-throated blue warblers are migratory and we are currently unable to track individuals from chick to adulthood to assess their change in condition and the effects of parental investment on reproductive performance because of high natal dispersal (recapture rate of chicks: <1%), which is a common limitation in studies of migratory animals.

After testing for the assumptions of the Trivers-Willard hypothesis, we examined the effects of natural variation in food availability on offspring sex ratio by estimating food availability within each territory (caterpillar density), which increases from low- to high-elevation forest (Rodenhouse et al. 2003; Cline et al. 2013). We also conducted a food supplementation experiment in low- and high-elevation forest to isolate the effects of food availability. Our previous work in this system showed that food-supplemented females provisioned their offspring more than control females at food-limited low elevations, indicating that females can adaptively respond to short-term changes in food resources that affect offspring rearing conditions (Kaiser et al. 2014). Moreover, food-supplemented males were in better body condition and had higher reproductive success (from extra-pair mating and double brooding) than control males, which led to greater variance in male reproductive success within the population (Kaiser et al. 2015, 2017). Sons might benefit from supplemental feeding if conditions during development ultimately influences a male's ability to obtain a high-quality territory and to attract social mates and extra-pair mates as a breeding adult. Thus, females provided supplemental food are predicted to produce more sons than daughters (supporting the Trivers-Willard hypothesis). Lastly, although adult male and female black-throated blue warblers do not differ in size (Holmes et al. 2020), we examined differences in the costs of rearing sons or daughters by comparing pre-fledging nestling mass among siblings and whether potential sex differences depended on food availability. If the costs and benefits of producing sons and daughters do not differ in this monomorphic species, females are predicted to produce equal sex ratios (supporting Fisher's hypothesis). For clarity, we use "females" to refer to adult breeding females and "daughters" to refer to their female offspring.

Methods

Study population

Black-throated blue warblers (*Setophaga caerulescens*) breed in mature, northern hardwood forests throughout eastern North America and migrate to the Greater Antilles for the non-breeding season (Holmes et al. 2020). We studied a marked population of black-throated blue warblers at the 3160 ha Hubbard Brook Experimental Forest in North Woodstock, New Hampshire, U.S.A. (43°56'N, 71°45'W). We collected data over five breeding seasons (May–August, 2007, 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) elevation forest (Rodenhouse et al. 2003). This species is sexually dichromatic but adults do not vary significantly in body size or mass (Holmes et al. 2020). Males establish and defend combined breeding and foraging territories in forested habitat with relatively dense understory vegetation. Females construct open-cup nests < 0.5 m high in understory vegetation and lay one egg per day (mean clutch size = 3.6, range = 2–5 eggs) (Holmes et al. 2020). Females incubate clutches without male assistance for approximately 12 days, and both sexes feed nestlings for approximately 9 days until fledging (Holmes et al. 2020). At Hubbard Brook, 30% of black-throated blue warbler pairs attempt second broods (i.e., double brood) when food resource conditions are favorable (Nagy and Holmes 2005; Townsend et al. 2013). Pairs are socially monogamous, with a small proportion of bigamous males, but extra-pair paternity rates are relatively high (56%) and decrease with food availability as males defending food-abundant territories invest more effort into mate guarding to maintain within-pair paternity (often within two broods) over pursuing extra-pair mates (Kaiser et al. 2015, 2017). However, the male mating strategy with the highest annual fitness return in this population includes fledging two broods containing within-pair young as well as siring extra-pair young (Kaiser et al. 2015). Food availability therefore affects both female and male fecundity by influencing double brooding and leads to greater variance in male reproductive success via within-pair and extra-pair paternity (Nagy and Holmes 2005; Kaiser et al. 2015, 2017; Germain et al. 2021). Additionally, annual survival probability is higher for males (0.51) than for females (0.40) (Sillett and Holmes 2002) and is higher for all adults in years with more abundant food (Sillett et al. 2000).

In each breeding season, we captured and marked adults and nestlings, mapped male territories, monitored nest attempts, and measured nestling provisioning rates. We captured adults using mist nets and marked individuals with a unique combination of three colored leg bands and one aluminum U.S. Geological Survey leg band. We determined the relative age of each adult as a yearling (second year, SY) or older adult (after-second year, ASY) using plumage characteristics (Holmes et al. 2020). We measured the length of the right tarsus to the nearest 0.01 mm and mass to the nearest 0.1 g. We mapped male territories relative to each plot's 50×50 m grid using the locations of singing males and territorial interactions among nearby conspecifics. Nests were located by following females carrying nest material and adults carrying food and searching the vegetation. We monitored nests every other day throughout all nest stages with daily checks near predicted hatch and fledge dates. We banded 6-day-old nestlings, collected approximately 30 µl of blood from the brachial vein, and measured their right tarsus and mass (as above). Total handling time was limited to 10 min. We returned nestlings to their nests immediately after processing. We used body size measurements to calculate the scaled mass index of females and nestlings (Peig and Green 2009, 2010) using age-specific means (tarsus length [mean ± SE]: adult female = 18.47 ± 0.06, $n = 93$, nestlings = 16.46 ± 0.06, $n = 259$). We stored blood samples in lysis buffer (White and Densmore III 1992) at 4 °C until genetic analyses were conducted. From 2009 to 2012, we measured female and male provisioning rates (visits per hour per nestling) from 2-hr video recordings of nests collected after dawn on day 7 of the nestling stage following previously described methods (Kaiser et al. 2014).

Habitat quality and caterpillar density

Habitat quality for black-throated blue warblers at Hubbard Brook is correlated with the abundance of Lepidoptera larvae, their primary food source, dense understory vegetation, and elevation (Rodenhouse et al. 2003; Cline et al. 2013; Kaiser et al. 2015; Holmes et al. 2020). Natural variation in food availability and reproductive success at the mid-elevation plot are, on average, similar to that on the high-elevation plot, but differ substantially from the low-elevation plot (Rodenhouse et al. 2003; Cline et al. 2013, p. 201; Kaiser et al. 2015); this pattern was true for years included in this study. Therefore, we combined data from mid- and high-elevation plots (hereafter, high-elevation habitat).

We calculated an index of food availability for each territory as a function of estimated caterpillar biomass per leaf, based on visual caterpillar surveys in the shrub layer (2007, 2009–2012), and the estimated abundance of understory leaves within each territory (Holmes et al. 1979; Sillett et al. 2004). We counted and measured caterpillars on 1000 leaves of striped maple (*Acer pensylvanicum*) and 1000 leaves of hobblebush (*Viburnum lantanoides*) (common foraging substrates for black-throated blue warblers) along four plot-wide transect surveys per study plot during four two-week surveys (1 June–31 July). Caterpillar measures were converted to wet biomass (mg) using length-mass regressions (Rogers et al. 1977). To determine the mean caterpillar biomass per leaf of each plant species in each survey period, we divided the average caterpillar biomass across transects (mg), by 1000 leaves. We estimated leaf abundances of each plant species on each territory with the Geospatial Modelling Environment (Beyer 2012) from interpolated surfaces of leaf density derived from understory leaf sampling (0–3 m height) conducted across each gridded study plot (Sillett et al. 2004) and territory boundaries digitized in ArcGIS 10 (ESRI 2011). We multiplied the two per-leaf quantities by leaf abundances within territories and summed each value to obtain an index of food availability for each territory. Additional details on the index of food availability are described in Kaiser et al. (2015). In analyses examining the effects of caterpillar density on offspring sex allocation, we used the estimate from the two-week survey coinciding with the female's fertile stage (i.e., nest building through egg laying) for each nest attempt.

Food supplementation experiment

Each year, following the establishment of breeding pairs, we randomly assigned 6–8 territories from each of the three study plots (low-elevation = low quality; mid- and high-elevation = high quality) to the food supplementation treatment. We monitored 15–20 control territories on each plot, which were separated from food-supplemented territories by at least one territory. We began supplemental feeding on first nest attempts 2–3 days after the onset of incubation and provided food daily throughout all nest stages: incubation, hatching, provisioning, post-fledging period of first broods, and egg laying, incubation, hatching, provisioning, and post-fledging period of second broods. We established feeding trays 1 m from nests and initially provided 5 g (37 kJ) of waxmoth larvae (Lepidoptera: *Galleria mellonella).* Once females were observed feeding from the tray, we moved the tray ~ 5 m from the nest and increased the amount of food provided to 7 g (52 kJ): 5 g of mealworms (Coleoptera: *Tenebrio monitor*) that were gut-loaded with cricket meal (Zilla Gut Load Cricket & Insect Food) to increase protein and calcium content, and 2 g of waxmoth larvae. During the nestling stage, we delivered 14 g (104 kJ) of food (10 g of mealworms and 4 g of waxmoth larvae) because adults regularly provisioned larvae to their young (observed on video recordings of parental provisioning). 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. Additional details on the food supplementation experiment are described in Kaiser et al. (2014).

In analyses examining the effects of food supplementation on offspring sex allocation, we only included nests where females had been provided supplemental food prior to and throughout their fertile stage to assess the effects of food on primary sex ratios. Given our experimental design, wherein supplemental feeding began 2–3 days after the onset of incubation, these nests were necessarily renesting attempts and second broods produced later in the season by pairs provided daily supplemental food at previous nest attempts and/or first broods. To control for possible offspring sex ratio bias due to seasonality, such as variance in natural food availability (Husby et al. 2006; Graham et al. 2011), we determined the genetic sex of nestlings from control broods only if their clutch initiation dates fell within the range of clutch initiation dates of food-supplemented nests (i.e., standardized clutch initiation dates for control and supplementally-fed nests). We did not determine the genetic sex of nestlings from broods initiated prior to this time.

Sexing methods

We determined the genetic sex of nestlings from blood samples obtained a few days prior to their fledging, using samples from complete broods (i.e., all eggs hatched and reached the sampling stage). The primary sex ratio of clutches at egg laying can differ from the secondary sex ratio of broods after hatching due to embryo mortality during incubation or offspring mortality in the nest and during the post-fledging period before offspring reach independence (Pryke and Rollins 2012). Hatching success was high (average hatching rate: 89.5%, $n = 271$ control broods 2007, 2009–2012) and nestling mortality was very uncommon, and we did not monitor mortality during the post-fledging period. Thus, we would expect little difference between the primary and secondary sex ratios unless sex-biased mortality occurs during the post-fledging period. Because we estimate sex ratios of complete broods, we report our estimates as primary sex ratios.

Molecular sexing was based on constant size differences between the CHD1*W* and CHD1*Z* introns and the presence of female-specific fragments (Fridolfsson and Ellegren 1999). We isolated genomic DNA from red blood cells using Qiagen DNeasy blood and tissue extraction kits (Qiagen, Valencia, CA). Offspring sex was determined by amplifying 1 μl of genomic DNA from each individual using this sex-linked marker with highly conserved primers, 2550 F and 2718R, flanking the intron in a 10 μl PCR (Fridolfsson and Ellegren 1999). The PCR consisted of 1 μl of DNA, 0.2 μl of 10 mM dNTPs, 0.20 μl of 10 μM forward and reverse primers, 1 μl of 10X PCR buffer (Sigma), 1.2 μl of 25 mM $MgCl_2$, 0.1 μl of 2.5 U μL^{-1} Taq polymerase (Invitrogen), and ddH_2O to bring the total volume to 10 μl. We ran the PCR under the following conditions: 3 min denaturation at 94° C, 35 cycles of 94° C for 30 s, 57° C for 30 s, and 72° C for 40 s. We visualized PCR products using gel electrophoresis on a 2% agarose gel stained with ethidium bromide. Both sexes carry the CHD1*Z* gene, but only females carry the CHD1*W* gene. Therefore, individuals with two bands were female (the heterogametic sex) and those with one band were male. To verify the accuracy of our methods, we assayed adult birds of known sex based on plumage characteristics ($n = 12$) and ran samples in duplicate.

Statistical analyses

Tests of the assumptions of the Trivers-Willard hypothesis

We first tested whether assumptions 1 and 3 (Table 1) of the Trivers-Willard hypothesis were met in this system; assumption 2 was met in a previous study (Kaiser et al. 2014). We examined the associations (control broods only) between (1) log caterpillar density and female scaled mass [Assumption 1: high food availability is associated with females in good condition, Table 1; $n = 50$] and (2) female provisioning rate and mean mass of nestlings in a brood [Assumption 3: increased female parental investment results in offspring in good condition, Table 1; $n = 42$] using linear models (LM). These data did not include females sampled more than once within or across years. All statistical analyses were conducted in the R statistical environment v.4.2.1 (R Core Team 2023). To minimize observer bias, blinded methods were used when all behavioral data were analyzed in association with the food supplementation experiment.

Population-wide offspring sex ratio bias

To investigate the possibility of offspring sex ratio bias at the population level, and considering separately both low- and high-quality habitats, we used the Neuhäuser test to compare the deviation of offspring sex ratio from expected 1:1 based on the within-brood differences between the proportions of sons and daughters (Neuhäuser 2004). The Neuhäuser test accounts for lack of independence among nestlings in a brood when testing for differences in sex ratios and is more robust (i.e., protects against type I error inflation) than alternatives (e.g., binomial test, Wilcoxon signed rank test) for quantifying bias in primary sex ratios when brood sizes vary (Neuhäuser 2004). We restricted our analyses to include only complete broods ($n = 79$).

Effects of food availability and parental provisioning on offspring sex ratio

We examined factors predicted to influence offspring sex allocation by constructing general linear models (GLM) with a binomial error distribution and logit link function using *lme4* (Bates et al. 2015). In each model, the response variable was a vector of the male and female offspring in each brood, which accounts for variation in brood size. In the model examining the association between per-territory food availability and offspring sex ratio (control broods only; $n = 64$), we included log caterpillar density, plot elevation (low elevation, high elevation), and female age class (yearling, older female). We tested for an interaction between log caterpillar density and plot elevation to examine whether the effect of food availability differed between low- and high-elevation habitat. Log caterpillar density was standardized ($\mu = 0$, SD $= 1$) to improve model convergence. We examined whether provisioning rates by females and their social mate differed based on the proportion of sons in the brood using linear mixed models (LMMs) for each parental sex separately and included parental identity as a random effect because both females and males were represented more than once in the dataset (females: $n = 42$, males: $n = 43$).

In the model testing the effects of food supplementation on offspring sex ratio, we included treatment (food-supplemented, control) as the primary factor of interest and accounted for potential effects of plot elevation, female age class, and female scaled mass ($n = 79$). We tested for an interaction between female scaled mass and treatment to examine whether maternal condition differed between treatments. We calculated the 95% confidence intervals for each fixed effect in GLMs as a measure of effect size. For an effect that is not significant (p value above 0.05 and 95% confidence interval spans zero), the breadth of the confidence interval indicates the likelihood of the effect size being very small (Colegrave 2003). Brood size did not differ significantly between treatments (Welch's t-test [mean ± SE]: t = -0.14, df = 25.52, P = 0.89; control = 3.6 ± 0.1 [n = 64], fed = 3.6 ± 0.1 [n = 15]), plot elevation (Welch's t-test: t = 0.13, df = 20.21, P = 0.90; low elevation = 3.6 ± 0.2 [n = 16], high elevation = 3.6 ± 0.1 [n = 63], or female age classes (Welch's t-test: t = -0.71, df = 56.22, P = 0.48; yearling = 3.6 ± 0.1 [n = 50]), older female = 3.5 ± 0.1 [n = 29]). Therefore, brood size should not bias results examining the influence of these fixed effects on offspring sex ratio.

Effect of food availability on pre-fledging nestling mass of sons and daughters

Lastly, we investigated whether daughters are costlier to rear than sons and whether potential differences depended on food availability. We used LMMs to examine whether scaled mass of 6-day-old nestlings (pre-fledging nestling mass) differed by nestling sex and food availability with nest identity included as a random effect. In the model examining the effect of per-territory food availability on scaled pre-fledging nestling mass (n = 176), we included as fixed effects: log caterpillar density, nestling sex, and plot elevation. We tested for interactions between (1) nestling sex and log caterpillar density to examine whether the effects of food availability differed between male and female nestlings, (2) nestling sex and plot elevation to examine whether the scaled pre-fledging mass of male and female nestlings differed between low- and high-elevation habitat, and (3) log caterpillar density and plot elevation. Log caterpillar density was standardized (μ = 0, SD = 1). In the model examining the effect of food supplementation on scaled pre-fledging nestling mass (n = 224), we included as fixed effects: nestling sex, treatment, and plot elevation. We tested for interactions between (1) nestling sex and treatment to examine whether the effect of treatment differed between male and female nestlings, (2) nestling sex and plot elevation, and (3) treatment and plot elevation. P-values for LMMs were estimated using *lmerTest* (Kuznetsova et al. 2017). We compared the ratio of variances in the scaled pre-fledging mass of male and female nestlings using an F test (male: n = 168, female: n = 124).

Table 2 Fixed effects from GLM examining the effect of natural variation in food availability on offspring sex ratio (control broods) in black-throated blue warblers at the Hubbard Brook Experimental Forest, NH. Significant P-values are given in bold

Model term [a]	ß ± SE	z	P	95% CI
[Males: Brood size] n = 64 broods				
Intercept	0.06 ± 0.23	0.25	0.80	-0.39, 0.51
Log Caterpillar density [b]	0.11 ± 0.17	0.63	0.53	-0.23, 0.45
Plot elevation (low)	-0.98 ± 0.44	-2.24	**0.02**	-1.88, -0.14
Female age class (yearling)	0.58 ± 0.31	1.91	0.056	-0.01, 1.19
Log Caterpillar density : Plot elevation	-0.56 ± 0.45	-1.26	0.21	-1.48, 0.29

[a] Log Caterpillar density was standardized to have sample mean = 0 and sample variance = 1

Results

Tests of the assumptions of the Trivers-Willard hypothesis

Several of our assumptions of the Trivers-Willard hypothesis were not supported in analyses of control broods (Table 1). Based on a limited sample size, we detected no significant relationship between log caterpillar density and female scaled mass (LM: β = -0.32 ± 0.18, $F_{1,48}$ = 3.13, P = 0.08, n = 50). We detected no significant association between female provisioning rate and mean brood mass (β = 0.05 ± 0.09, $F_{1,40}$ = 0.28, P = 0.60, n = 42).

Population-wide offspring sex ratio bias

We sexed 283 offspring from 79 complete broods; 162 offspring were male. The population-wide offspring sex ratio was significantly male-biased (Neuhäuser test: mean ± SE: 0.575 ± 0.001, z = 2.23, P = 0.026, n = 79 broods). Offspring sex ratios were significantly male-biased in high-elevation forest (0.591 ± 0.001, z = 2.39, P = 0.017, n = 63), but did not differ from parity in low-elevation forest (0.509 ± 0.004, z = 0.13, P = 0.90, n = 16).

Effects of food availability and parental provisioning on offspring sex ratio

Food availability (i.e., log caterpillar density) was not significantly associated with offspring sex ratio (Table 2; Fig. 1a). Offspring sex ratios were more male biased in the broods of older (after-second year) females and in high-elevation forest relative to broods of yearling (second year females) and in low-elevation forest (control broods only). However, we found no significant difference in offspring sex ratios between food-supplemented broods and control broods in either low- or high-elevation forest (Table 3; Fig. 1b). All

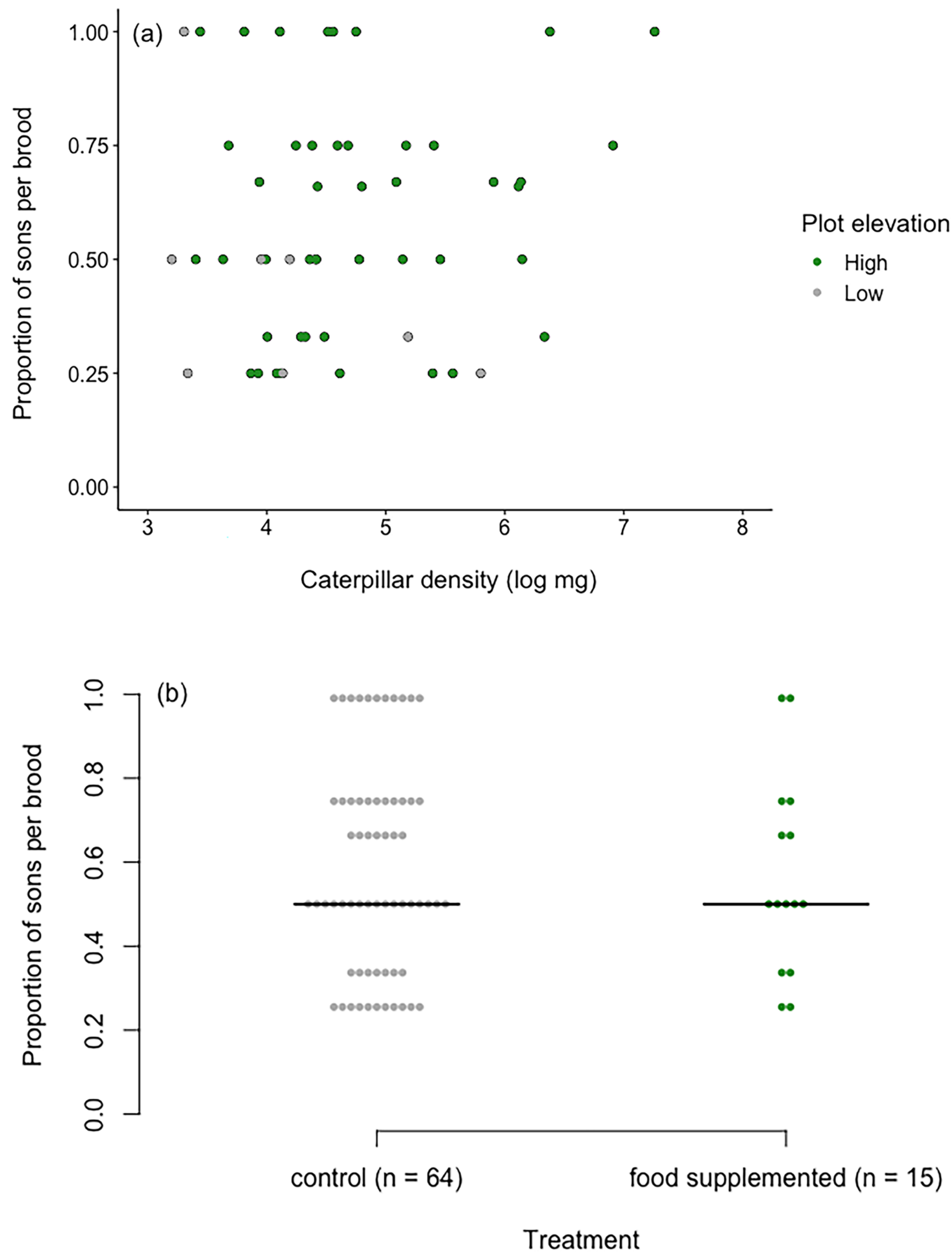

Fig. 1 Offspring sex ratio estimates according to **(a)** per-territory food availability ($P=0.53$, $n=64$ broods) and **(b)** food supplementation (proportion of sons, $P=0.39$, $n=79$ broods) in black-throated blue warblers at the Hubbard Brook Experimental Forest, NH. Points in **(b)** represent the proportion of sons in each brood and lines represent the 50th quartiles

terms had effect sizes near zero and CIs overlapping zero (Fig. 2a).

Given that we did not detect an effect of food supplementation on offspring sex ratio, we assessed post hoc whether our experiment had the statistical power to detect a biologically relevant weak effect using *pwr* (Champely 2020). We compared our effect sizes (beta coefficients) and degrees of freedom (Table 3) to published effect sizes from studies of birds examining the effect of several different social and environmental factors on offspring sex ratio bias (West and Sheldon 2002). Only 3–18% of the variance in offspring sex ratios were explained by ecological conditions in these published studies and the effect sizes were extremely small (West and Sheldon 2002). Thus, type II error rates might be high in many published experimental studies examining the effect of ecological conditions on offspring sex ratios.

Table 3 Fixed effects from GLM examining the effects of food supplementation on offspring sex ratio in black-throated blue warblers at the Hubbard Brook Experimental Forest, NH

Model term [a]	ß ± SE	z	P	95% CI
[Males: Brood size] $n = 79$ broods				
Intercept	0.42 ± 0.25	1.66	0.09	-0.07, 0.92
Treatment (food-supplemented)	-0.27 ± 0.32	-0.85	0.39	-0.90, 0.36
Plot elevation (low)	-0.43 ± 0.33	-1.32	0.19	-1.08, 0.21
Female scaled mass [b]	0.25 ± 0.15	1.64	0.10	-0.05, 0.55
Female age class (yearling)	0.16 ± 0.29	0.57	0.57	-0.40, 0.73
Female scaled mass : Treatment	-0.17 ± 0.33	-0.51	0.61	-0.82, 0.49

[a] Female scaled mass was standardized to have sample mean = 0 and sample variance = 1

However, with our sample size of 79 broods, our experiment had a 95% power to detect a weak effect size. We therefore would have had the statistical power to detect comparatively small effects of food availability on offspring sex ratio bias with our experiment.

We did not detect sex-differential investment in offspring by females or their social mate. We found no statistical difference in parental provisioning rates based on the proportion of sons in a brood (LMM; females: 0.44 ± 0.74, df = 39.97, $t = 0.59$, $P = 0.56$; males: -0.62 ± 0.45, df = 15.84, $t = -1.40$, $P = 0.18$).

Effects of food availability on pre-fledging nestling mass

We did not detect sex-specific costs for sons and daughters reared under either low or high food availability. In the model examining the effect of per-territory food availability on scaled pre-fledging nestling mass, male and female siblings did not differ (LMM: -0.02 ± 0.09, df = 151.11, $t = -0.18$, $P = 0.86$) and variation in scaled pre-fledging mass was not associated with log caterpillar density (0.11 ± 0.11, df = 83.99, $t = 1.04$, $P = 0.30$), plot elevation (0.27 ± 0.28, df = 70.57, $t = 0.98$, $P = 0.33$), or the interactions between nestling sex and log caterpillar density (-0.02 ± 0.10, df = 138.24, $t = -0.26$, $P = 0.80$), nestling sex and plot elevation (0.04 ± 0.27, df = 140.89, $t = 0.14$, $P = 0.89$), or log caterpillar density and plot elevation (0.16 ± 0.16, df = 44.77, $t = 1.02$, $P = 0.31$). In the model examining the effect of food supplementation on scaled pre-fledging mass, male and female siblings did not differ (LMM: 0.003 ± 0.09, df = 193.31, $t = 0.04$, $P = 0.97$) and variation in scaled pre-fledging mass was not associated with treatment (0.12 ± 0.19, df = 105.70, $t = 0.65$, $P = 0.52$), plot elevation (-0.03 ± 0.21, df = 110.74, $t = -0.15$, $P = 0.88$), or the interactions between nestling sex and treatment (-0.15 ± 0.19, df = 182.65, $t = -0.81$, $P = 0.42$), nestling sex and plot elevation (0.09 ± 0.21, df = 185.29, $t = 0.44$, $P = 0.66$), or treatment and plot elevation (-0.47 ± 0.53, df = 65.44, $t = -0.89$, $P = 0.38$). All terms had effect sizes near zero and CIs overlapping zero (Fig. 2b). Moreover, the variance in scaled pre-fledging mass was similar for male and female siblings (F test: $F_{167,123} = 1.17$, $P = 0.36$).

Discussion

The key prediction of the Trivers-Willard hypothesis – that females with access to high food availability should overproduce sons – was not supported in this study of black-throated blue warblers. The population-wide offspring sex ratio was significantly male biased, likely driven by a bias in producing sons at higher elevation (i.e., higher quality habitat). However, we found no detectable effect of food availability (from natural variation or supplemental feeding) on offspring sex ratios. We also found that the relative costs of producing sons or daughters does not appear to differ based on differences in food availability in the rearing environment. Biologically relevant effect sizes associating ecological conditions and offspring sex ratio bias reported in the avian literature are extremely small (West and Sheldon 2002; McNew et al. 2020). However, our sample size would have been sufficient to detect these weak effects. Thus, our findings indicate that black-throated blue warblers do not manipulate offspring sex ratios in response to variation in food availability.

Trivers and Willard (1973) reasoned that a female in good condition should produce higher quality offspring relative to a female in poor condition. Underlying this line of logic are five implicit assumptions (Table 1). Should these assumptions be met, females are predicted to produce more of the sex that receives the greatest increase in reproductive potential from increased parental investment afforded by high food availability and/or good maternal condition (Cockburn et al. 2002). In this study, most assumptions of the Trivers-Willard model were not met or, because black-throated blue warblers are migratory, could not be tested. We did not find an association between food availability and maternal condition (no support for assumption 1), although this was based on a limited sample size. As our index of maternal condition, we chose scaled mass, which has been shown to perform as the best predictor of variation in energy reserves (Peig and Green 2010). Scaled mass is often applied in bird studies to measure body condition, but it might measure only one aspect of overall body condition (Labocha and Hayes 2012; Wilder et al. 2016). Ultimately,

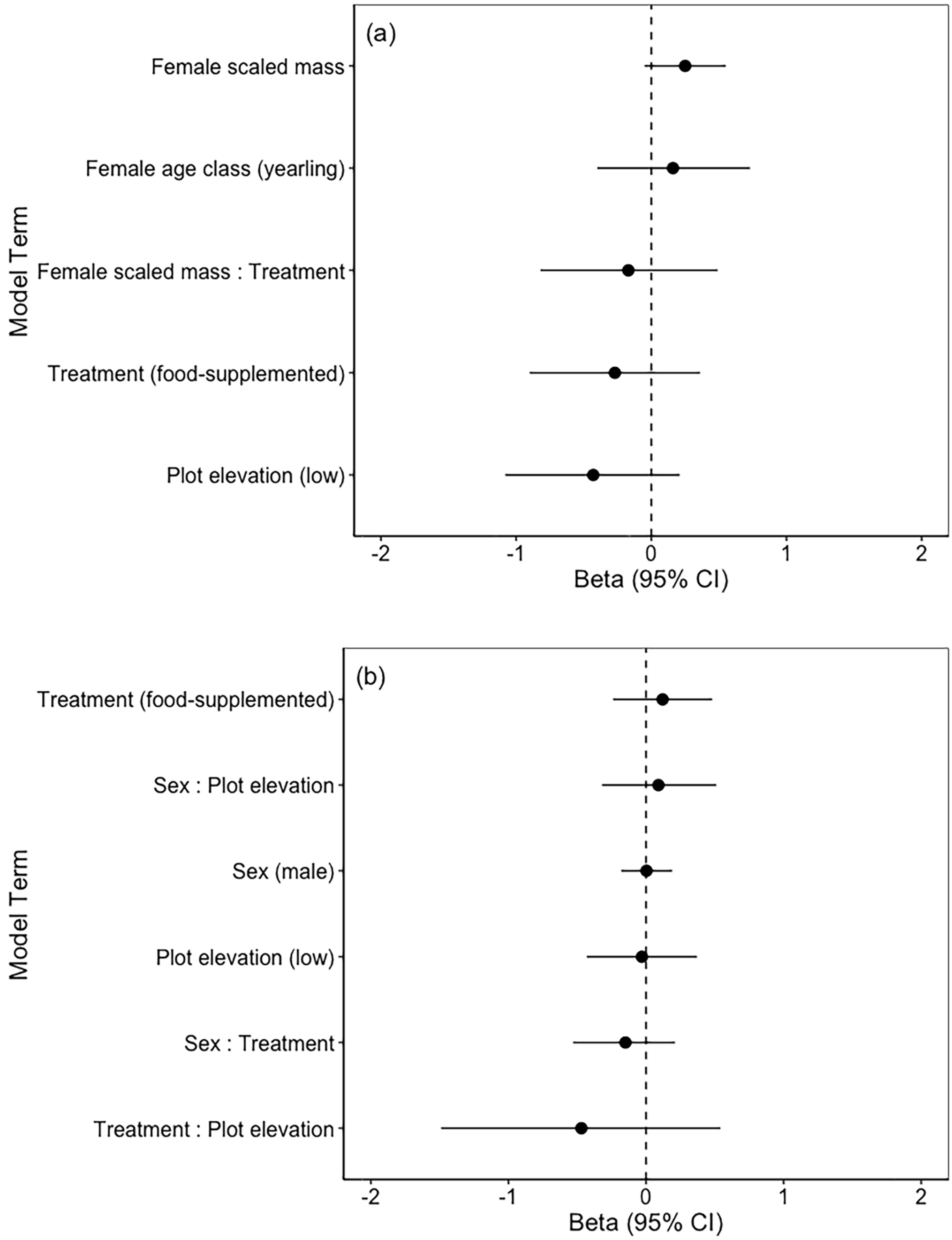

Fig. 2 Effect size plots from models examining the effects of food supplementation on **(a)** offspring sex ratio (GLM, $n=69$ broods) and **(b)** scaled pre-fledging nestling mass (LMM, $n=224$ nestlings from 65 broods) in black-throated blue warblers at the Hubbard Brook Experimental Forest, NH. Points represent variable estimates and whiskers depict 95% CIs. Dotted line represents 0, and variables are considered significant if CIs do not overlap zero

this measure of female condition should be highly variable and translate into the ability to respond adaptively to environmental conditions (Navara 2018). We previously showed that females increase their provisioning rates (e.g., parental investment) when provided supplemental food (Kaiser et al. 2014) (supporting assumption 2). Females might act as a flexible mediator between the environment and offspring condition. However, we found no relationship between female provisioning rate and the condition of offspring (scaled pre-fledging mass) (no support for assumption 3). This was not surprising given that our previous work showed that higher male provisioning rate, rather than adjustments in female provisioning rate, were correlated with heavier broods (Kaiser 2013). Although males deliver prey less frequently, they compensate by bringing in larger prey loads, which has a greater relative effect on brood mass than female provisioning. Lastly, the inherent limitations of studying a migratory animal with high natal dispersal (Holmes et al. 2020) prevented us from associating different levels of parental investment with how the condition of sons and daughters translates to condition as adults (assumption 4), or the respective reproductive performance of sons and daughters (assumption 5).

The relationship between offspring rearing condition, such as food availability, and their reproductive performance as adults is poorly understood. Although direct tests of assumption 5 is not possible in most migratory species, we know that food availability is a strong driver of variance in reproductive performance for both sexes in our population. Variance in reproductive success is assumed to be greater for males than for females in polygynous mating systems and socially monogamous mating systems with high rates of extra-pair paternity (Clutton-Brock 1988; Lukas and Clutton-Brock 2014). In our population, polygyny occurs at low levels (0–15%; (Holmes et al. 2020), but > 50% of the males gain extra-pair paternity (Kaiser et al. 2015). Extra-pair paternity can increase the variance in male reproductive success, and male black-throated blue warblers that gain extra-pair paternity are also less likely to be cuckolded (Webster et al. 2001), further increasing the variance in male reproductive success (Moller and Birkhead 1994; Webster et al. 1995; Møller and Ninni 1998; Reid et al. 2014). Most variance in male reproductive success is generated by differences in within-pair paternity from double brooding (Kaiser et al. 2015, 2017). Black-throated blue warblers can raise two broods in a season, but only one-third of the population is successful at double brooding when food resources are sufficient later in the breeding season on high quality territories (Webster et al. 2001; Nagy and Holmes 2005; Townsend et al. 2013; Kaiser et al. 2015, 2017). The number of broods produced by a male's social mate accounts for more total variance in male reproductive success than the number of extra-pair mates that a male acquires (Germain et al. 2021). Thus, food availability affects variance in both male and female reproductive success by affecting whether or not pairs double brood (Townsend et al. 2013; Germain et al. 2021). This suggests that the sex-specific variance in reproductive performance might not differ enough in this population to drive biases in offspring sex ratios.

We also found no evidence that one sex is more susceptible to low food availability and therefore costlier to produce and raise, at least through the nestling stage. Provisioning rates did not differ based on the proportion of sons in the brood nor did sons and daughters differ in scaled pre-fledging mass or variance in scaled pre-fledging mass. However, we did not directly measure developmental costs or mortality of offspring. Previous studies have measured growth rates to quantify differential costs during early development (Spelt and Pichegru 2017; Khwaja et al. 2018) and used embryo and nestling mortality as indicators of differences in developmental costs between sons and daughters (Kato et al. 2017; Alonso et al. 2018). We also have not measured post-fledgling survival in this species, which can differ among males and females in monomorphic species (Green and Cockburn 2001; Dittmar et al. 2016). In this study, we chose not to measure growth rate because of the risk of nest abandonment by females and we did not determine the sex of unhatched eggs or nestlings that died to directly quantify sex-specific mortality. Generally, the larger sex is predicted to be the costlier sex to rear (Merkling et al. 2015; Santoro et al. 2015), but no strong pattern has emerged for species lacking sexual size-dimorphism (Bradbury and Blakey 1998; Magrath et al. 2002). Studies that directly examine developmental costs in species with negligible size differences between the sexes will be important to better justify predictions of offspring sex ratio bias under low food availability.

Although we did not find a direct link between food availability and offspring sex ratio, we did find that offspring sex ratio was significantly male biased in high quality habitat at higher elevations. One possibility for this male bias is the variation in nutrient content across elevations. At Hubbard Brook, foliar and caterpillar nitrogen content increase with elevation (Erelli et al. 1998). Birds breeding at high elevations had access to more abundant, high nutrient food. Several studies have provided evidence that females adjust sex ratios in response to the nutritional content of food, rather than access to food (Navara 2018). Females may bias offspring sex ratios based on pre-laying nutrient availability if nutrient deficiencies early in life increase sex-specific developmental costs and mortality. For example, in captive zebra finches (*Taeniopygia guttata*) females fed diets high in nutrient quality produced more sons (the larger sex) than females fed low-nutrient diets (Bradbury and Blakey 1998; McGraw et al. 2005). Furthermore, daughters had higher mortality rates (51.5%) when reared on nutrient-restrictive diets relative to sons (7.3%) (Kilner 1998; Pryke and Rollins 2012). These studies suggest that the nutrient content of the female's pre-laying diet rather than food availability *per se* may influence offspring sex allocation, potentially to reduce the risk of nestling mortality (i.e., cost of rearing environment) (Kilner 1998; Pryke and Rollins 2012). In our study, although food-supplemented females had a predictable source of food during egg laying, mealworms and waxworms could have provided lower nutritional content than caterpillars, their primary food source.

An alternative explanation for our finding male-biased offspring sex ratios in high-quality habitat is competition over limited resources (Clark 1978). According to the Local Resources Competition (LRC) Hypothesis, when resource competition is low (i.e., high-quality habitat), females should produce offspring of the less-dispersing sex, by favoring males (in birds). In contrast, when resource competition is high (i.e., low-quality habitat), females should produce offspring of the more-dispersing sex to reduce resource competition. High natal dispersal precludes our ability to determine which sex disperses more in black-throated blue warblers to test the LRC hypothesis. However, if we assume males are less dispersive as in most other passerines, consistent with the LRC prediction females produced a male-biased sex ratio in high-elevation forest (high-quality habitat). Empirical support for resource competition influencing offspring sex ratio has been found in great tits (*Parus major*) competing for nest sites (Song et al. 2016), but few studies have tested the LRC hypothesis in birds.

Several studies in birds have attempted to test hypotheses linking food availability and/or maternal condition with offspring sex ratio with inconsistent results. For example, 4 of 23 (17%) bird studies that examined offspring sex ratios in relation to some measure of food availability found no significant effect, and 6 of 19 (32%) studies that examined offspring sex ratios in relation to maternal condition found no significant effect (reviewed in Navara 2018). The number of studies that reported male-biased broods when food was limited was nearly equal to the number of studies that showed female-biased broods under the same conditions. Likewise, the direction of the significant effects of maternal condition on offspring sex ratio varied. This makes it difficult to predict the direction of offspring sex ratio bias under different breeding conditions and to determine whether reported patterns are examples of facultative sex manipulation and adaptive. Moreover, publication bias towards significant results may lead to an overestimation of the strength of the links between breeding conditions and offspring sex ratio (Palmer 2000; West and Sheldon 2002). Nevertheless, the number of studies showing a significant effect suggests that it is important to consider the effects of environmental variables on offspring sex ratio patterns.

Clearly, multiple environmental and social factors have the potential to drive sex allocation and it is unlikely that any single factor will explain species-level patterns. These factors are often correlated (e.g., maternal condition can be associated with nutrient and food availability), which could obscure patterns. In addition to the hypothesis we tested, several other hypotheses have been proposed that predict biased offspring sex ratios based on some aspect of the breeding environment: mate quality (Griffith et al. 2003), territory quality (Dubois et al. 2006), tidal flooding (Benvenuti et al. 2018), rainfall (McNew et al. 2020), cost of reproduction (Lindén and Møller 1989), laying order (Dijkstra et al. 1990), seasonality (Daan et al. 1996), male condition (Booksmythe et al. 2017), male provisioning (Rathburn and Montgomerie 2003), and differential mortality (Slagsvold et al. 1986). Although not all these factors have strong empirical support, experimental studies that are designed to distinguish among the key predictions of a combination of adaptive hypotheses will be critical for understanding how and if females manipulate offspring sex ratios in response to interacting factors and selective pressures. Future studies should explicitly investigate the assumptions of competing hypotheses to better inform the associated predictions.

Conclusions

By experimentally manipulating food availability in low- and high-elevation forest, we were able to assess whether female black-throated blue warblers adjust offspring sex allocation based on food availability in the rearing environment. Although biased offspring sex ratios have been associated with aspects of environmental quality, we found that female black-throated blue warblers do not appear to be under selection to adjust offspring sex ratios in response to food availability when the rearing environment does not differentially affect the relative costs of producing sons and daughters. Our results did not support the Trivers-Willard hypotheses, but several assumptions of this hypothesis were not met. Hypotheses on offspring sex ratio bias have many underlying assumptions that are rarely tested, or cannot feasibly be tested, which makes generating predictions and synthesizing empirical evidence difficult. Future studies are needed that evaluate assumptions on the relative developmental costs and future reproductive benefits of producing sons and daughters under different resource conditions in animals that migrate, lack sexual size-dimorphism, and pursue extra-pair mating.

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Author contributions SAK conceived the study, designed the methodology, and collected the data, KCG conducted molecular sexing, SAK and KCG contributed equally to analyzing the data and wrote the first draft of the manuscript. SAK, TSS, and MSW acquired funding, provided resources, and contributed to the study design. All authors commented on previous versions of the manuscript and gave final approval for publication.

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Data Availability Statistical analyses reported in this article can be reproduced using the data and code available from the Environmental Data Initiative: Kaiser, S.A., K.C. Grabenstein, T.S. Sillett, and M.S. Webster. 2023. Data and code from "No evidence of sex ratio manipulation by black-throated blue warblers in response to food availability" Kaiser et al. 2023 Behavioral Ecology and Sociobiology ver 1. Environmental Data Initiative. https://doi.org/10.6073/pasta/37bcca63dea73a0b1386c7a134dedc6b (Accessed 2023-11-02).

Declarations

Competing interests The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval 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 ABS/ASAB 'Guidelines for the treatment of animals in behavioral research'. Sampling and processing had no discernable negative impacts on individuals. Behavioral observations did not disrupt the normal activities of individuals.

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