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RESEARCH ARTICLE

Human recreation decreases antibody titre in bird nestlings: an overlooked transgenerational effect of disturbance

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ABSTRACT

Outdoor recreational activities are booming and most animals perceive humans as predators, which triggers behavioural and/or physiological reactions [e.g. heart rate increase, activation of the hypothalamic–pituitary–adrenal (HPA) axis]. Physiological stress reactions have been shown to affect the immune system of an animal and therefore may also affect the amount of maternal antibodies a female transmits to her offspring. A few studies have revealed that the presence of predators affects the amount of maternal antibodies deposited into eggs of birds. In this study, using Eurasian blue and great tit offspring (*Cyanistes caeruleus* and *Parus major*) as model species, we experimentally tested whether human recreation induces changes in the amount of circulating antibodies in young nestlings and whether this effect is modulated by habitat and competition. Moreover, we investigated whether these variations in antibody titre in turn have an impact on hatching success and offspring growth. Nestlings of great tit females that had been disturbed by experimental human recreation during egg laying had lower antibody titres compared with control nestlings. Antibody titre of nestling blue tits showed a negative correlation with the presence of great tits, rather than with human disturbance. The hatching success was positively correlated with the average amount of antibodies in great tit nestlings, independent of the treatment. Antibody titre in the first days of life in both species was positively correlated with body mass, but this relationship disappeared at fledging and was independent of treatment. We suggest that human recreation may have caused a stress-driven activation of the HPA axis in breeding females, chronically increasing their circulating corticosterone, which is known to have an immunosuppressive function. Either, lower amounts of antibodies are transmitted to nestlings or impaired transfer mechanisms lead to lower amounts of immunoglobulins in the eggs. Human disturbance could, therefore, have negative effects on nestling survival at early life-stages, when nestlings are heavily reliant on maternal antibodies, and in turn lead to lower breeding success and parental fitness. This is a so far overlooked effect of disturbance on early life in birds.

KEY WORDS: *Cyanistes caeruleus*, Human disturbance, Immunology, Outdoor activities, *Parus major*

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INTRODUCTION

Human sports activities and recreation often occur in nature and may negatively impact wildlife (Bötsch et al., 2017, 2018; Larson et al., 2016; Steven et al., 2011). Humans are often perceived as predators by wildlife (Frid and Dill, 2002), which usually react with behavioural (e.g. flight response; Blumstein, 2010) and/or physiological responses, such as the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Almasi et al., 2015; Fowler, 1999; Thiel et al., 2008). If these behavioural and/or physiological responses occur frequently, they may have long-term impacts on physiology, such as excessive energy expenditure, high levels of baseline glucocorticoids and immunosuppression, which in turn can compromise health and fitness (Tablado and Jenni, 2017). If stress reactions occur in critical life stages, such as gestation or egg laying, they can lead to transgenerational effects, negatively affecting offspring (Alonso-Alvarez et al., 2007; Champagne and Meaney, 2006; Naguib and Gil, 2005). In the case of birds, stressful events occurring during the egg-laying phase may therefore have an important impact on nestlings through, for example, stressed mothers depositing higher levels of the main glucocorticoid hormone corticosterone (Hayward and Wingfield, 2004; Love et al., 2008; Rubolini et al., 2005; Saino et al., 2005) and/or altered levels of antibodies and other (non-specific) immune components in the eggs (Hargitai et al., 2006; Morosinotto et al., 2013; Müller et al., 2004; Saino et al., 2002; Staszewski et al., 2007). Maternal antibodies transferred to the nestling represent the mother's repertoire of IgY antibodies that are circulating in her blood at the time of egg production, which have been produced following exposure to different antigens (Lemke et al., 2003, 2004). Freshly hatched nestlings depend almost entirely on these maternal antibodies for their immune responses for the first weeks of their life (Buechler et al., 2002; Gasparini et al., 2009; Grindstaff et al., 2003; Pihlaja et al., 2006). Therefore, the amount of maternal antibodies deposited into the eggs positively correlates with nestling survival and body condition (Grindstaff, 2008; Pihlaja et al., 2006).

The aim of this study was to experimentally test in Eurasian blue tits *Cyanistes caeruleus* (Linnaeus 1758) (henceforth blue tits) and great tits *Parus major* Linnaeus 1758 whether human recreational activities during the early breeding season (i.e. egg-laying period) affect antibody titre in the following generation (measured in nestlings a few days after hatching), which would be caused by physiological changes in laying females. We also controlled for the effect of other factors possibly modulating these physiological responses such as vegetation cover (reducing detection of humans by birds and/or bird risk perception) or other sources of stress such as competitors. Moreover, we examined whether the amount of circulating antibodies in young nestlings was associated with hatching success and nestling growth, as low amounts of antibodies could lead to reduced survival of embryos or slower growth of nestlings. We predicted that nestlings hatched in areas with higher human frequentation have a lower amount of antibodies compared

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with broods in control areas with no human visitation. We also expected that the reduced amount of antibodies would be correlated with a reduced hatching success and lower body mass compared with control nestlings.

MATERIALS AND METHODS

Study area and experimental design

The study was carried out in the breeding season of 2014 and 2015 in the Forêt domaniale de Chaux in France (47°05′N, 05°40′E), which is a forest consisting primarily of pedunculate oaks (*Quercus robur*) and European hornbeam (*Carpinus betulus*) and whose harvest is managed by the Office National des Forêts (ONF). We worked in 12 plots (mean size 9.2 ha, range: 7.5–13 ha), which fulfilled the following criteria: (1) homogeneous vegetation structure, (2) well separated from each other (>600 m) to avoid spill-over effects, (3) no timber harvesting in the study plots during the entire study period, and (4) location far away from urban settlements (>9 km) to reduce other sources of human disturbance; during our daily presence in the study area for several hours, we encountered <1 human per month (see Bötsch et al., 2017). In early February 2014, we installed 210 nest boxes (Schwegler, Type B1, with 32 mm entrance diameter) for small cavity nesters (mainly tit species) at a density of about two nest-boxes per ha (i.e. not exceeding the natural breeding density of tits; Krebs, 1971). After the first breeding season, in autumn 2014, we cleaned and removed the nest boxes and installed them again in February 2015, to have the same experimental setup for the two study years.

Each plot was divided into two and each split-plot either received an experimental-disturbance treatment (during early spring; 7 March to 22 April in 2014 and 2015; see also Fig. 1) or served as control. The treatment consisted of mimicking a common human recreational activity (i.e. people hiking in the forest) by groups of 2–3 field assistants walking back and forth through the split-plots on a regular mower-pattern transect (distance between walking lines 20 m; for details, see Bötsch et al., 2017). This treatment was applied 1–3 times every day, equally throughout disturbed split-plots. The field assistants carried a loudspeaker (Hama, smartphone speaker, power 3 W, with a Samsung digital audio player F3) broadcasting human conversation (e.g. from TV shows to audio books) at an average volume level of 60 dB at 1 m distance to reproduce normal hiking conversation (Byrne et al., 1994; Hacki, 1996). We varied the direction of the mower-pattern transects by 90 deg between visits, as well as the time of day of the visit, to maintain unpredictability. Because of limited field assistant numbers in 2014, we could only apply the treatment to six (split-)plots, while in 2015 the treatment was applied to all 12 (split-)plots. The six split-plots which were 'disturbed' in 2014 became control split-plots the next year and vice versa. This experiment was approved by the local authorities and the French ringing scheme Centre de Recherches sur la Biologie des Populations d'Oiseaux (C.R.B.P.O.; permit number 2014157-0012 of the Direction Régionale de l'Environnement, de l'Aménagement et du Logement de Franche-Comté and permit number 15006 for blue tits and great tits for 2014–2016 from the C.R.B.P.O.; for details, see also Bötsch et al., 2017).

Blood sampling

Each year from 20 April onwards, all 210 nest boxes were checked every second week. Nest boxes with complete clutches were checked daily around the estimated hatching date to determine the exact hatching date. Blue tit and great tit nestlings were blood sampled at the age of 6 days, when they were large enough to tolerate blood sampling but still had an underdeveloped immune system, and thus still maintained maternal antibody titres (Grindstaff, 2008; Hasselquist and Nilsson, 2009; King et al., 2010). It is possible that at the age of 6 days, nestlings might have already started to produce their own antibodies (depending on the level of development of the bursa of Fabricius), and lost some of their maternal antibody titre (King et al., 2010); however, there is strong evidence that at this early stage of life the proportion of self-produced antibodies is extremely low because the immune system is still immature (Davison et al., 2011; Grindstaff, 2008; Grindstaff et al., 2006; King et al., 2010; Staszewski et al., 2007). Moreover, maternal antibodies at hatching correlate strongly with the total amount of circulating antibodies several days after hatching, as shown by Pihlaja et al. (2006), where for magpie the correlation holds still at day 10. Therefore, we assumed that by measuring antibody titre in 6 day old nestlings, we could obtain a good approximation of the difference between treatments in maternally transmitted antibodies.

At day 6 (mean±s.d.=6.2±0.6 days, range: 4–8 days), all nestlings of a brood were counted and weighed to the nearest 0.1 g with a digital balance and a random subsample of 5 nestlings per brood were blood sampled through vein puncturing with a

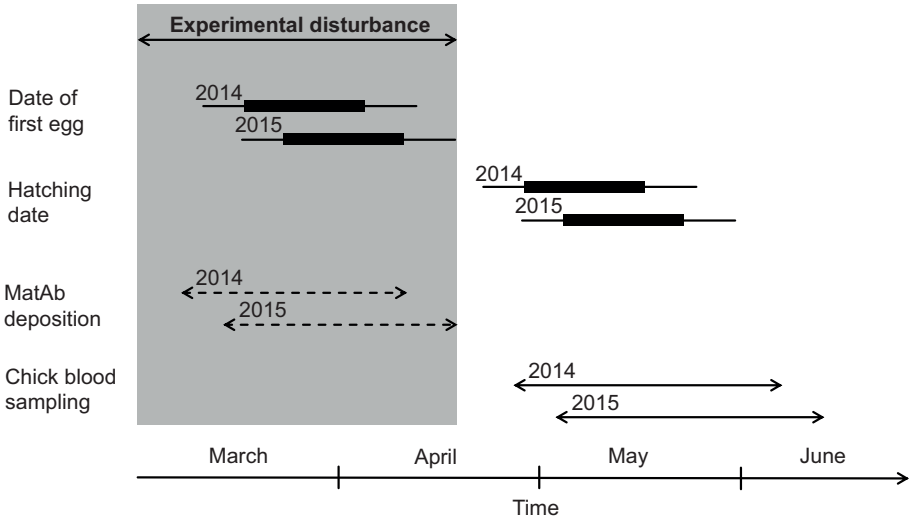

Fig. 1. Diagram of the temporal overlap of experimental disturbance and breeding stages (egg laying and hatching). Note that the deposition of the maternal antibodies (MatAb) in eggs overlapped with the disturbance phase, whereas hatching and the following feeding period did not. Breeding in the second study year (2015) was about 1 week later than that in the first study year (2014).

0.3 mm needle at the metatarsus and collecting the effluent blood with a heparinised capillary tube. In nestlings where blood sampling was successful, we collected up to 40 µl and directly centrifuged in the field for 5 min at 8000 rpm (Hettich, EBA 3S) to separate the plasma from the cells. Both blood cells and plasma were stored in liquid nitrogen or deep freezers (−20°C) until analysis.

When nestlings were about 15 days old (13–16 days), they were weighed again and ringed with an aluminium ring from the C.R.B.P.O. The number of ringed nestlings per brood served as a proxy for the number of fledglings, as nestling mortality at this stage is assumed to be low (Lindén et al., 1992; Oddie, 2000). In 2015, we additionally marked blood-sampled nestlings with small, coloured elastic bands and therefore could identify them individually when ringing. Therefore, the analysis of body mass increment of individual nestlings is based on 2015 data only.

Maternal antibody measurement

Antibodies were measured with an ELISA (after Karell et al., 2008), carried out in 96-well microplates, which were coated with an anti-chicken IgG (Sigma C-6409) diluted 1:180 in a 0.05 mol l^{-1} carbonate buffer (pH 9.6) and incubated overnight at 4°C. Afterwards, the plates were washed 3 times with phosphate-buffered saline (PBS)/Tween 20 and blocked (for at least 1 h at room temperature) with 1% BSA (bovine serum albumin) diluted in 0.01 mol l^{-1} PBS/Tween 20 (hereafter referred to as BSA-PBS/Tween 20). After washing, 100 µl of the diluted test plasma was added in duplicate to the wells. The plasma samples (3 µl) of blue tits were diluted 1:100 and those of great tits 1:500. On each plate, we added in duplicate a standard dilution series of a chicken plasma pool: pure buffer as a negative control and seven dilutions from 1:10,000 up to 1:640,000 (diluent: BSA-PBS/Tween20). All plasma sample measures were then expressed relative to this standard in units per µl. Two wells were filled with the BSA-PBS/Tween 20 buffer as blanks, and two wells were filled with a 1:160,000 diluted chicken plasma pool as internal controls. The plates were incubated for 3 h at room temperature and afterwards washed again 3 times with 250 µl PBS/Tween 20. Then, 100 µl of 1:3000 diluted (BSA-PBS/Tween 20) peroxidase-conjugated rabbit anti-chicken IgG (Sigma A9046) was added to each well, except the blanks, where 100 µl BSA-PBS/Tween 20 buffer was added instead. The plates were incubated overnight at 4°C and again washed 3 times with 250 µl PBS/Tween 20. Then, 100 µl of the substrate solution was added, consisting of 20 ml citrate buffer (pH 4), 80 µl of 1:40 diluted 30% hydrogen peroxide (diluted in distilled water) and 200 µl ABTS [2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)]. The plate was then put on a plate shaker for 15 min and the absorbance measured with an ELISA plate reader at 405 nm (Bio-Rad, Benchmark Microplate Reader). For all consecutive analyses, the mean of the duplicate measures was computed.

Pooled plasma samples of blue tits (linear range 1:50–1:400) and great tits (1:500–1:1000) produced a dose–response curve that paralleled the pooled chicken IgG standard curve. All samples were analysed on 52 plates (96-well plates). Intra- and inter-assay coefficients of variation were 3.9% and 28.3% in 2014 and 2.9% and 29.7% in 2015. Because of the relatively high inter-assay variation, we always include batch (group of plates; up to four plates were run in parallel) as a random factor in the models. Note that samples were assigned to the plates according to nest box number, which excluded any bias according to treatment (similar amounts of disturbed and undisturbed samples per plate).

Vegetation mapping

In June 2015, we conducted a vegetation survey. With a stratified random sampling, we distributed one survey point per 0.5 ha (n=210) and measured the following habitat variables: ground cover (%) on a 2×2 m area, shrub cover (%) on a 3×3 m area, number of trees (diameter at breast height >5 cm) per species, standing deadwood on an 8×8 m area and canopy cover (%, by looking straight up to the canopy and estimating the amount of sky covered by the canopy in the observer's visual field). The vegetation measures were averaged within each split-plot, and from these means we computed a principal component analysis (PCA). We used only the first axis score of the PCA for further analyses, because it explained 65% of the variation, and because the second axis was correlated with ground cover, which is unlikely to modulate the effect of human disturbance on the birds (see Table S1).

Statistical analyses

Only first broods with at least one egg laid during the experimental disturbance period were used (see Fig. 1). Predated broods (2014: 7 control and 3 treatment broods; 2015: 6 control and 10 treatment broods) were excluded from all analyses as they do not reflect the direct effect of human disturbance. All analyses were done using the lme4 package in R 3.3.0 (Bates et al., 2015; https://www.R-project.org/). To investigate the potential effect of experimental disturbance on antibody titre in great tit and blue tit nestlings, we used two linear mixed models (Table 1). Antibody measures were log-transformed

Table 1. Dependence of antibody titre on human disturbance and other parameters

	Great tit			Blue tit		
Variable	Estimate	95% CrI	PP	Estimate	95% CrI	PP
Intercept	5.360	5.051; 5.670	–	3.982	3.360; 4.589	–
First principal component (vegetation)	0.031	−0.109; 0.175	0.67	−0.071	−0.292; 0.144	0.74
Disturbance (disturbed)	−0.095	−0.242; 0.050	0.90	0.007	−0.254; 0.272	0.52
Julian date of the first egg	0.172	0.069; 0.277	>0.99	0.164	0.009; 0.321	0.98
Difference in body mass to heaviest sibling in g	−0.215	−0.257; −0.173	>0.99	−0.102	−0.173; −0.031	>0.99
Nestling age	0.062	−0.024; 0.145	0.93	0.105	−0.083;0.293	0.86
Relative GT occupancy	−0.082	−0.196; 0.029	0.93	−0.197	−0.503;0.115	0.89
Total number of occupied neighbouring nest boxes	0.010	−0.079; 0.082	0.51	0.050	−0.080;0.180	0.77
Year (2014)	−0.148	−0.713; 0.410	0.70	0.026	−0.873;0.963	0.53
First principal component (vegetation)×disturbance (disturbed)	0.168	−0.001; 0.337	0.98	0.094	−0.145;0.336	0.78

Model estimates with their corresponding 95% credible intervals (CrI) are given for blue tits and great tits. Posterior probabilities (PP) can take values between 0.5 and 1. The reference categories are nests from control split-plots in 2015. The factor level of each categorical variable is given in parentheses. Results are based on 297 great tit and 106 blue tit nestlings out of 111 and 38 broods, respectively. GT, great tit.
Distribution: normal; random factors: nest box ID (with random slope disturbance), split-plot ID nested within plot ID and plate ID nested within batch ID.

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Table 2. Effect of nestling antibody titre and other parameters on hatching success

	Great tit			Blue tit		
Variable	Estimate	95% CrI	PP	Estimate	95% CrI	PP
Intercept	2.507	2.023; 2.990	–	2.151	1.385; 2.895	–
First principal component (vegetation)	−0.079	−0.381; 0.223	0.70	−0.007	−0.493; 0.464	0.51
Mean antibody titre in units μl^{-1}	0.265	0.017; 0.510	0.98	−0.076	−0.393; 0.240	0.68
Relative GT occupancy	−0.182	−0.514; 0.147	0.86	−0.349	−1.005; 0.336	0.84
Julian date of the first egg	−0.021	−0.363; 0.309	0.55	0.025	−0.314; 0.362	0.56
Year (2014)	0.690	−0.471; 1.859	0.87	−0.661	−2.211; 0.967	0.79

Model estimates with their corresponding 95% credible intervals (CrI) are given for great tits and blue tits. Posterior probabilities (PP) can take values between 0.5 and 1. Results are based on 102 great tit and 35 blue tit broods. GT, great tit.
Distribution: binomial, link function logit; random factors: number of sampled chicks per nest, split-plot ID nested within plot ID and plate ID nested within batch ID.

(natural logarithm) to fulfil model assumptions. We then tested for the effect of the two-level factor disturbance (disturbed versus control split-plots). As the effect of disturbance could be obscured by the cover provided by vegetation, we also included as explanatory variables the first principal component of the vegetation PCA [first principal component (vegetation)] and the interaction between disturbance and vegetation. Furthermore, we tested for the effect of laying date of the first egg (Julian date of the first egg), nestling age at sampling (in days) and the difference in body mass between the measured nestling and its heaviest sibling [difference in body mass (in g) from heaviest sibling] as a surrogate for hatching order. Given that competition for nesting sites may also be a strong biotic stressor, we accounted for the effect of competition stress by including the total number of occupied nest boxes (independent of the species) within a 100 m radius (total number of occupied neighbouring nest boxes), as a nest box-specific measure of breeding density. In addition, as great tits are dominant over blue tits and other tit species, we also tested for the proportion of nest boxes per split-plot that were occupied by great tits (i.e. number of nest boxes occupied by great tits divided by all occupied nest boxes in a split-plot; relative GT occupancy). Finally, we included year as a factor into the models.

In the above-mentioned two models, we accounted for the non-independence of the data within broods by including the random factor brood ID, nested within split-plot ID. We also included plot ID to further account for spatial autocorrelation. Additionally, we allowed the effect of disturbance to vary among plots by including the variable disturbance as a random slope within the random factor plot ID. To account for the non-independence of antibody measures between plates and batches (several plates per lab run), we included a random factor plate ID in the first two models, nested within batch ID.

For the analyses investigating the relationship between antibody titre and hatching success in both great tits and blue tits, we used two generalised linear mixed models with a binomial error distribution (Table 2), in which hatching success was modelled as the number of hatched nestlings (at day 6) among the total number of eggs (clutch size). We then tested for the effect of antibodies (mean antibody titre in units μl^{-1}) and we also controlled for the effect of relative GT occupancy, Julian date of the first egg, first principal component (vegetation) and year. In order to avoid over-parameterisation, due to the lower sample size for these models, variables like nestling age and total number of occupied neighbouring nest boxes that were not found to have a substantial effect in the previous models (Table 1) were not included. Because of the potential introduction of bias while computing the mean antibody titre if only one nestling was sampled within a brood, we removed for these analyses all nests where only one nestling was sampled (seven out of 109 great tit nests and three out of 38 blue tit nests). Additionally, we accounted for the number of successfully sampled nestlings within a brood as an additional random factor (see Table 2). As for the previous models, we accounted for the non-independence of the data by including a random factor plate ID nested within batch ID and a random factor split-plot ID nested within plot ID.

In order to examine the relationship between antibodies and nestling growth (Table 3), we used nestling mass as the dependent variable and modelled how nestling mass varied with age and antibodies by including an interaction between the natural logarithm of nestling age and the antibody titre [antibody titre in units $\mu l^{-1}\times$ ln (nestling age)]. We also accounted for the effect of relative GT occupancy, Julian date of the first egg, the difference in body mass to heaviest sibling in g, as a surrogate for hatching order, and vegetation [first principal component (vegetation)]. Moreover, we

Table 3. Dependence of body mass increase from day 6 to day 13–16 on antibody titre and other factors

	Great tit			Blue tit		
Variable	Estimate	95% CrI	PP	Estimate	95% CrI	PP
Intercept	−7.367	−7.770; −6.960	–	−3.100	−3.823; −2.387	–
Antibody titre in units μl^{-1}	2.027	1.618; 2.435	>0.99	1.428	0.705; 2.141	>0.99
ln(nestling age)	9.715	9.544; 9.886	>0.99	5.605	5.304; 5.916	>0.99
Minutes since sunrise	−0.174	−0.657; 0.315	0.76	0.102	−0.708; 0.898	0.60
Relative GT occupancy	0.105	−0.048; 0.259	0.91	0.115	−0.169; 0.401	0.79
Julian date of the first egg	0.283	0.125; 0.444	>0.99	0.032	−0.255; 0.320	0.59
Number of fledglings	0.149	−0.004; 0.306	0.97	−0.081	−0.345; 0.171	0.73
Difference in body mass to heaviest sibling in g	−0.433	−0.535; −0.333	>0.99	−0.111	−0.317; 0.100	0.85
Antibody titre in units $\mu l^{-1}\times$ln(nestling age)	−0.801	−0.979; −0.627	>0.99	−0.596	−0.911; −0.288	>0.99
Minutes since sunrise×ln(nestling age)	0.190	−0.023; 0.405	0.96	−0.078	−0.425; 0.277	0.67

Estimates of the body mass development models depending on antibody titre with their corresponding 95% credible intervals (CrI) for great and blue tits. Data used are only from 2015 when nestlings were marked and followed individually. Posterior probabilities (PP) can take values between 0.5 and 1. Body mass development results are based on 239 great tit (461 measures) and 30 blue tit (59 measures) nestlings from 97 and 18 broods, respectively.
Distribution: normal; random factors: chick ID nested within nest box ID nested within split-plot ID nested within plot ID.

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also tested for the effect of brood size on growth by including the number of fledged nestlings (number of fledglings), and controlled for the effect of time of day (when weighing the nestlings) on nestling mass (minutes since sunrise) both alone and in interaction with the natural logarithm of nestling age [minutes since sunrise×ln (nestling age)]. As in the previous model, to account for the non-independence of multiple measures within nestlings (i.e. at different ages), we included as random factors nestling ID nested within brood ID, nested within split-plot ID, nested within plot ID. As these data were available only for 2015, it was not necessary to introduce the year effect.

Model parameters and their respective 95% credible intervals (CrI) were calculated using a Bayesian framework. We simulated 10,000 random samples from the joint posterior distribution of the model parameters using the function sim (which incorporates uninformative priors) from the package arm (https://CRAN.R-project.org/package=arm). From these simulated data, we used the 2.5% and 97.5% quantiles as the lower and upper bands of our 95% CrI (Korner-Nievergelt et al., 2015). To assess the impact of each variable, we computed the corresponding posterior probabilities (PPs; Tables 1–3; Table S2), which can take values between 0.5 and 1, with larger values representing a stronger effect. PPs are calculated as the mean of the difference (to zero or between factor levels) of the 10,000 random samples which are different from zero. If a given variable is continuous, the PP depicts the strength of the given slope being different from zero, whereas if a variable is categorical, it depicts the difference to the reference category. With the model estimates, it is possible to calculate PPs of slope differences between each other and not compared with zero (we did that for Figs 2B and 4A,B).

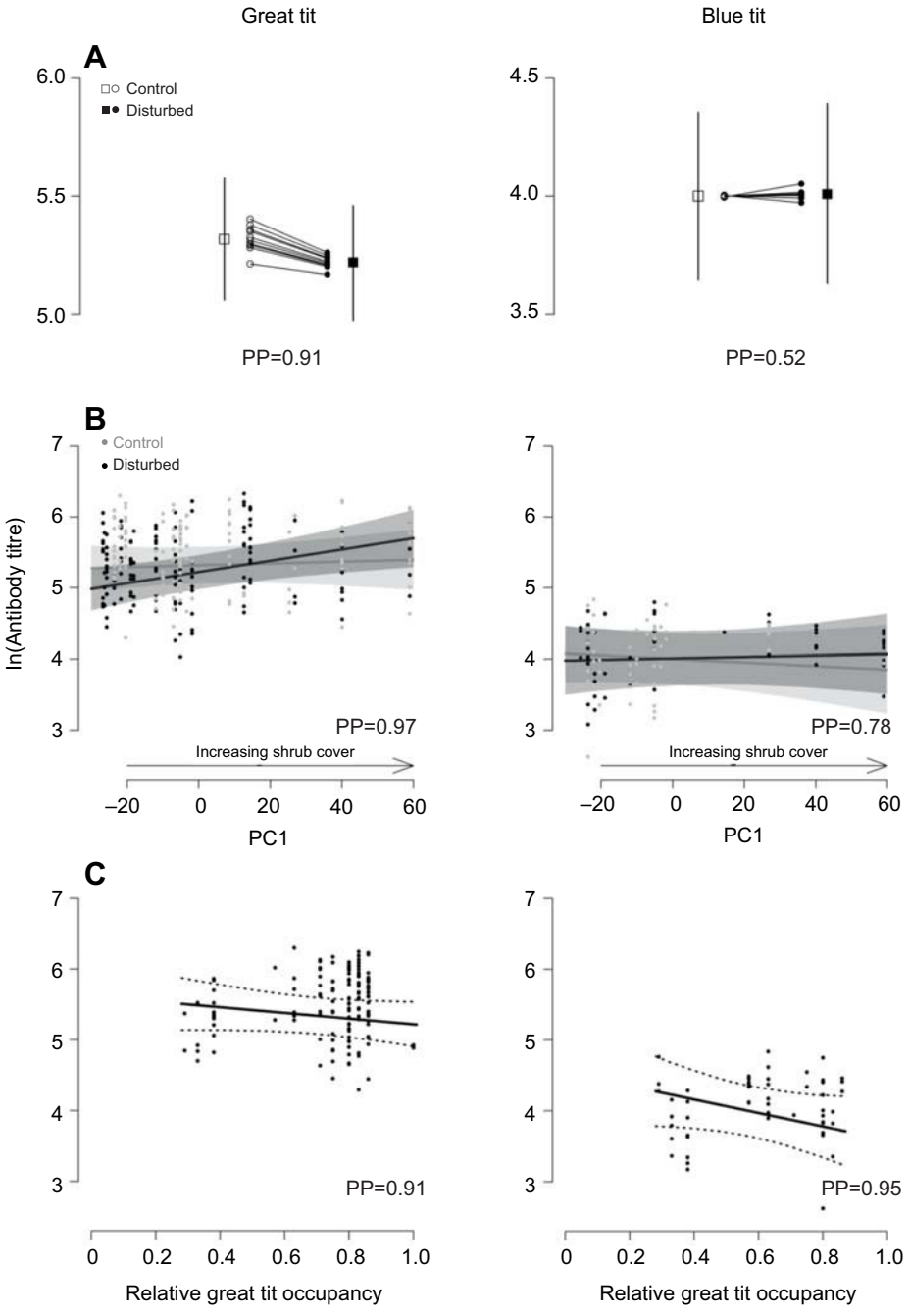

Fig. 2. Effect on antibody titre of experimental disturbance and modulating factors for an average year. (A) Antibody titre (units μl^{-1}) in great tit and blue tit nestlings (n=297 great tit and 106 blue tit nestlings out of 111 and 38 broods, respectively). (B,C) Modulating influence of vegetation density (B) and of competition for nesting sites (C). In A, squares and lines represent mean estimates±95% credible interval (CrI); circles are individual plot estimates (for the control and disturbed split-plots). In B and C, solid lines indicate model estimates (mean); shaded polygons and dotted lines indicate the ±95% CrIs; and dots are the raw data points (in C, only the relationship for control plots is shown). In all three cases, the posterior probability (PP) indicates the strength of the effect. The higher the probability (from 0.5 to 1), the higher the certainty of a true effect of the variable tested.

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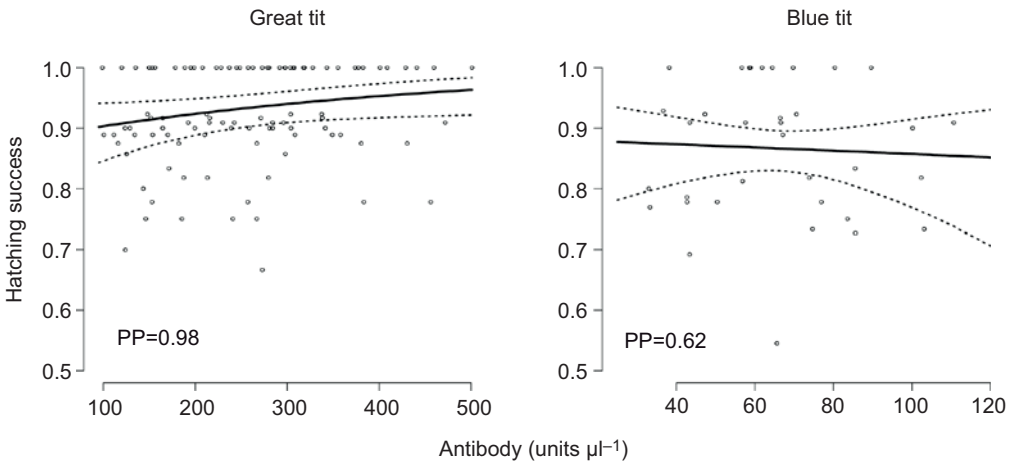

Fig. 3. Effect of antibody titre on hatching success. Shown are model estimates (solid lines) with corresponding 95% CrIs (dotted lines) for an average year. The *x*-axis corresponds to the mean antibody titre per brood (great tit: *n*=102 broods, blue tit: *n*=35 broods). PP indicates the posterior probability (0.5 to 1) that slopes differ from zero. Open circles are the raw data points. Note the different *x*-axes ranges.

RESULTS

The experimental disturbance resulted in a reduction of antibodies in recently hatched nestlings, compared with control broods, in great tits but not in blue tits (Fig. 2A, Table 1). The interaction between disturbance and the first principal component (vegetation), which is highly correlated with shrub-cover (Table S1), showed that vegetation density modulated the impact of disturbance on transmission of antibodies to offspring in great tits (Table 1, Fig. 2B). That is, in disturbed split-plots, but not in controls, circulating antibodies of young great tit nestlings were especially low in plots where the vegetation cover was less dense (Fig. 2B). In the case of the blue tits, we did not find an effect of experimental disturbance (Fig. 2A), but there was a negative effect of the relative density of breeding great tits on antibody titre of young blue tit nestlings, which was also present, although less relevant, in great tits (Fig. 2C, Table 1).

For both species, Julian date of the first egg and for great tits also nestling age were positively correlated with antibody titre, while hatching order (i.e. difference in body mass to the heaviest sibling) showed a negative correlation (Table 1).

Hatching success of great tits, but not of blue tits, was positively related to antibody titre of the hatched nestlings (Fig. 3 and Table 2). For both species, the relative density of breeding great tits was slightly negatively correlated with hatching success (Table 2).

For both species, antibody titre of 6 day old nestlings was positively correlated with nestling body mass at the same early age (Fig. 4A,B and Table 3). This positive relationship was not present anymore when examining the correlation of antibody titre (measured at day 6) with body mass shortly before fledging (Fig. 4A,B). This translated to a lower body mass increment in nestlings with higher initial antibody titres, as can be seen when comparing the slopes of the different weight development curves for different antibody titres (Fig. 4). As expected, body mass increase also depended on nestling age, brood size, season and rank in the size hierarchy in great tits.

DISCUSSION

In this study, we showed that experimental human disturbance during egg laying reduced antibody titre in great tit nestlings, which represents a transgenerational effect. The effect of human disturbance was, however, not homogeneous throughout space, but depended on vegetation density. We found that a higher density of shrub vegetation reduced the negative impact of disturbance on nestling antibody titre. Vegetation may have acted as a protective shield, buffering human disturbance (Tablado and Jenni, 2017).

We hypothesise that the most likely mechanism to explain the transgenerational effect of the experimental disturbance on young nestling circulating antibodies is that human disturbance acted as a biological stressor for laying females, leading to consistently increased levels of circulating corticosterone, which in turn reduced their antibody titre through its immunosuppressive action (Rubolini et al., 2005; Saino et al., 2003). A reduced female antibody titre, or possible impaired transfer mechanisms into the egg, would result in fewer antibodies (maternal antibodies) in their eggs, and

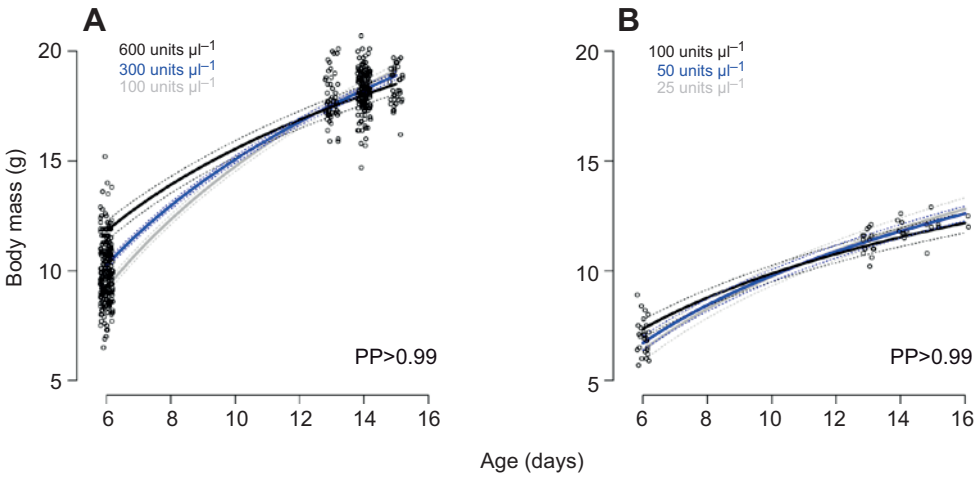

Fig. 4. Body mass development from day 6 until day 13–16 versus antibody titre. (A) Great tits [*n*=239 great tit nestlings (461 measures) out of 97 broods]. (B) Blue tits [30 blue tit nestlings (59 measures) out of 18 broods]. Shown are model estimates (mean)±95% CrI for three selected species-specific antibody titres. PP indicates the posterior probability that the relationship between body mass and age (growth curve) varies with differing maternal antibody titre. Circles are the raw data points.

subsequently fewer antibodies in young nestlings. We cannot confirm the exact mechanism because we decided not to enter the control (undisturbed) plots during egg laying in order to avoid any human disturbance-induced brood abandonment (Kania, 1992), which would have invalidated our experiment, and therefore did not capture laying females (or collect faeces samples) to measure their corticosterone levels or antibody titre. In a similar way, we avoided measuring corticosterone or antibodies in eggs to reduce the invasiveness of the study and to leave clutch/brood size unchanged. However, there is strong evidence that corticosterone, which is released under stressful situations, including disturbance by humans (Almasi et al., 2015; Müllner et al., 2004), has immunosuppressive effects (e.g. Rubolini et al., 2005; Saino et al., 2003; Stier et al., 2009), leading to reduced amounts of circulating immunoglobulins (Bourgeon and Raclot, 2006; Gao et al., 2016; Hargitai et al., 2009). Therefore, this physiological cascade is a likely mechanism through which our experimental disturbance could have resulted in lower antibody titres transmitted from laying females to their eggs.

In the case of blue tits, we did not find an effect of the disturbance treatment or its interaction with vegetation on young nestling circulating antibody titre; however, we did find a negative effect of the relative abundance of great tits. The entrance diameter of our nest boxes permitted both species to enter, and great tits are dominant over blue tits and may prevent the occupation of nest boxes by blue tits (Barrientos et al., 2015; Löhrl, 1977). A plausible explanation for the pattern observed in blue tits could be that great tits are an important biological stressor for blue tits, and therefore the presence of great tits may act as a modulating factor, which obscures the negative effect of our experimental disturbance.

Predator abundance could have been lower in the disturbed than in undisturbed split-plots, because many predators avoid humans (Berger, 2007; Muhly et al., 2011). However, if this was the case, humans would have acted as a shield from predators and the expected effect would have been the opposite of what we found. Therefore, we believe that potential changes in predator pressure were not a significant issue in our study. Another potential issue concerns settlement decision of tits; the experimental disturbance could have affected this as split-plots with an experimental disturbance could have been less attractive and therefore avoided by the birds. The presence of humans has been shown to lead sometimes to reduced numbers of birds (Bötsch et al., 2017). Such non-random settlement according to individual quality may have resulted in differences in reproductive parameters. However, additional analyses revealed no differences between control and treatment groups in hatching date, clutch size, number of hatchlings and fledglings, and nestling body mass (Table S2). Therefore, our experimental disturbance (2–3 passages per day) was probably not sufficient to affect parental investment or settlement.

Hatching success for great tits was higher in broods in which 6 day old nestlings had higher antibody titres. Because we expect that antibody titre at an early age is representative of the amount of maternally transmitted antibodies, this increase in hatching success with increasing circulating antibodies could imply that maternal antibodies protect from infections, which would otherwise cause the death of the embryo or the freshly hatched young (Gasparini et al., 2001; Tschirren et al., 2009). Note that we counted the number of hatchlings at day 6, and therefore we do not know whether failed eggs or the death of freshly hatched nestlings caused the difference between clutch size and number of hatchlings. Another explanation would be that maternal antibodies are correlated with other non-measured variables that may affect hatching probability. For example, stressed mothers may have laid lower quality eggs, with higher yolk corticosterone, modified other egg components or incubated less well than non-stressed mothers (Hayward and Wingfield, 2004; Cyr and Romero, 2007; DuRant et al., 2013).

In addition, we found that antibodies at day 6 positively correlated with body mass, probably as a result of nestlings having more maternal antibodies, allowing them to invest more into growth (Brommer, 2004; Soler et al., 2003). However, at fledging, there was no correlation between body mass and antibody titre at day 6, because lighter nestlings caught up in body mass, and hence had a higher body mass increment and reached a similar body mass near fledging, as also found by Tschirren et al. (2009). This result coincides with the findings of Ismail et al. (2015), who interpreted the lower body mass increment as a cost of having more maternal antibodies. However, in our case, nestlings with larger amounts of antibodies were also the largest nestlings at day 6 and therefore needed to gain less mass to reach fledging body mass than smaller nestlings (see Fig. 4A,B). During our two study years, weather conditions were favourable during the breeding season, which allowed nestlings with lower body mass at day 6 to catch up and reach optimal body mass at fledging, and therefore fledging success and body condition at fledging were high even in large broods (blue tits up to 16 fledglings).

Whether human recreation negatively affects birds transgenerationally, through changes in maternal antibody deposition, certainly also depends on several other modulators. These include the intensity of disturbance, the type of human recreation activity, the mother's stress tolerance (e.g. habituation towards humans) and, as we showed, vegetation density and inter-specific interactions. Dense vegetation may buffer the disturbance impact, which has also been shown in the case of behavioural escape responses to disturbance (e.g. Thiel et al., 2007). There are also factors other than disturbance, driving variation in antibody titre, such as body condition of the mother (e.g. food availability), clutch size, hatching order (as in this study; see Table 1), sex, date (as in this study; see Table 1) or the health status of the mother and the nestlings in general (Boulinier and Staszewski, 2008; Grindstaff et al., 2003; Hasselquist and Nilsson, 2009; Klasing and Leshchinsky, 1998; Lobato et al., 2008). Although we could not rule out that other factors also affect nestling antibodies, this does not invalidate our results, as the fact of finding an effect of experimental disturbance, despite all the potential sources of 'noise', reinforces our findings.

In the present study, we showed for great tits that human disturbance in the critical life stage of egg production may result in lower antibody titre in the next generation and that this is linked to hatching rate and body mass of young nestlings. Although we did not specifically test whether maternal antibodies had an effect on nestling survival, they most likely do so as shown by Pihlaja et al. (2006), and this impact could be particularly detrimental in cases where disturbance continues through the entire breeding season. Up to now, mainly direct effects of human disturbance on reproduction have been shown, such as prevention of chick feeding and interruption of incubation, or indirect effects such as noise (Safina and Burger, 1983; Schroeder et al., 2012; Zanette et al., 2011). Here, we propose that disturbance during egg laying results in reduced antibody titre deposited in eggs, and therefore in recently hatched nestlings. This is a so far overlooked transgenerational effect of human disturbance on wildlife and therefore protected areas, with prohibited human access, are necessary refuges for wildlife.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: Y.B., Z.T., B.A., L.J.; Methodology: Z.T., B.A., L.J.; Validation: Z.T., B.A.; Formal analysis: Y.B., Z.T.; Investigation: Y.B.; Data curation: Y.B.; Writing - original draft: Y.B., Z.T., L.J.; Writing - review & editing: Y.B., Z.T., B.A., L.J.; Supervision: Z.T., L.J.; Project administration: L.J.

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APPENDIX

Human recreation decreases antibody titres in bird nestlings: an overlooked transgenerational effect of disturbance

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Table S1: Principal component analysis (PCA) of 23 habitat variables. Shown are the first three principal components with the cumulative variance (last line). Because only three variables had loadings > 0.1, the remaining 20 variables are not shown.

	PC1	PC2	PC3
Mean canopy cover	-0.193	-0.263	-0.943
Mean shrub cover	0.953	0.177	-0.245
Mean ground vegetation cover	-0.231	0.948	-0.216
Cumulative variance	65.1%	95.7%	99.8%

Table S2: Year-specific model estimates with their corresponding 95% credible intervals in parenthesis and posterior probabilities (PP). For each species and year five different models have been calculated.

		Great tit				Blue tit			
		2014		2015		2014		2015	
		Estimate (95% CrI)	PP	Estimate (95% CrI)	PP	Estimate (95% CrI)	PP	Estimate (95% CrI)	PP
Hatching date [Julian]	Intercept	111.7 (106.8; 116.6)	-	121.0 (119.3; 122.6)	-	116.9 (108.9; 124.8)	-	122.3 (119.5; 125.1)	-
	Disturbance (disturbed)	1.9 (-4.3; 7.9)	0.74	-0.5 (-2.2; 1.1)	0.74	1.3 (-9.1; 11.8)	0.61	0.4 (-3.4; 4.2)	0.60
Clutch size (log)	Intercept	2.5 (2.3; 2.7)	-	2.3 (2.2; 2.4)	-	2.4 (2.2; 2.6)	-	2.4 (2.3; 2.6)	-
	Disturbance (disturbed)	-0.1 (-0.4; 0.2)	0.79	-0.03 (-0.2; 0.1)	0.71	0.08 (-0.2; 0.4)	0.70	0.06 (-0.2; 0.3)	0.70
Number of hatchlings (log)	Intercept	2.5 (2.3; 2.7)	-	2.2 (2.1; 2.3)	-	2.3 (2.1; 2.5)	-	2.3 (2.1; 2.5)	-
	Disturbance (disturbed)	-0.1 (-0.4; 0.2)	0.76	-0.09 (-0.2; 0.05)	0.90	0.01 (-0.3; 0.3)	0.53	0.08 (-0.2; 0.3)	0.72
Number of fledglings (log)	Intercept	2.4 (2.2; 2.7)	-	2.2 (2.1; 2.3)	-	2.2 (2.0; 2.5)	-	2.3 (2.1; 2.5)	-
	Disturbance (disturbed)	-0.08 (-0.4; 0.2)	0.71	-0.06 (-0.2; 0.08)	0.81	0.06 (-0.3; 0.4)	0.64	0.06 (-0.2; 0.3)	0.67
Nestling body mass [g]	Intercept	9.8 (9.0; 10.7)	-	10.1 (9.8; 10.4)	-	7.0 (6.5; 7.4)	-	7.1 (6.6; 7.6)	-
	Disturbance (disturbed)	-0.3 (-1.6; 0.9)	0.70	-0.1 (-0.6; 0.3)	0.70	-0.2 (-0.7; 0.4)	0.70	-0.4 (-1.1; 0.3)	0.86

Distribution: normal or poisson; random factors: (nest box ID nested within (only for the last model)) split-plot ID nested within plot ID.