Environmental constraints can explain clutch size differences between urban and forest blue tits (*Cyanistes caeruleus*): insights from an egg removal experiment

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Abstract

Urban environments are expanding globally, presenting novel ecological challenges to which species might not be well adapted. Understanding whether species responses to urban living are adaptive or maladaptive is critical to predicting the future impacts of urbanisation on biodiversity. Urban breeding birds exhibit reduced reproductive investment (clutch size) compared to neighbouring non-urban populations. However, whether this reduction is an adaptive response or a result of physiological constraints is unclear. Here, we investigated the ability of urban and forest blue tits (*Cyanistes caeruleus*) to lay new eggs following an egg removal manipulation. Consistent with the expectation that constraints limit clutch size, egg removal did not induce urban females to lay replacement eggs. Meanwhile, forest birds laid approximately two replacement eggs after egg removal. Additionally, we found that the size of replacement eggs from forest females declined over the lay sequence, a pattern not observed in urban clutches. Hatchlings from experimental nests were lighter in both habitats, with lighter hatchlings having reduced survival. Furthermore, as urban blue tits did not lay replacement eggs, egg removal resulted in a brood reduction in the city and nestlings from urban experimental nests had higher survival than those from urban control nests. Overall, our results suggest cities place constraints on egg production in urban birds. Urban females may experience energetic or nutrient limitations that restricts egg formation and/or exacerbates the trade-off between survival and egg production. Additionally, females may be misjudging urban habitat quality, due to time constraints when laying, and produce a clutch too large to be sustained in the city.

Key words: Urbanisation · Clutch size · Breeding constraints · Adaptation

Introduction

Urban land coverage is rapidly expanding, increasing from 450.97 thousand km^2 in 1990 to 747.05 thousand km^2 in 2010, and now represents nearly 1% of the global land surface (1,2). Urban environments have a distinct set of ecological features, characterised by increased ambient temperatures (3,4), a high abundance of non-native species (5,6), increased habitat fragmentation (7,8), increased pollution (light, chemical, and noise) (9,10), and changes in the quality, composition, and availability of food (11–13). Urban environmental conditions create novel ecological and evolutionary pressures to which species might not be well adapted, potentially compromising the persistence of wildlife globally. Understanding adaptive and/or maladaptive biological changes associated with urbanisation is, therefore, crucial to determine the current and future impact of urbanisation on biodiversity.

Studies on birds, which are among the best-studied organisms in urban ecology and evolution, provide robust evidence for a reduction in reproductive investment (i.e. clutch size) and success in urban breeding populations compared to their non-urban counterparts (14–18). For example, in great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*), the clutch sizes of urban breeding birds tend to be 10-20% smaller than those nesting in neighbouring forests (19–21). Meta-analyses that include multiple species also provide evidence for smaller clutch sizes in urban populations compared to neighbouring non-urban populations (15,18,22). However, it remains unclear whether the small clutches of urban birds represent a constraint imposed upon females by the urban environment when laying or reflect an adaptation to city living.

Cities could impose constraints on birds during egg production, limiting the female's ability to invest in a larger clutch (constraints hypothesis; (23–27)). Egg production is metabolically demanding, with the energy required for egg-laying ranging between 13-41% above the basal metabolic rate in passerines (28–30). For example, the basal metabolic rate of great tits increases by 22% during ovarian follicle development (31). In small passerines that produce large clutches, the resources required for egg production far exceed what females can store endogenously (32–34). Therefore, birds need to source the energy and nutrients for egg formation (including proteins, antioxidants, omega-3 polyunsaturated fatty acids, and calcium), from their diet when laying, with invertebrates often being the most nutrient-rich food items (25,35–37). Habitat fragmentation, non-native plant species, pollution, and increased pesticide use in the city reduces the quality and quantity of invertebrate prey (12,13,38). City birds may attempt to compensate for the reduced availability of invertebrates by exploiting the abundant and predictable human-provisioned food from refuse and bird

feeders (39). However, anthropogenic food, despite being energy-rich, is nutritionally poor and contains limited proteins, antioxidants, and omega-3 polyunsaturated fatty acids (10,40). Thus, nutrient-constrained urban birds may be already laying at their maximum, being unable to source sufficient resources to invest in the formation of additional eggs that result in viable offspring. Under the constraint's hypothesis, the observed clutch size does not maximise the female's fitness payoffs (via maximising offspring recruitment and female survival). If females were freed from the constraint, they would immediately upregulate egg production (26,27).

Alternatively, the small clutches of urban birds may be an adaptive response to city living. Here, rather than being constrained by the available resources when laying, birds may be using environmental cues (e.g., food availability) when making clutch size decisions to predict the conditions the brood will experience later in the reproductive cycle (adaptive hypothesis; (41–45)).Therefore, the small clutch sizes of urban birds may be an adaptive response to match the number of young the parents can adequately provision given the reduced high-quality food available in the local environment (41,46,47). Previous research reveals that urban birds have smaller broods and fledge fewer offspring than their non-urban counterparts (15,20). Small clutch sizes could be an adaptation to city living, allowing urban parents to invest more resources into fewer nestlings, thereby maximising the number of offspring recruited (22). Although large clutches result in more offspring, this may increase sibling competition over access to resources (48,49). Therefore, offspring from large urban broods could be more likely to be malnourished, with limited survival prospects (50). If the observed clutch size maximises the female's fitness payoffs, any deviation from the observed clutch size should result in fewer offspring recruited into the population (46).

To determine whether clutch size is constrained by the environment when laying or is adapted to maximise the number of offspring recruited, previous studies have removed eggs from the clutch and observed the re-laying ability of females. These egg removal experiments reveal that some species (known as indeterminate layers) can replace eggs in response to egg removal (51). In indeterminate layers, if the observed clutch size is adapted to maximise the number of offspring recruited, then females should lay replacement eggs following egg removal in an attempt to match the optimal clutch size for the habitat (52,53). Alternatively, if laying females are constrained during laying (e.g., by diet quality as explained above), then this should limit the number and size of replacement eggs produced in response to experimental egg removal (26,54–56). Only a few studies have compared if females differentially respond to egg removal between environments and none have done so in the urban habitat.

Here, we investigate the constraints and adaptive strategy hypotheses to explain differences in clutch size between urban and forest populations of blue tits. Blue tits evolved as a cavity-nesting forest species but have readily colonised urban environments (57,58). Due to their prevalence in cities, willingness to use provisioned nest boxes, and ability to tolerate human disturbance, blue tits are an ideal study system for investigating how urbanisation influences reproductive decisions. Blue tits are moderately indeterminate layers, tending to lay replacement eggs in response to egg removal (51,58,59). Thus, the number of eggs that females produce can be experimentally manipulated by removing eggs from the clutch before the female initiates incubation. Previous research on great tits reveals females lay two replacement eggs following the removal of the first four eggs (54,60). Blue tits are close relatives of great tits and, if they are not immediately constrained, should exhibit a similar response to egg removal (51,52).

Specifically, we removed the first four eggs laid in city and forest breeding blue tit nests and observed how this manipulation affected the number and quality of eggs laid. Subsequently, we examined how egg removal influenced offspring quality at hatching and their fledging probability. If differences in clutch size between habitats are caused by constraints on egg production, then we predict that, following the egg removal manipulation, urban females should lay fewer and smaller additional eggs (and, therefore, produce smaller hatchlings) than forest females. However, fledging probability and growth rates should be higher in experimental nests than in control nests if females do not replace the removed eggs, as, here, egg removal would also result in a brood reduction. Under the adaptive hypothesis, we predict that urban and forest females should lay a similar number of additional eggs of equal size following the egg removal manipulation, with no difference in hatchling bodyweight between treatment groups and habitats. Additionally, there should be no difference in fledging probability and growth rates between treatment groups and habitats. However, we predict that the number of offspring fledged will be higher in control nests in both habitats if the observed clutch size reflects an adaptive strategy.

Methods

Study populations

We monitored one urban and one forest nest-box population of blue tits in Scotland during the breeding season of 2022 (breeding season: April 1st to June 30th; nest-box: made with woodcrete, 260H × 170W × 180D, hole diameter: 32mm, Schwegler, Germany). The urban site

is a city centre park in Glasgow (Kelvingrove Park, N=28 nest-boxes included in the current study, coordinates = [55.869, −4.2851]). In Kelvingrove, the environment contains open land, and small shrubs, with tree species comprising non-native species, including Turkey oak (*Quercus cerris*), laurel (*Laurus nobilis*), and ornamental cherry (*Prunus spp.*). The forest site is located at Loch Lomond, 40km north of Glasgow, and is an ancient native oak woodland (Scottish Centre for Ecology and the Natural Environment (SCENE), N=33 nests included in the current study, coordinates = [56.129,−4.6145]). The dominant tree species at SCENE include oak (*Quercus spp.*), birch (*Betula spp.*), and sycamore (*Acer pseudoplatanus*) (39,61,62)

Experimental design

Assignment of treatment groups

From April 1st, we visited nest-boxes twice weekly to monitor nest-building activity. We increased the frequency of visits to every two days once the blue tits started lining the nest-cup to ensure we accurately recorded the first egg date. We visited nests after 11:00 to ensure sufficient time for the blue tit to lay (in passerines, most egg-laying occurs shortly after sunrise (63)). Once a new clutch was detected, it was alternatively assigned to the control or experimental group, following a 1:1 ratio to reduce the risk of any differences in phenology occurring between treatment groups. For nests included in the study, we found no difference in the first egg-laying date between treatment groups ($x^2_{df=1}=0.124$, $P=0.725$). In all nests, once a new egg was found, we marked eggs using a non-soluble marker pen, with a number corresponding to the eggs position in the laying sequence. We photographed every egg (used to calculate egg volume; details below) in both control and experimental nests, including a measuring chart in each photograph. Control nests had no eggs removed, with the photographing and marking of eggs being the only time we disturbed control females during egg production (Figure 1). In experimental nests, we removed the first four eggs from the nest on the morning they were laid. Eggs removed from the experimental nests were also weighed (± 0.010g) using digital scales. At the urban site, we included 14 control and 14 experimental clutches. At the forest site, we included 17 control and 16 experimental clutches.

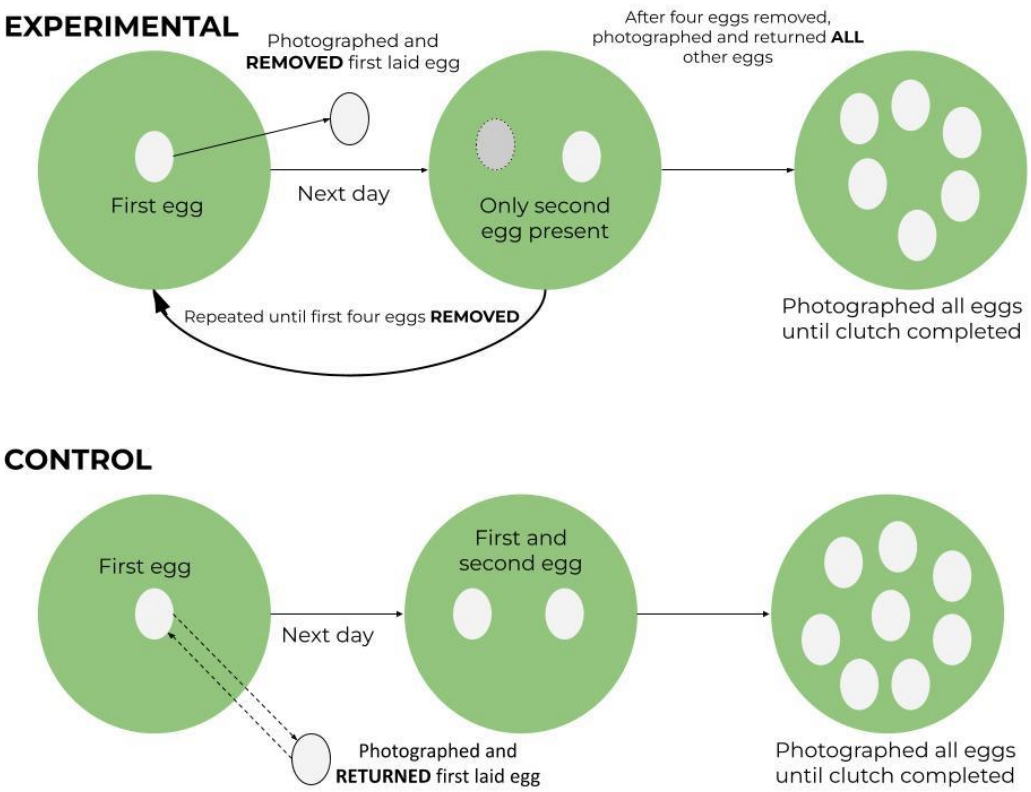

Figure 1. Overview of the treatment groups. The first four eggs were photographed, marked, and removed from experimental nests on the day they were laid. After the fourth egg was removed, all subsequent laid eggs remained in the nest. No eggs were removed from control nests, with eggs only being handled when photographed and marked.

When the female stopped laying for two days, and the eggs were warm to touch, we assumed the female had completed the clutch and started incubating (64). Assuming a minimum incubation length of 14 days from clutch completion (65), 13 days after clutch completion, we started nest-box visits every two days to record the exact hatch date.

Nestlings were weighed (± 0.1g) on days two, six, and 12 after hatching. On day six, nestlings were fitted with a British Trust for Ornithology (BTO) metal ring with a unique ID number to establish individual identities. We also measured tarsus and wing lengths (± 0.1mm) 12 days after hatching. Nest-boxes were checked >21 days after hatching to identify and record dead and fledged nestlings.

Egg volume measurements

We used egg volume as a proxy of egg size, which reflects the level of pre-natal investment into the young before incubation and may dictate offspring quality at hatching (66,67). We used an Olympus TG-6 digital camera to photograph eggs in the field. We photographed eggs on a

20×20 cm measuring chart at a 90° angle to the egg's long axis, and there were no adjustments to lens distortion. We photographed each egg four times, rotating the egg along its long axis between photographs. As blue tits exhibit substantial intra-specific variation in egg shape (68), commonly used methods to estimate volume, such as Hoyt's equation (*Volume* = $k \times length \times breadth^2$), are difficult to generalise across different study populations (69). Hoyt's equation uses *k* (a species-specific variable), egg length, and breadth to estimate egg volume but assumes that the shape of the measured egg exactly matches that of the test population (70). Therefore, to calculate egg volume, we used IMAGEJ and the egg measurement plugin developed by Troscianko (71). For a given length along the eggs long axis, IMAGEJ calculated the radius (*r*) of the egg using equation 1.

$$1)\quad r = \frac{a e^{\frac{-l^2}{2b^2} + \frac{cl}{b^2} - \frac{c^2}{2b^2}} \sqrt{1 - l\sqrt{l}}}{\pi b}$$

Where *a* is the width (mm) of the egg, *b* is the spread of distribution (the standard deviation in a normal distribution), *c* is where the peak of the distribution is located along the egg's length (the mean in a normal distribution), and *l* is the length of the egg (mm). The plugin uses a least-squares function to fit the model to the shape of the egg and requires anchor points around the outer edge of the egg in the image. Using the multipoint selection tool in IMAGEJ, we selected 12 anchor points around the edge of the eggs surface, making sure we selected the tip and base of the egg. For each egg, we calculated volume from three separate images, producing three volume measurements per egg. In total, 1677 images of 559 eggs were analysed. For each egg, we took the mean of the three volume measurements, resulting in one mean volume measurement per egg. We used the R package *rptR v.0.9.22* (72) to quantify the repeatability of the volume measurements calculated in IMAGEJ (see methods S1 for further detail). The repeatability of the volume measurements between images was low (*repeatability [95% Confidence Interval 'CI'] = 0.347 [0.279, 0.434]*). However, individual females exhibited consistent egg size within clutches (*repeatability [95% CI] = 0.527 [0.414, 0.616]*) and mean egg volume across the three images per egg was strongly correlated with egg weight (using removed eggs, N = 86 eggs, Pearson's correlation coefficient *r [95% CI] = 0.748 [0.637, 0.828])*, confirming that our measurement of egg volume was capturing biologically relevant variation.

Data analysis

General modelling procedure

We performed all statistical analysis and data visualisation in Rstudio v.1.4.1106 (73) using R v.4.2.0 (74).We used the R package *lme4 v.1.1* (75) to build linear models and generalised and linear mixed models to explain variation in the investigated reproductive traits (see below). We initially built a global model for each reproductive trait, containing all explanatory variables and interactions with the potential to explain variation in that trait. Using the package *lmtest v.0.9* (76), we used likelihood-ratio tests (LRTs) to determine the statistical significance of each model predictor. To test the statistical significance of each term in the global model, we sequentially dropped predictors from the model and compared the model without the focal predictor against the global model using LRTs. We then derived final models by backwardly eliminating non-significant interactions. We did not apply model simplification of single effect predictors. In several models (details below), we mean centred continuous predictors. In all models, date (either hatch date or laying date) was expressed as the number of days since the 1st of January. Date was initially fitted as both a quadratic and linear term before quadratic terms were dropped from all the models if not significant. We used the R package *performance v.0.9.1* (77) to assess the normality of residuals, homogeneity of variance, and the collinearity between fixed effects. We used the package *ggplot2 v.3.3.6* (78) to visualise the model predictions and raw data.

Global models

Number of eggs laid

We used linear models to explain variation in the number of eggs laid by a female, including the first egg-laying date (mean-centred) and the habitat (2-level factor, "urban" versus "forest") × treatment group (2-level factor, "experimental" versus "control") interaction as fixed effects. A total of 61 clutches were initially included in the analyses, with nine clutches removed from the final dataset because the female abandoned the clutch before incubation, and we could not determine if the clutch was completed. We then created additional post-hoc within-habitat models for the city and forest, including the first egg-laying date (the day the first laid egg was recorded in the nest; mean-centred) and treatment group as fixed effects. There were 20 additional clutches external to the experiment in the forest (hereafter referred to as "super controls"). Therefore, in the forest, we created another within-habitat model, including the treatment group (2-level factor: "control", or "super controls") and first egg-laying date as fixed effects. Finally, we created a post-hoc model where the super control and control treatments were grouped into one control group. The first egg-laying date (mean-

centred) and the habitat × treatment group ("super control + control", or "experimental") interaction were included as fixed effects in the final post-hoc model.

Egg volume

To determine whether there were differences in egg volume between habitats and treatment groups, we initially built a linear mixed model with egg volume as a response variable and the following explanatory variables: the total number of eggs laid by each female (mean-centred), egg-laying date (the exact day each egg was laid; mean-centred), and the interaction between habitat and treatment group. Clutch ID (a 70-level factor) was included as a random effect intercept. Additionally, we assessed whether the egg removal treatment differentially affected the total volume of eggs produced by a female between habitats. We calculated the total volume of egg produced by a female by taking the mean egg volume per nest box and multiplying it by the number of eggs laid. We then used a linear model to analyse the relationship between the total volume of egg produced and the following explanatory variables: first egg laying date (mean-centred), and the interaction between habitat and treatment group. Finally, we created within-habitat models for the city and forest, with the total volume of eggs produced as the response variable and the first egg-laying date (mean-centred) and treatment group as fixed effects.

Variation in egg volume throughout the laying sequence

We then created two additional models testing the effect of laying order (fitted either as a categorical or continuous variable) on egg volume. In small, income-breeding birds, laying order influences egg composition and size (79,80) and egg investment is functionally biased within the clutch (81). Therefore, to account for the effect of laying order on egg volume, we included eggs one to nine in the laying sequence, as only three city birds produced a clutch larger than nine eggs. We first divided eggs into two laying order groups: eggs one to three; or eggs four to nine. Evidence suggests blue tits acquire the resources for egg formation daily (25,34); however, in small passerines the yolk rings for the first three eggs develop two to three days before the first egg is laid (82).Therefore, females may not recognise the effect of the egg removal treatment until the fourth egg in the lay sequence, after having synthesised the yolks for the first three eggs before the experimental manipulation. The global linear mixed model for the effect of laying order group on egg volume included the following explanatory variables: egg laying date (mean-centred), the total number of eggs laid by each female (mean-centred), and the three-way interaction between laying order group (2-level factor), habitat, and

treatment group. We then created an additional model testing the effect of laying order (fitted as a continuous variable) on egg volume to determine if the investment into egg quality changes over the lay sequence. The global linear mixed model contained egg volume as the response variable and egg laying date (mean-centred), the total number of eggs laid by each female (mean-centred), and the three-way interaction between the position of the egg in the lay sequence, habitat, and treatment group as fixed effects. We included Clutch ID (a 70-level factor) as a random effect in both volume models.

Nestling bodyweight

We used a linear mixed model to determine whether egg removal differentially affected nestling body weight between habitats two-days after hatching. As previous research suggests there may be a relationship egg size and nestling bodyweight at hatching (66,83), the habitat or treatment group with the largest eggs should also produce heavier nestlings at hatching. We included nestling bodyweight two days after hatching (the first-time nestlings were weighed) as the response variable with the number of siblings (equivalent to the number of hatched eggs; mean-centred), hatch date (mean-centred), time of day (2-level factor: "morning" or "afternoon"), and the two-way interaction between habitat and treatment group as fixed effects. Clutch ID (a 58-level factor) was included as a random effect. We also modelled nestling bodyweight on days 6 and 12 after hatching (see methods S1 for more detail).

The effect of hatchling bodyweight on nestling survival

To assess whether nestling body weight two days after hatching influenced the probability of a nestling surviving until fledging, the nestling's survival probability (2-level factor: "fledged" or "dead") was included as a response variable in a binomial generalised linear mixed model. We included the following explanatory variables: hatch date (mean-centred), number of siblings (mean-centred), time of day, and the three-way interaction between the treatment group, nestling body weight two days after hatching, and habitat. As part of a different study, some hatchlings were cross-fostered and, hence, for the analysis presented here, we included the clutch ID of hatching (a 58-level factor) and clutch ID of rearing (a 58-level factor) as random effect intercepts.

Nestling survival

To test the effect of habitat, treatment group, and age on nestling survival, we created two separate models: one with the number of nestlings as the response variable, the other with the

proportion of nestlings over brood size as the response variable. We used a linear mixed model to determine whether the effect of the treatment group on the number of nestlings depended on habitat and nestling age (4-level factor: “day two”, “six”, “12”, or “fledged”). We included the number of nestlings as the response variable while the number of eggs incubated (mean-centred), hatch date (mean-centred), and the three-way interaction between nestling age, habitat, and treatment group were included as fixed effects. We modelled the probability of nestling survival (i.e., the probability that a nestling was alive or dead at each measured time-point) using a binomial generalised linear mixed model. We included survival probability as the response variable while hatch date (mean-centred) and the three-way interaction between nestling age, habitat, and treatment group were included as fixed effects. Clutch ID (a 58-level factor) was included as a random effect intercept in both models.

Results

The effect of egg removal on the female’s ability to replace eggs in the city and forest

Initially, we found no differential effect of egg removal on the female’s ability to relay eggs between habitats, represented by the absence of a location × treatment group interaction ($x^2_{df=1}=2.069$, $P=0.150$; Table S1). However, visually, there was a clear difference in the ability of experimental and control females to replace the removed eggs in the urban and forest habitats (Figure S1), suggesting that the lack of statistical support of the interaction may have been due to the small sample size. Following this, we created within-habitat models for the city and forest. In the city, treatment group had no effect on the number of eggs laid (model estimate ± SE=0.472 ± 0.594 eggs, $x^2_{df=1}=0.701$, $P=0.403$; Table S2). In the forest, birds in the experimental group laid more eggs than those in the control group (model estimate ± SE=1.632 ± 0.600 eggs, $x^2_{df=1}=7.267$, $P=0.007$; Table S3). We then compared the number of eggs laid between control, experimental, and super control nests external to the experiment in the forest. We found no difference in the number of eggs produced by control and super control nests (model estimate ± SE=-0.681 ± 0.450 eggs, $x^2_{df=1}=2.409$, $P=0.121$; Table S4). As there were no differences in the number of eggs produced between control and super control nests, we grouped super controls as controls. After grouping, we found a significant interaction between treatment group and location ($x^2_{df=1}=4.132$, $P=0.050$; Table S5; Figure 2). Here, forest experimental females laid approximately two more eggs than forest control females (Figure 2). Meanwhile, experimental and control females did not differ in the number of eggs they laid in the city (Figure 2). In both habitats, the number of eggs laid declined over the breeding season ($x^2_{df=1}=24.498$, $P<0.001$; Table S5).

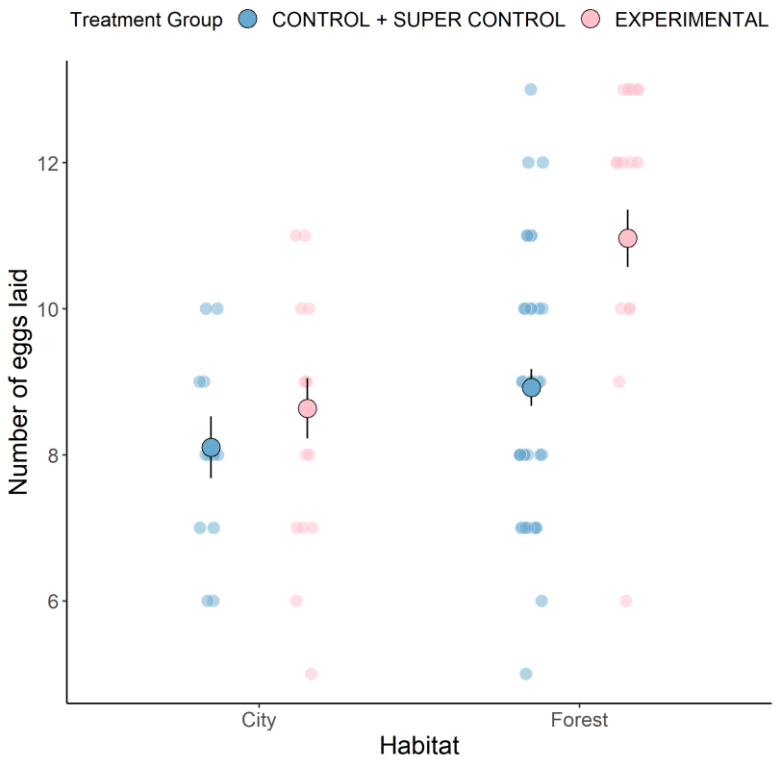

Figure 2. The effect of egg removal on the number of eggs laid by the females in the city and the forest. Large dots represent model predictions ± standard error. Small dots are the raw data points. N=81 clutches.

The effect of egg removal on egg size

Egg volume did not depend on the treatment group × habitat interaction when laying order was not included in the model ($x^2_{df=1}=2.154$, $P=0.341$; Table S6). Additionally, when fitted as single effects (in contrast to when they were included in the interaction), habitat ($x^2_{df=1}=3.460$, $P=0.063$; Table S7, Figure 3a) and treatment group ($x^2_{df=1}=0.016$, $P=0.899$; Table S7, Figure 3a) did not affect egg volume when laying order was not included in the model. The total volume of egg material produced (mm^3) did not depend on the interaction between location and treatment group ($x^2_{df=1}=1.149$, $P=0.284$; Table S8-S11; see Results S1 for further detail).

The effect of laying order, treatment group, and habitat on egg volume

When eggs were grouped according to their laying order position (1. eggs one to three or 2. four to nine), there was no three-way interaction between the laying order group, treatment group, and habitat ($x^2_{df=1}=3.167$, $P=0.075$; Table S12-S13; see Results S2 for further detail). We then further investigated the effect of laying order on egg volume by fitting laying order as a continuous variable. When laying order was fitted continuously, we found a three-way interaction between laying order, treatment group, and habitat ($x^2_{df=1}=3.923$, $P=0.047$; Table S14, Figure 3b). There was no difference in egg volume across the lay sequence and between treatment groups in the city, the same holds for control nests in the forest. Meanwhile, egg volume declined over the lay sequence in experimental nests from the forest (Figure 3b).

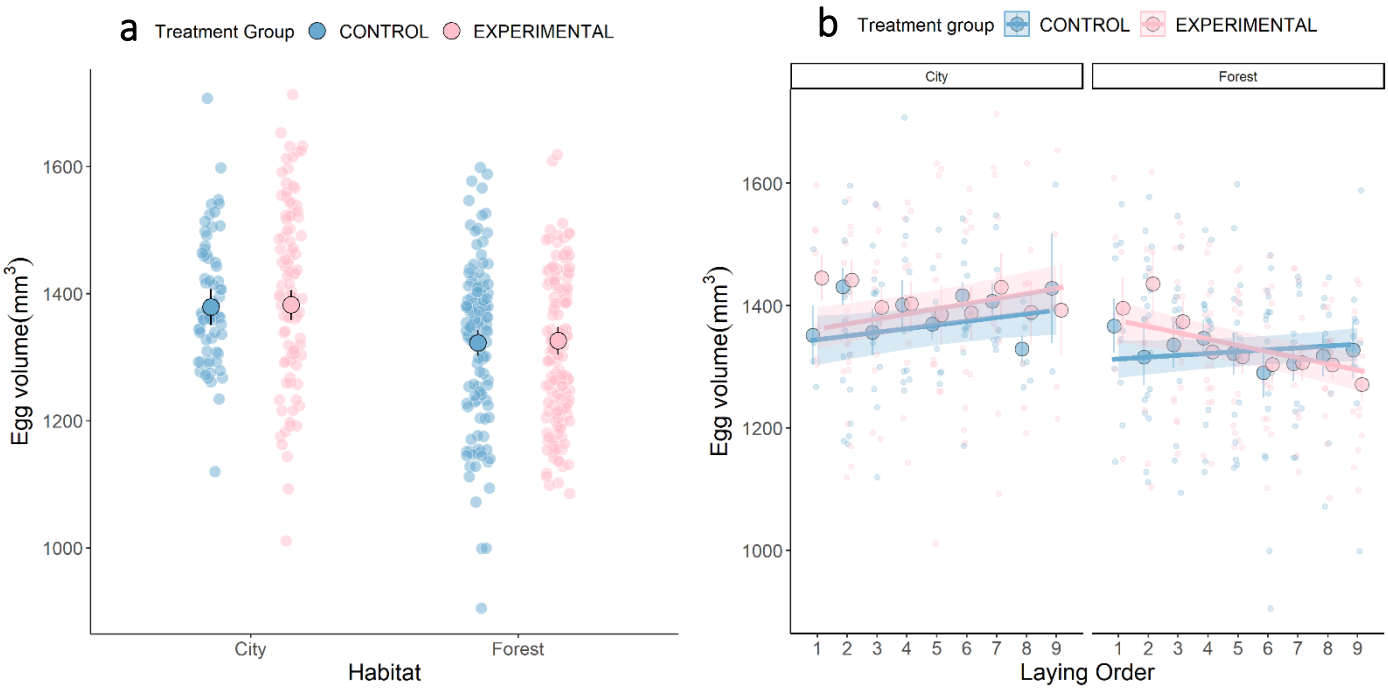

Figure 3. a) The effect of the treatment group on egg volume in the forest and city. Large dots represent model predictions from the minimal model ± standard error. Small dots are the raw data points. N=460 eggs. **b)** The effect of the treatment group and laying order (eggs one to nine) on egg volume in the forest and city. Large dots represent raw means ± standard error. Straight lines and ribbons represent model predictions ± standard error. Small dots are the raw data points. N=460 eggs.

The effect of egg removal on hatchling bodyweight

There was no differential effect of the egg removal treatment on nestling body weight two days after hatching between habitats, as represented by the absence of a treatment group × habitat interaction ($x^2_{df=1}=0.132$, $P=0.717$; Table S15). However, we found that nestlings in experimental nests were lighter than those in control nests two days after hatching in both habitats ($x^2_{df=1}=4.117$, $P=0.042$; Table S16, Figure 4a). We found that the nestlings hatch date did not affect body weight ($x^2_{df=1}=0.182$, $P=0.670$; Table S16). Additionally, there were no

differences in hatch date across habitats ($x^2_{df=1}=0.190$, $P=0.663$) and between treatment groups ($x^2_{df=1}=0.180$, $P=0.184$). On days six and 12 after hatching, there was an interaction between nestling age, treatment group, and habitat ($x^2_{df=1}=18.895$, $P<0.001$; Table S17). In the forest, there were no differences in nestling bodyweight between treatment groups on days six and 12, the same holds for both treatment groups in the city on day six. Meanwhile, in the city, nestlings in experimental nests were heavier than those in control nests 12 days after hatching.

The effect of hatchling bodyweight on survival

We found no differential effect of nestling weight on survival two days after hatching between treatment groups and habitats, as represented by the absence of a three-way interaction between nestling bodyweight two days after hatching, treatment group, and habitat ($x^2_{df=1}=0.256$, $P=0.611$; Table S18). In both treatment groups and habitats, the likelihood of a nestling surviving until fledging increased with body mass two days after hatching ($x^2_{df=1}=10.647$, $P=0.001$; Table S19; Figure 4b).

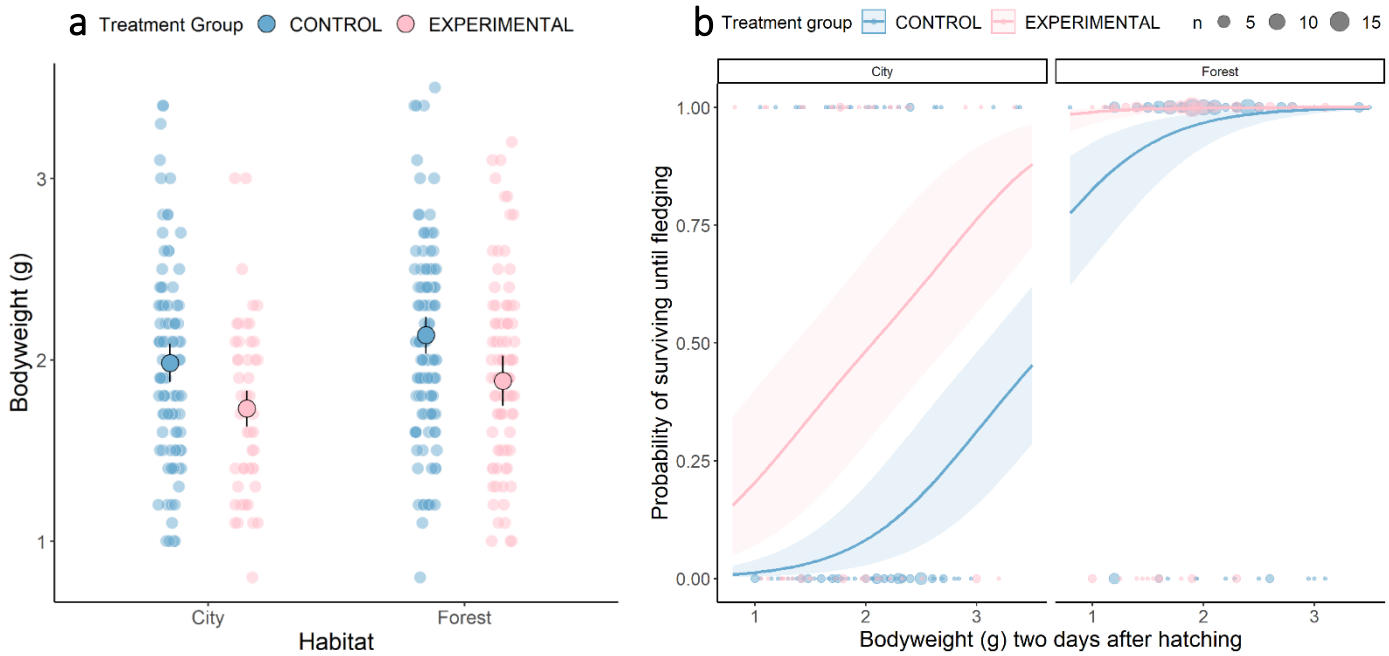

Figure 4.a) The effect of the habitat and treatment group on nestling weight (g) two days after hatching. Large dots represent minimal model predictions ± standard error. Small dots are the raw data points. N=372 nestlings. **b)** The effect of nestling bodyweight two days after hatching on the probability of survival until fledging in both treatment groups and habitats of rearing. Lines and associated ribbons represent the minimal model predictions ± standard errors. Small dots represent raw data points. Dot size indicates the number of nestlings of each body weight. N=364 nestlings.

The effect of egg removal on nestling survival between habitats

We found that the number of nestlings in the brood depended on the three-way interaction between treatment group, habitat, and nestling age ($x^2_{df=3}=18.530$, $P<0.001$; Table S20, Figure 5a). Control broods in the forest had more nestlings than experimental broods at each measured time point. Meanwhile, in the city, on days two and six after hatching, control broods had more nestlings in the brood than experimental nests. However, on day 12 and fledging, there was no difference between treatment groups in the number of nestlings alive in the city. Additionally, we found that nestling survival depended on the three-way interaction between treatment group, habitat, and nestling age ($x^2_{df=3}=8.761$, $P=0.034$; Table S21, Figure 5b). There was no difference in nestling survival between treatment groups across all time points in the forest. In the city, on days two to six after hatching, there was no difference in nestling survival. Meanwhile, on day 12 and fledging, nestling survival was higher in experimental nests than in control nests in the city.

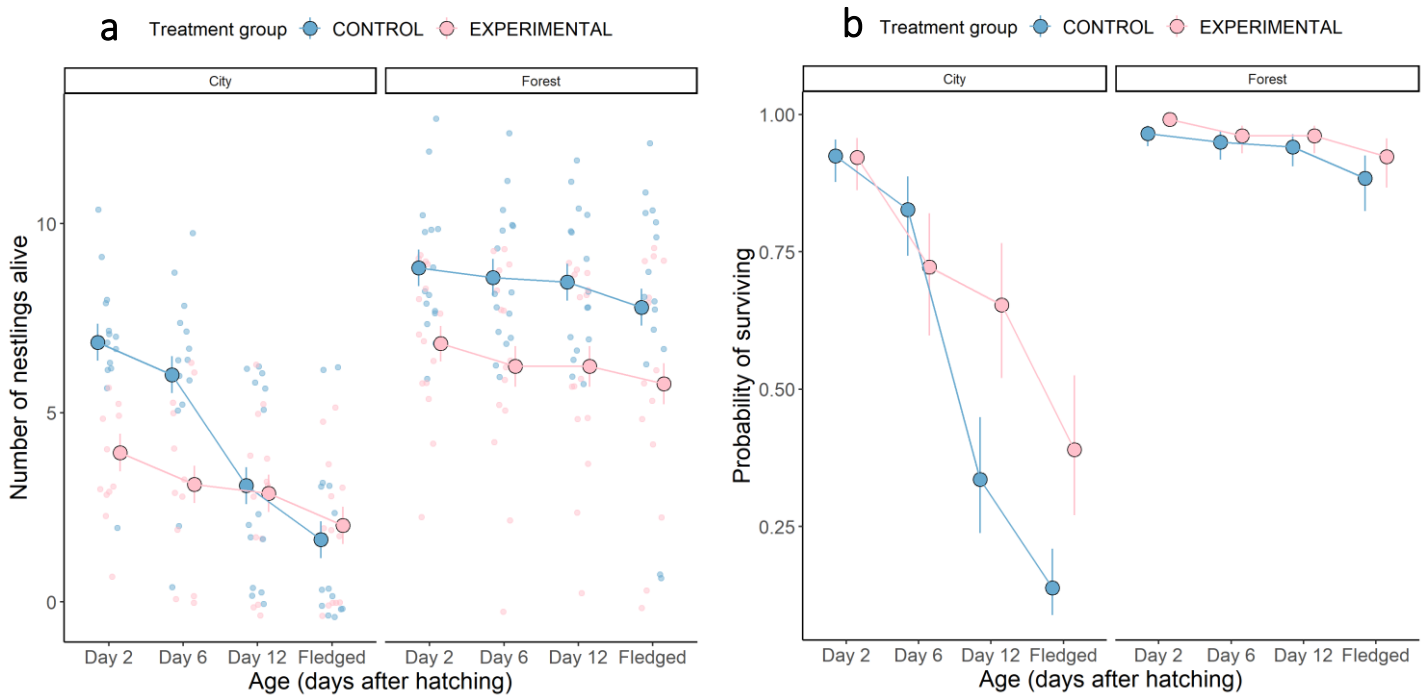

Figure 5. a) The effect of treatment group, habitat, and age on the number of offspring alive. Large dots and associated bars represent model predictions ± standard errors. Small dots represent raw data points. N=60 broods. **b)** The effect of treatment group, habitat, and age on nestling survival. Large dots represent model predictions ± standard error. N=60 broods.

Discussion

To test if the small clutches of urban birds reflected constraints imposed upon females by the urban environment when laying or represented an adaptation to urban living, we investigated the ability of females to replace eggs following egg removal in both the city and the forest. If

the differences in clutch size between habitats were due to constraints on egg production, we predicted urban females would produce fewer or smaller eggs (and, subsequently, have lighter offspring two days after hatching) than forest females following egg removal. Additionally, under the constraints hypothesis, if egg removal resulted in a brood reduction, nestling survival should be higher in experimental than control nests, with this difference being more pronounced on the city. Meanwhile, under the adaptive hypothesis, the number and size of additional eggs (and offspring two days after hatching) produced after egg removal should have been similar between urban and forest females. Additionally, there should be no difference in fledgling probability between treatment groups and across habitats. However, the number of offspring fledged should be greater in control than experimental nests in both habitats if the observed clutch size is an adaptive strategy. In line with the constraint's hypothesis, we found that city birds did not replace the removed eggs while, in line with the adaptive strategy hypothesis, forest birds produced an additional two eggs following egg removal. If urban blue tits were not limited in some way during egg production, then, as observed in the forest and previous studies, females should have laid additional eggs after egg removal to maximise the number of viable offspring produced (26,52,54,60). As urban females did not lay replacement eggs, there was no difference in egg volume across the lay sequence between city treatment groups. Conversely, in experimental forest nests, egg volume declined over the lay sequence. As predicted by the constraint's hypothesis, this decline in egg size likely reflects the inability of laying females to maintain egg quality when egg production is experimentally upregulated (26,84). Nestlings from experimental nests were also lighter than those in control nests in both habitats two days after hatching. In experimental forest nests, this reduced body weight may reflect the female's inability to maintain offspring quality in response to increased laying effort. However, experimental nestlings were also lighter in the city, despite urban females exhibiting no decline in egg volume across the lay sequence. Even at this early-stage body weight is important, with heavier nestlings being more likely to fledge in both habitats. In both habitats, experimental nests reared fewer offspring than control nests as we did not return the removed eggs before the female initiated incubation. Thus, in the forest, where females laid additional eggs, the increased costs of egg production are confounded with reduced brood-rearing costs. Meanwhile, there was no difference in the number of eggs laid between treatment groups in the city. Any effect of egg removal on nestling growth rate and survival in the city is likely due to the brood reduction. Although there were no differences in nestling survival and growth rate between forest treatment groups, this was not the case in the city. Following the artificial brood reduction in experimental city nests, nestlings were heavier at day 12 and were more likely to fledge than their control counterparts. Therefore, urban birds

could be producing clutches that are too large to be sustained in the city. Overall, these results suggest the environmental forces operating on clutch size differ between the city and the forest. Urban conditions are likely imposing constraints on females when laying, preventing increased investment in egg production, such as energy and nutrient limitations (15,26,85). Alternatively, the small clutches of city birds could be an adaptation to maximise the mother's survival if producing large clutches incur a fitness cost (i.e., sacrificing somatic maintenance or future fecundity) (86–88). We discuss these alternative explanations of our finding below, including the limitations of this research and future directions to further understand the evolution of clutch size in anthropogenic environments.

The effects of energy and nutrient limitation on egg production

The energetic costs associated with egg production may be increased in the city, preventing urban birds from replacing eggs after egg removal. As the environment and resources available to urban birds could be of low quality, city females may differentially allocate energy between activities compared to forest birds. During the pre-laying period, individuals divide energy between nutrient acquisition, thermoregulation, territory defence, mate attraction, and egg production (89–91). Individuals cannot maximise their investment in all tasks, i.e., trade-offs occur between tasks (92,93). For example, the energy costs associated with thermoregulation may limit the energy allocated to egg production (94). In great tits, the ability to replace eggs following egg removal is temperature constrained, with females failing to upregulate egg production in years with lower spring temperatures (54). Low temperatures increase the daily energy expenditure of females during egg laying as they expend more energy on thermoregulation (55,95,96). However, in our study it is unlikely temperature prevented urban birds from replacing eggs. Urban areas are generally 1-3°C warmer than surrounding natural areas due to the heat-island effect (3,4). Thus, urban birds should spend less energy thermoregulating than forest birds due to the warm city climate. If anything, urban birds should have increased the energy allocated to egg production and laid replacement eggs.

Alternatively, urban blue tits may not upregulate egg production after egg removal due to being constrained by the poor nutrient quality of anthropogenic food. In small passerines that produce large clutches, the reserves required for egg production exceed what females can store endogenously (32–34,97). Thus, they must acquire the nutrients for egg production daily from their diet when laying (80,98,99). Breeding females need to source sufficient resources to deposit in the egg during this period, including sulphur-rich proteins (25,100), antioxidants (36,101,102), fatty acids (37,103), and calcium (33,35,104,105). However, in the city, there

could be lower availability of these resources when compared to the forest, as invertebrate prey may be less abundant (13,47). Thus, urban females may expend more energy on nutrient acquisition than forest birds, limiting the energy they can allocate to egg production (106). Urban blue tits may compensate for the reduced natural food availability by exploiting abundant and predictable human-provisioned food (10,39,107). However, this food is nutritionally poor, with limited carotenoids, proteins, vitamins, and omega-3 polyunsaturated fatty acids (108,109)Therefore, following egg removal, urban females may have been unable to source sufficient high-quality resources to invest in the production of additional eggs (44,110–112).

Given females deplete endogenous stores of biomolecules by reallocating them to the egg, the mother may face a trade-off between allocating resources for somatic maintenance and egg production, which could underlie the costs of egg production (54,86,93). During laying, females have increased susceptibility to oxidative stress (113,114), disease (115), and parasite infection (60,116), with parents investing more in egg production and having fewer resources to allocate to immune function (117). The effects of nutrient limitation during egg laying on female condition and survival may have been operating differently between habitats. On one hand, the nutrient requirements for the female's somatic maintenance may be fulfilled in the forest. Thus, forest females may invest any additionally acquired resources into upregulating egg production at little cost to their survival. On the other hand, as nutrient limitation may be higher in the city (118,119), urban females in poor condition could be selectively investing in somatic maintenance at the expense of egg quantity (40,120–122). Thus, after egg removal, urban females may have been too exhausted to form additional eggs, as doing so would reduce survival or future fecundity (26,88).

The trade-off between clutch size and egg size

As the resources available to the breeding female are limited, individuals producing larger clutches may compensate by producing smaller eggs (123–126). However, we found that mean egg size did not differ between habitats, despite urban birds producing smaller clutches than their forest counterparts. Not all eggs are equal within the clutch, and the level of investment may be biased across the laying sequence, leading to intra-clutch differences in embryonic development, physiology, and nestling growth (26,127–129). In both treatment groups in the urban site, we found that egg volume exhibited no change over the lay sequence: the same holds for control clutches in the forest. Despite this, previous studies find egg size increases over the lay sequence in small passerines, reflecting increased protein content in the last laid

eggs (26,67), and this may compensate for the detrimental effects of asynchronous hatching (79,130,131). In our experimental forest nests, egg volume declined over the lay sequence. These results are in line with previous studies finding females have a limited ability to maintain egg quality when laying above their usual clutch size (26,56,84,91). Urban birds may be adaptively producing small clutches to maintain an equal investment across the laying sequence. If urban birds laid replacement eggs, this could detrimentally impact egg and offspring quality, which they may be unable to compensate for during nestling rearing due to the limited food availability for offspring provisioning in urban areas (26,39,132). Thus, in cities, females may terminate laying earlier than forest birds before they pass their critical physiological threshold where egg size and clutch size are traded off against each other (26,130,131). Meanwhile, forest blue tits might be able to upregulate egg production following egg removal, as they can compensate for any detrimental effects of egg size on offspring quality later in the reproductive cycle.

Egg size, nestling weight, and survival

As parents can directly affect their nestling's body weight by maximising investment in egg quality (133–136), one may predict that an increase in egg size may be advantageous, especially in the city where resources are limited in the nestling-rearing phase, as large eggs should result in chicks with increased nutrient reserves that facilitate rapid growth after hatching (66,67,137–139). However, we found no difference in nestling body weight two days after hatching between the urban and forest sites, but experimental nestlings were lighter than control nestlings in both habitats. In experimental forest nests, where blue tits upregulated egg production, the lighter body mass of nestlings two days after hatching may reflect the reduced investment into the last laid eggs (84). However, experimental nestlings were also lighter in the city, despite no difference in the number of eggs laid between city treatment groups. We did not return the removed eggs to the female in experimental nests, with experimental nestlings always coming from beyond egg four in the laying sequence. As the investment in the clutch is biased, even if egg volume did not differ across the lay sequence in the city, the nutrients required for nestling development may be allocated differentially between eggs (128). Even though the protein content of the eggs may increase over the lay sequence, other nutrients may decrease, such as vitamin E and carotenoids (140,141). Thus, from egg five onwards in the laying sequence, the yolk content may be of lower quality than in eggs one to four, resulting in lighter nestlings.

We found that the nestling's body weight two days after hatching predicted nestling survival, with heavier individuals more likely to fledge. Therefore, the level of pre-natal investment may dictate whether a nestling recruits into the population (91,136), especially in the city where we found nestling survival was lower than in the forest. This pattern is also observed in white-browed sparrow weavers (*Plocepasser mahali*), where variation in egg volume has fitness implications for the offspring, as nestling mass at hatching predicts survival until fledging (139). Nestlings that are heavier at hatching may have higher fitness prospects, with the increased nutrient reserves from the egg improving growth rates, boosting immunocompetence, and allowing them to be more competitive at feeding (133,142–144). A meta-analysis of 283 studies by Krist (67) found that juvenile survival was strongly correlated with egg size, and the effects of egg size on survival persisted into the post-fledging stage. However, the benefits of large eggs may be confined to early in the post-hatching period as increased egg size does not always result in increased growth rates or survival (48,145–148). To our knowledge, no study has attempted to link how egg size impacts other components of fitness in urban and non-urban bird populations.

4.4 Maladaptive responses to the city environment

In the city, nestling survival was greater in experimental nests, where the parents reared fewer nestlings as we did not replace the removed eggs, than in control nests. Therefore, our results suggest urban females may be making maladaptive decisions when laying as they could maximise offspring survival by producing a smaller clutch. The clutches of urban birds may be too large due to the lack of barriers preventing gene flow from the forest. Clutch size is a heritable trait (e.g., in great tits, $h = 0.48$), often under directional selection to increase (21,149–152). Immigration between the city and forest may have homogenised gene pools (153,154), preventing the evolution of adaptive clutch size by gene flow if the two populations have a different fitness optimum for clutch size (155,156). Alternatively, cities may function as ecological traps (10). Although cities may have more resources and stable conditions during the winter than in the forest, leading birds to preferentially settle here, the poor food quality and exposure to pollution may impair nestling development (39,157). Therefore, urban birds may be misinterpreting habitat quality when settling and, subsequently, producing clutches that are too large to be sustained. These maladaptive decisions occur as females are time constrained during laying and quickly assess habitat quality based on limited knowledge of the environment (158–160).

Limitations and future investigations

This study has limitations that could influence the interpretation of the results. First, most females were not individually identified. We were, therefore, unable to assess how parental quality and age affected the ability of females to upregulate egg production. Low-quality or young individuals that naturally produce smaller clutches may be pushed into the city if free territories in the forest are no longer available (10,106,161). Indeed, laying following egg removal may be more common in the forest if females are more experienced at breeding (60,162,163). Therefore, in the future, we need to determine if the parental quality or the age structure of the city and forest populations differ and if that further explains differences in clutch size between urban and forest habitats. Second, this study consists of only one urban and one forest population, with no quantitative assessment of the urban environment. Comparative work across cities is needed to see if our results generalise across study populations. Species may also exhibit different responses to egg removal if the factor limiting egg production varies inter-specifically. Information on the costs of egg production should be obtained from urban and natural populations of different species filling various niches. Third, the number of fledglings is only one component of fitness. Ideally, we should have measured the number of offspring recruited or the parent's lifetime reproductive success. Similarly, we were also unable to assess the parent's survival. Indeed, the fitness costs of egg production may be paid by the mother rather than by the offspring (54). Fourth, future work should compare the costs of each stage of the reproductive cycle (egg laying, incubation, and nestling rearing) between urban and forest birds, and assess the consequences of these costs, as there may be interactive effects between reproductive phases that operate differently between the city and the forest (53). Finally, a common garden or transplant experiment would be valuable to further consolidate our findings and confirm that the small clutches of urban birds reflect a constraint.

Conclusion

Our study provides experimental support for blue tits being more constrained during egg production in urban than forest habitats. Urban birds may experience greater energetic or nutrient constraints than forest birds that either immediately restricts egg formation or exacerbates the trade-off between somatic maintenance and egg production in urban breeding females. Additionally, urban birds may produce small clutches as an increase in clutch size would be traded off against egg size, the detrimental effects of which city birds cannot compensate for during the nestling rearing stage. Our results also suggest urban birds produce clutches too large to be sustained in the city. Egg removal in urban experimental nests resulted in a brood reduction, and the nestlings in the reduced broods had higher survival prospects than

those in control nests. Thus, the clutch sizes of urban birds may be maladaptive, either due to gene flow between the forest and the city preventing the evolution of clutch size or through birds misjudging the quality of the urban environment as they are time constrained when laying. Overall, our results emphasise a need to incorporate the environmental constraints and fitness costs associated with egg production when attempting to explain variation in reproductive investment and success for birds breeding in anthropogenically modified landscapes.

Ethics Statement

All work involving nest disturbance, egg removal, and cross-fostering was covered by the license 207317 issued by NatureScot to DMD. Permission for bird ringing was granted by the British Trust for Ornithology, with licenses to DMD (permit number: C6822), CJB, and PC-L.

Supplementary Information

Supplementary material is available online in the associated supplement files.

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