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Microbiome affects egg carotenoid investment, nestling development and adult oxidative costs of reproduction in Great tits

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Summary

1. Parasites influence allocation trade-offs between reproduction and self-maintenance and consequently shape host life-history traits. The host microbiome includes pathogenic and commensal micro-organisms that are remarkable in their diversity and ubiquity. However, experimental studies investigating whether the microbiome shapes host reproduction are still lacking.
2. In this study, we tested whether the microbiome affects three important components of bird reproduction, namely (i) the maternal transfer of anti-microbial compounds to the eggs, (ii) the development of nestlings and (iii) the trade-off between reproduction and self-maintenance, here measured by the oxidative costs of reproduction.
3. We experimentally modified the microbiome of wild breeding Great tits (*Parus major*) by spraying nests with liquid solution that either favoured or inhibited bacterial growth compared to a control. These treatments modified the bacterial communities in the nests and on adult feathers.
4. We found that females from the treatment that decreased bacterial densities in the nests laid eggs with less carotenoids than females from the control, while we found no significant effect of increasing bacterial densities and modifying community composition compared to the control. Nestlings exposed to decreased bacterial densities grew faster and had longer tarsus length at fledging. Moreover, our analyses revealed that the relationship between investment in reproduction and oxidative damage was affected by the treatments. Adults raising larger clutches suffered higher oxidative damage in control nests, whereas this oxidative cost of reproduction was not detected when we modified bird microbiome.
5. Our study provides experimental evidence for an effect of the microbiome on egg carotenoid investment, nestling development and oxidative cost of reproduction and thus highlights the major effect that the microbiome may have on the evolution of host life-history strategies.

Key-words: life-history traits, maternal effects, microbial communities, nestling growth, oxidative stress

Introduction

Micro-organisms constitute the major part of the earth biomass, largely outnumbering animal and plant cells. Whereas commensal micro-organisms are often involved in host digestion or nutrient synthesis (Stevens & Hume 1998), exposure to pathogenic micro-organisms can be costly since they can reduce reproductive success and survival (Sheldon & Verhulst 1996; Clayton & Moore 1997). The numerous micro-organisms constituting the host microbiome, defined as the total microbial community in

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contact with the host (Morgan, Segata & Huttenhower 2013), could play an important role in the evolution of host life-history traits (Ezenwa *et al*. 2012; McFall-Ngai *et al*. 2013), although experimental evidence from natural systems is still lacking (Ezenwa *et al*. 2012).

Studies investigating host-micro-organisms interactions have largely been performed by focussing on one or a few micro-organisms, aiming to define it as pathogen or symbiotic with regard to the host (Sheldon & Verhulst 1996; Clayton & Moore 1997; Schmid-Hempel 2011). However, hosts are never exposed to only one micro-organism (Balmer & Tanner 2011; Alizon, de Roode & Michalakis 2013), and competition and cooperation between the dozens to hundreds of micro-organisms forming the host microbiome (Faust *et al*. 2012; Human Microbiome Project Consortium 2012; Morgan, Segata & Huttenhower 2013) can have major consequences for the hosts (Alizon, de Roode & Michalakis 2013; Morgan, Segata & Huttenhower 2013). In this experimental study, we thus considered that the host is affected by a community of micro-organisms, the resulting effect on host being determined by the outcome of interactions between micro-organisms within the microbiome.

Micro-organisms in bird nests are highly diverse (Goodenough & Stallwood 2010) and can infect embryos, reducing egg viability (Cook *et al*. 2003). Moreover, it has been suggested that nest micro-organisms can affect nestlings during their growth. For instance, Corsican blue tits (*Cyanistes caeruleus*) and Sandwich terns (*Sterna sandvicensis*) use aromatic plants in their nests that reduce bacterial diversity and abundance in the nests and favour nestlings' health (Mennerat *et al*. 2009a,b; Møller *et al*. 2013). Moreover, González-Braojos *et al*. (2012) found a negative correlation between nest bacterial densities and nestling condition. Since nestling condition at fledging (i.e. tarsus length and/or body mass) positively correlates with survival and recruitment (Tinbergen & Boerlijst 1990; Lindén, Gustafsson & Pärt 1992; Both, Visser & Verboven 1999; Heeb *et al*. 1999), the nest microbiome can be expected to have significant effects on reproductive success in birds. However, experimental evidence for an effect of the microbiome on nestling development is still lacking.

It has been proposed that female birds could protect embryos and hatchlings from pathogenic micro-organisms by allocating antimicrobial compounds to the eggs (Mousseau & Fox 1998; Saino *et al*. 2002b). Two of the most abundant antimicrobial substances in egg albumen are ovotransferrin and lysozymes (Shawkey *et al*. 2008). Ovotransferrin chelates compounds required for microbial growth (Li-Chan, Powrie & Nakai 1995), whereas lysozymes lyse the polysaccharide walls of Gram-positive bacteria (Rogers & Perkins 1969). Moreover, egg yolk contains carotenoids that are considered important immune-stimulants and antioxidants in birds (Blount *et al*. 2003; Alonso-Alvarez *et al*. 2004a; but see Costantini & Møller 2008). Egg carotenoids have been suggested to be essential to offspring growth and the development of their immune system (Møller, Biard & Blount 2000; Saino *et al*. 2003; Romano *et al*. 2008; Newbrey & Reed 2009; Tanvez *et al*. 2009). For instance, carotenoid supplementation in nestlings of Blue and Great tits has positive effects on body condition at fledging (Biard, Surai & Møller 2007) and increases fledging success (Marri & Richner 2014). Antimicrobial compounds are likely to be costly to produce and use (Van de Crommenacker *et al*. 2010), and carotenoids have been suggested to be limited since they can only be acquired through the diet and because their use for immunity trades off against their use as antioxidants and/or pigments in sexual coloration (Von Schantz *et al*. 1999; Møller, Biard & Blount 2000). Moreover, supplementing mothers in carotenoids during laying increase female carotenoid investment in eggs and have positive effects on nestlings (Berthouly, Helfenstein & Richner 2007; Biard, Surai & Møller 2007; Berthouly, Cassier & Richner 2008). We thus expected females to adjust their investment in antimicrobial compounds and carotenoids in eggs depending on their own microbial exposure and the infection risk for embryos and nestlings.

Reproductive investments, from the production of eggs to the raising of offspring, are physiologically demanding activities that can result in an increase in the metabolic rate, the production of excessive amount of free radical and reactive species, and thus oxidative stress (Stearns 1992; Oppliger, Christe & Richner 1996; Alonso-Alvarez *et al*. 2004b; Harshman & Zera 2007; Monaghan, Metcalfe & Torres 2009; Costantini *et al*. 2010; Royle, Smiseth & Kölliker 2012; Metcalfe & Monaghan 2013). In addition to being demanding in terms of energy and antioxidant resources to overcome oxidative stress, reproduction is also associated with increased susceptibility to infection by parasites (Sheldon & Verhulst 1996; Mennerat *et al*. 2009b; Christe *et al*. 2012; Van de Crommenacker *et al*. 2012). This is because, in addition to direct competition between immunity and reproduction for energy (Sheldon & Verhulst 1996), infections by pathogens trigger an inflammatory response, which leads to the production of large amounts of free radicals that may generate oxidative stress (Bedard & Krause 2007; Sorci & Faivre 2009). Hence, since reproductive investment is traded off against immune response and down-regulation of oxidative stress (Sheldon & Verhulst 1996; Harshman & Zera 2007), the effects of pathogenic micro-organisms on hosts are expected to increase with reproductive investment (Mennerat *et al*. 2009b; Christe *et al*. 2012; Van de Crommenacker *et al*. 2012). More specifically, we expected that the effect of pathogenic micro-organisms on host oxidative stress should increase with host reproductive investment.

In this study, we experimentally modified the bacterial communities in nests of wild breeding Great tits to test the effects of the microbiome on (i) the maternal transfer of carotenoids and immune factors to the eggs, (ii) the growth and condition at fledging of nestlings and (iii) the oxidative costs of reproduction for adults. Nests were sprayed with liquid solutions that either favoured or inhibited

bacterial growth compared to a control. These treatments also affected bacterial communities on adult feathers (Jacob *et al*. 2014a). First, we expected that females should modify their investment in immune factors in eggs depending on the bacterial communities they faced. Secondly, we predicted that modifications of nest microbiome should affect nestling growth, size at fledging and immunity (Mennerat *et al*. 2009a,b; Møller *et al*. 2013). If maternally transferred compounds in the eggs serve to protect nestlings from micro-organisms, we expected to find interactions between egg compounds and treatments on nestling development. Finally, concerning the oxidative costs of reproduction, our aim in this study was not to investigate whether investment in reproduction leads to oxidative costs, but to test the hypothesis that oxidative costs of reproduction depend on microbial exposure. The effects of micro-organisms depend on the host level of reproductive investment (Mennerat *et al*. 2009b; Christe *et al*. 2012; Van de Crommenacker *et al*. 2012). We thus predicted that an experimental increase in host exposure to micro-organisms should intensify the oxidative costs of reproduction (i.e. the relationship between reproductive investment and oxidative damage), whereas inhibiting micro-organisms should alleviate these costs. Oxidative damage can arise when antioxidant capacities and repair mechanisms are insufficient to overcome free radicals and reactive species generated by oxidative stress (Monaghan, Metcalfe & Torres 2009). In this study, we thus used oxidative damage to cell membrane lipids as a marker of oxidative stress consequences (Monaghan, Metcalfe & Torres 2009).

Materials and methods

EXPERIMENTAL DESIGN

The study was carried out on a Great tit population breeding in nest boxes close to Toulouse, France (43°39′N, 1°54′E), during the 2011 and 2012 reproductive seasons. In the winter, old nest material was removed from the nest boxes and the insides of the boxes were scraped with a hard brush. Nest boxes were visited daily from the beginning of March to detect the start of nest building.

We randomly assigned the nests to three treatments that consisted in spraying liquid substances in the centre of each nest cup, after carefully removing the eggs or the nestlings, every 2 days from the beginning of nest building until the nestlings fledged (mean volume 1·7 ± 0·02 mL; Jacob *et al*. 2014a). First, to favour the bacterial growth in the nests, we used TSB (tryptic soy broth, 40 mg L^{-1} in sterilized distilled water; Sigma, Saint Louis, MO, USA), a liquid general growth medium for heterotrophic micro-organisms that is commonly used in microbiology. Secondly, we used nisin, a bacteriostatic solution used for food conservation (Harris, Fleming & Klaenhammer 1992; Economou *et al*. 2009; 7 g nisin (900 IU mg^{-1}; B&K Technology Group, Xiamen, China) *in* 50 mM EDTA) to inhibit bacterial growth in the nests. TSB and nisin were diluted in water, and humidity can favour microbial growth (Cook *et al*. 2005; Wang, Firestone & Beissinger 2011). Consequently, we used water as a control in order to attain similar humidity levels in all treatments and ensure that any differences in host phenotype between the treatments resulted from differences in bacterial communities (for details see Jacob *et al*. 2014a). The total number of times treatments were applied to the nests was 16·6 ± 0·3 (mean ± SE) with no significant differences between treatments ($F_{2,52}$ = 2·25; P = 0·12). During incubation, nests were treated only on days 1, 5 and 9 after the start of incubation in order to limit the risks of nest desertion. A total of 54 nests were included in our study (17 nests in the TSB treatment, 17 in nisin and 20 in control). Nestling growth could not be measured in six nests, leading to 16 nests in the TSB treatment, 15 in the nisin and 17 in the control. Nests from the three treatments did not differ significantly in laying date ($F_{2,48}$ = 0·20; P = 0·82) and clutch size ($F_{2,48}$ = 1·24; P = 0·30). Since treatments were sprayed onto the nests every 2 days, the number of treatment applications increased with clutch size ($F_{1,48}$ = 9·22; P = 0·003). As a result, we included the number of treatment applications as a covariate in all analyses.

To measure the effects of the treatments on nest bacterial communities, we collected two samples of nest material using sterilized tweezers at a standardized position in the centre of the nest cup at day 9 of incubation. One sample was placed in a sterile 1·5-mL Eppendorf tube filled with 1 mL of phosphate buffered saline (PBS) for DNA extraction. A second sample was put into PBS with 20% glycerol to limit crystallization and cellular death and was used to perform culture-based analyses (Jacob *et al*. 2014a). Samples were kept in ice in the field and stored at −20 °C until laboratory analyses.

All sampling and manipulations were made after systematically washing hands and material with 70% ethanol in order to avoid cross contaminations. All manipulations were performed according to French legislation, and permits were obtained from DREAL (Direction Régionale de l'Environnement, de l'Aménagement et du Logement) and CRBPO (Centre de Recherches sur la Biologie des Populations d'Oiseaux; ringing permit No. 565).

NESTLING AND ADULT MEASUREMENTS

Nests were visited daily to detect the day of hatching. Following Tschirren & Richner (2006), we measured nestling mass three times during their nesting phase using an electronic balance (±0·01 g). A first measure was performed at the beginning of nestling growth (day 3 post-hatching), a second at day 9, and finally at day 14, at the end of nestling growth. At day 14, we also measured tarsus length with a calliper (±0·1 mm) and nestling immune response via an injection of phytohaemagglutinin (PHA-P; Sigma), a measure that covers multiple aspects of nestling immunocompetence (Martin *et al*. 2006). We injected 0·02 mL of sterile phosphate-buffered saline containing 0·1 mg of PHA-P in the left wing patagium. Patagium thickness was measured before and 24 h (±1 h) after the injection with a micrometre (type 2046FB-60; Mitotuyo, Kawasaki, Japan), standardized by measuring 5 s after applying the micrometre (Brinkhof *et al*. 1999). We used the difference of patagium thickness as a measure of nestling PHA response (Smits, Bortolotti & Tella 1999).

Adult Great tits were trapped in the nest boxes around day 10 post-hatching. We measured tarsus length to the nearest 0·01 mm using a calliper, body mass with an electronic balance (±0·01 g) and wing length with a ruler (±0·1 mm). In order to measure how modifications of the nest microbiome affected microbial communities on adult feathers, we took two samples of feathers close to the left tarsus (on the ventral part in contact with nest material during incubation), each one comprising 10 feathers, and placed them in PBS and PBS plus glycerol. We collected blood from all adults from the brachial vein with heparinized capillaries. Blood samples were placed in 1·5-mL Eppendorf tubes, kept on ice in the field and centrifuged at 16 100 ***g*** for 5 min the day of sampling. Resulting plasma samples were stored at −20 °C until oxidative damage analyses. Due to technical problems, blood samples from nine females and three males were not available for oxidative damage analyses, leading to 85 adults in the analyses (45 females and 40

males) from 51 nests (16 nests in the TSB treatment, 16 in nisin and 19 in control). We found no significant differences in adult tarsus length, wing length and body mass between the treatments (tarsus length: $F_{2,51} = 1{\cdot}48$; $P = 0{\cdot}24$; wing length: $F_{2,51} = 2{\cdot}06$; $P = 0{\cdot}14$; body mass: $F_{2,51} = 2{\cdot}74$; $P = 0{\cdot}08$).

MICROBIAL TREATMENT EFFECTS

As published elsewhere, we found that the treatments applied to the nests led to significant modifications of bacterial communities in nests and on feathers (Jacob *et al.* 2014a), and we present an overview of these effects below. Briefly, we used culture-based methods to measure the density of bacterial communities in the nests and on feathers (tryptic soy agar for total cultivable bacteria and feather meal agar for cultivable keratinolytic bacteria; Jacob *et al.* 2014a). ARISA was used to measure the composition of bacterial communities. ARISA is a molecular method that consists in amplifying highly variable regions of the bacterial ribosomal operon and measuring the length of the amplified fragments to obtain profiles in which each peak corresponds to an operational taxonomic unit (OTU; for details see Jacob *et al.* 2014a).

The TSB treatment led to an increase in total and keratinolytic bacterial densities in the nests (total: estimate ± SE = 0·87 ± 0·38; d.f. = 32; $t = 2{\cdot}30$; $P = 0{\cdot}028$; keratinolytic: estimate ± SE = 1·36 ± 0·62; d.f. = 32; $t = 2{\cdot}19$; $P = 0{\cdot}036$; fig. 1 in Jacob *et al.* 2014a) and on Great tit feathers (total: estimate ± SE = 1·64 ± 0·61; d.f. = 36; $t = 2{\cdot}69$; $P = 0{\cdot}011$; keratinolytic: estimate ± SE = 2·53 ± 0·62; d.f. = 37; $t = 4{\cdot}90$; $P < 0{\cdot}001$; fig. 1 in Jacob *et al.* 2014a), and to a modification in bacterial community composition (nests: d.f. = 30; $F = 3{\cdot}59$; $P = 0{\cdot}005$; feathers: d.f. = 37; $F = 7{\cdot}26$; $P = 0{\cdot}002$; Jacob *et al.* 2014a). The nisin treatment led to a decrease in total and keratinolytic bacterial densities in the nests (total: estimate ± SE = −1·70 ± 0·46; d.f. = 33; $t = -3{\cdot}67$; $P < 0{\cdot}001$; keratinolytic: estimate ± SE = −2·68 ± 0·73; d.f. = 33; $t = -3{\cdot}65$; $P < 0{\cdot}001$; fig. 1 in Jacob *et al.* 2014a) and to an increase in bacterial loads on feathers (total: estimate ± SE = 0·87 ± 0·39; d.f. = 34; $t = 2{\cdot}20$; $P = 0{\cdot}035$; keratinolytic: estimate ± SE = 0·99 ± 0·41; d.f. = 35; $t = 2{\cdot}43$; $P = 0{\cdot}021$; fig. 1 in Jacob *et al.* 2014a). However, the nisin treatment did not significantly affect community composition (nests: d.f. = 30; $F = 0{\cdot}65$; $P = 0{\cdot}72$; feathers: d.f. = 35; $F = 0{\cdot}51$; $P = 0{\cdot}77$; Jacob *et al.* 2014a).

ADULT OXIDATIVE DAMAGE ANALYSES

Plasmatic concentrations of malondialdehyde in adults, a product of cell membrane lipid peroxidation caused by oxidative stress (Monaghan, Metcalfe & Torres 2009), were assessed using HPLC with fluorescence detection, as described previously (Losdat *et al.* 2011) with some modifications (see Data S1, Supporting information).

MATERNAL IMMUNE FACTOR MEASUREMENTS

Two eggs per clutch were sampled within 24 h after they had been laid. In order to assess potential intra clutch variation in maternal immune factors along the laying sequence (Shawkey *et al.* 2008; D'Alba *et al.* 2010), we sampled one early laid egg (rank 1 or 2) and one egg in the middle of the laying sequence (rank 4 or 5). Eggs were cracked open the day of sampling, and albumen and yolk were stored separately at −20 °C until laboratory analyses. Ovotransferrin concentrations in egg albumen were measured using the method described by Shawkey *et al.* (2008). Briefly, this colorimetric method allows estimating the amount of iron needed to saturate ovotransferrin, a measure that suitably reflects ovotransferrin concentration in the albumen samples (Yamanishi *et al.* 2002; Shawkey *et al.* 2008). Lysozyme concentrations in egg albumen were measured using the protocol described by Lee & Yang (2002), consisting in measuring the kinetic of *Micrococcus lysodeiktikus* degradation by lysozymes in a sample. Finally, yolk carotenoid concentrations were measured using the colorimetric method described by Fredriksson, Elwinger & Pickova (2006). Yolk carotenoids were extracted from a yolk sample (14·8 ± 1·7 mg) with 300 μL of acetone–hexane (2 : 1; v : v). Carotenoid concentration (μg mL^{-1}) was assessed as the absorbance of the resulting solution measured at 450 nm relatively to the mass sampled. All egg samples were analysed twice to investigate assays repeatability, and all three assays were suitably repeatable (ovotransferrin: $r = 0{\cdot}74$; $P < 0{\cdot}001$; lysozymes: $r = 0{\cdot}82$; $P < 0{\cdot}001$; carotenoids: $r = 0{\cdot}95$, $P < 0{\cdot}001$). Ovotransferrin, lysozymes and carotenoids in eggs were measured blindly to the treatment by SJ, NP and FRP, respectively.

STATISTICAL ANALYSES

Female body condition was estimated through the regression of body mass on tarsus length (body mass = 2·13 + 6·76*tarsus length; $r^2 = 0{\cdot}67$; $F_{1,48} = 14{\cdot}08$; $P < 0{\cdot}001$; Schulte-Hostedde *et al.* 2005). We used wing length as a second index of female phenotype since residual body mass and wing length were not significantly correlated ($T = 1{\cdot}75$; d.f. = 47; $P = 0{\cdot}09$). Analyses of the effects of the treatments on maternal immune factors in eggs were performed using linear mixed models. Since we sampled two eggs per clutch, we declared the nest as a random factor and included the following factors and covariates: treatment, egg rank in the laying sequence, year, date, female body condition and female wing length. We computed estimates relative to the control treatment in the models. Figures show partial residuals after correction for egg rank and female condition when these factors remained significant in the final models.

For the analyses of nestling growth, condition at fledging and immunity, we used the mean concentrations of maternally transferred antimicrobial compounds and carotenoids assessed in the two sampled eggs as proxies for the mean investment in the whole clutch. The amount of maternally transferred compounds is expected to vary according to the laying sequence (Shawkey *et al.* 2008; D'Alba *et al.* 2010). Since we only had information on egg content for two eggs in the clutch, we used the means per nest for nestling growth, condition and immunity as dependent variables in linear models including treatment, year, date, number of nestlings, concentrations of ovotransferrin, lysozymes and carotenoids, female body condition, female wing length and number of treatments applied to the nests as factors and covariates. We analysed separately nestling early growth, measured as nestling mass gain between day 3 and 9, and later growth between day 9 and 14 (Tschirren & Richner 2006). We included nestling early mass gain (between day 3 and 9) in the model testing for treatment effects on mass gain between day 9 and 14 (Table 2), thus accounting for the fact that nestling later growth is likely to be affected by early growth. Moreover, in order to investigate whether egg compounds played a role in nestling protection from nest micro-organisms, we included the interactions between egg compounds and the microbial treatments. In order to make sure that these interactions did not fail to reach significance (see below) because of low degrees of freedom when all three interaction were included in the models, we ran additional models with a restricted set of parameters (i.e. interactions between treatments and egg compounds, date, year, number of nestlings and number of times treatments were sprayed in the nests). These models yielded qualitatively similar results, and tables show results from factor selection in complete models. Figures show partial residuals after correction for egg contents and female condition when those factors remained significant in the final models.

To analyse the factors that influence oxidative damage in adults, we built linear mixed models including nests as a random factor and the following variables as fixed factors and covariates: treatment, year, number of treatments applied to the nest before adult capture, date of capture, adult tarsus length, wing length, body mass and sex, and the treatment by clutch size interaction. Variables were log-transformed when needed to reach normality. Clutch size ranged from 7 to 13 eggs, but only two nests contained seven eggs and three nests contained 13 eggs, so that not all three treatments were represented in these extremes. We thus pooled extreme clutch sizes with the closest values. We present results with pooled extremes, but analyses without pooling lead to qualitatively similar conclusions.

We applied a backward selection procedure to all models, removing non-significant terms using a stepwise elimination procedure with a significance level set at $\alpha = 0{\cdot}05$, unless they appeared in a significant interaction term. An AIC model selection approach led to qualitatively similar results, and tables show final models after backward selection procedure. All analyses were performed using R software (version 2.15.2; R Development Core Team 2005).

Results

FEMALE INVESTMENT IN EGGS

We found a significant effect of the treatments on the amount of carotenoids in eggs ($F_{2,50} = 6{\cdot}6$; $P = 0{\cdot}003$; Table 1; Fig. 1). Females exposed to decreased bacterial densities in nests (nisin) transferred lower concentrations of carotenoids into their eggs compared to females in the control group ($-0{\cdot}29 \pm 0{\cdot}10$; d.f. = 50; $t = -2{\cdot}91$; $P = 0{\cdot}005$). However, we found no significant effect of the TSB treatment, which favoured bacterial growth, on levels of maternally transferred yolk carotenoids ($0{\cdot}07 \pm 0{\cdot}10$; d.f. = 50; $t = 0{\cdot}69$; $P = 0{\cdot}49$). Lysozyme and ovotransferrin concentrations did not significantly differ between the treatments (Table 1). We found that egg carotenoid content was negatively correlated with female wing length (Table 1) and that female body condition tended to be negatively correlated with egg lysozyme concentration (Table 1). Finally, carotenoid and ovotransferrin concentrations, but not lysozymes, significantly decreased with laying sequence (carotenoids: $0{\cdot}94 \pm 0{\cdot}06$ and $0{\cdot}74 \pm 0{\cdot}04$ μg mL^{-1} in first and later laid eggs, respectively; ovotransferrin: $4{\cdot}60 \pm 0{\cdot}29$ and $3{\cdot}83 \pm 0{\cdot}27$ mg mL^{-1}; Table 1).

Table 1. Analyses of factors affecting carotenoids, lysozymes and ovotransferrin concentrations in eggs. Final models after backward selection are shown

	Estimate ± SE	d.f.	*F*	*P*
Egg carotenoids				
Treatment				
Nisin	−0·29 ± 0·10	2, 50	6·6	0·003
TSB	0·07 ± 0·10			
Egg rank	−0·22 ± 0·07	1, 50	10·2	0·002
Date	0·01 ± 0·01	1, 50	6·2	0·016
Year	−0·41 ± 0·09	1, 50	22·3	<0·001
Female wing length	−0·95 ± 0·30	1, 50	10·1	0·003
Egg lysozymes				
Female body condition	−0·4 ± 0·02	1, 51	3·7	0·059
Egg ovotransferrin				
Egg rank	−0·77 ± 0·36	1, 51	4·7	0·034
Year	1·23 ± 0·42	1, 51	8·7	0·005

TSB, tryptic soy broth.

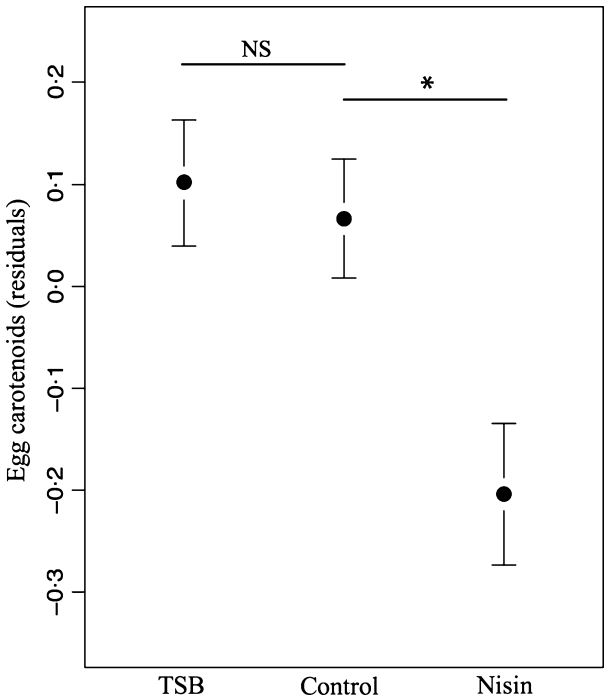

Fig. 1. Effects of microbiome modifications on egg carotenoid content (μg mL^{-1}). Egg carotenoids are expressed as partial residuals after statistically controlling for other significant factors (Table 1) and are thus centred on zero (see Materials and methods).

NESTLING GROWTH AND CONDITION AT FLEDGING

Experimental modifications of nest microbiome had significant effects on nestling mass gain between day 3 and 9 ($F_{2,41} = 5{\cdot}5$; $P = 0{\cdot}008$, Table 2) and tarsus length at fledging ($F_{2,35} = 4{\cdot}8$; $P = 0{\cdot}015$; Table 3), but not between day 9 and 14 ($F_{2,37} = 0{\cdot}42$; $P = 0{\cdot}66$, Table 2). Nestlings from the nisin treatment, exposed to reduced nest microbial densities, showed greater early growth ($1{\cdot}04 \pm 0{\cdot}33$; d.f. = 41; $t = 3{\cdot}14$; $P = 0{\cdot}003$; mass at day 9: TSB: $13{\cdot}86 \pm 0{\cdot}34$ g; control: $13{\cdot}18 \pm 0{\cdot}33$ g; nisin: $14{\cdot}22 \pm 0{\cdot}36$ g; Table 2), but this effect on nestling mass was temporary since we found no significant difference between treatments in nestling mass at fledging ($F_{2,41} = 0{\cdot}29$; $P = 0{\cdot}75$). However, nestlings from the nisin treatment showed longer tarsi at fledging ($0{\cdot}05 \pm 0{\cdot}01$; d.f. = 35; $t = 3{\cdot}84$; $P = 0{\cdot}004$; TSB: $2{\cdot}25 \pm 0{\cdot}01$ g; control: $2{\cdot}24 \pm 0{\cdot}01$ g; nisin: $2{\cdot}27 \pm 0{\cdot}01$ g; Fig. 2). In contrast, we found no significant effect of the TSB treatment on nestling growth and tarsus length (Tables 2 and 3; Fig. 2).

We found that nestling tarsus length was positively correlated with carotenoids, lysozymes and ovotransferrin in eggs (Table 3; Fig. 3). However, the relationships between

Table 2. Analyses of factors affecting nestling early and later growth. Final models after backward selection are shown

	Estimate ± SE	d.f.	*F*	*P*
Mass gain between day 3 and 9				
Treatment				
Nisin	1·04 ± 0·33	2, 41	5·5	0·008
TSB	0·12 ± 0·32			
Date	0·04 ± 0·02	1, 41	4·1	0·049
Year	−1·87 ± 0·36	1, 41	27·7	<0·001
Number of nestlings	−0·25 ± 0·11	1, 41	5·6	0·023
Mass gain between day 9 and 14				
Treatment				
Nisin	−0·35 ± 0·40	2, 37	0·42	0·66
TSB	−0·22 ± 0·37			
Year	−0·26 ± 0·38	1, 37	0·46	0·50
Number of nestlings	0·12 ± 0·12	1, 37	1·01	0·32
Nestling mass gain (day 3–9)	−0·97 ± 0·17	1, 37	33·6	<0·001

TSB, tryptic soy broth.

Table 3. Analyses of factors affecting nestling condition at fledging and phytohaemagglutinin (PHA) response. Final models after backward selection are shown

	Estimate ± SE	d.f.	*F*	*P*
Tarsus length at day 14				
Treatment				
Nisin	0·05 ± 0·02	2, 35	4·8	0·015
TSB	0·01 ± 0·01			
Year	−0·04 ± 0·01	1, 35	9·2	0·004
Carotenoids	0·06 ± 0·02	1, 35	7·0	0·012
Lysozymes	0·37 ± 0·14	1, 35	6·9	0·012
Ovotransferrin	0·01 ± 0·01	1, 35	6·2	0·018
Female wing length	0·08 ± 0·05	1, 35	3·3	0·078
PHA response				
Date	−0·49 ± 0·20	1, 34	6·2	0·018
Year	−17·18 ± 3·10	1, 34	30·6	<0·001

TSB, tryptic soy broth.

concentrations of egg compounds and nestling growth and size at fledging did not differ across treatments (all interactions: $P > 0{\cdot}10$) and these interactions were thus removed from the models during backward selection. Nestling mass at day 14 was not affected by any of the factors and covariates included (all $P > 0{\cdot}21$). Finally, we found no significant effect of the microbial treatments on nestling PHA response (Table 3).

ADULT OXIDATIVE DAMAGE

Analyses of adult oxidative damages revealed a significant interaction between treatments and clutch size on parental levels of oxidative damage ($F_{2,45} = 5{\cdot}32$; $P = 0{\cdot}008$; Table 4, Fig. 4). In the control treatment, we found that birds having laid larger clutches suffered greater oxidative damage (slope: 0·37 ± 0·14; $F_{1,17} = 2{\cdot}58$; $P = 0{\cdot}02$; Fig. 4). In contrast, in the TSB and nisin treatments that modified bird microbiome, we found that clutch size and levels of oxidative damage did not significantly covary (TSB slope: 0·13 ± 0·16; $F_{1,14} = 0{\cdot}83$; $P = 0{\cdot}42$; nisin slope: −0·26 ± 0·16; $F_{1,14} = -1{\cdot}65$; $P = 0{\cdot}12$; Fig. 4). Since the treatment by clutch size interaction did not significantly differ between the sexes ($F_{2,28} = 0{\cdot}36$; $P = 0{\cdot}70$), the effect observed in males probably arose through their investment in nestling care. Accordingly, we also found a significant interaction between treatments and fledgling number on levels of oxidative damage ($F_{2,42} = 3{\cdot}45$; $P = 0{\cdot}04$). Finally, we found no significant interactions between treatments and egg contents, nestling growth or condition at fledging on adult oxidative damage (all $P > 0{\cdot}30$).

Discussion

In this study, we experimentally modified the microbiome of wild Great tits during breeding. The TSB treatment

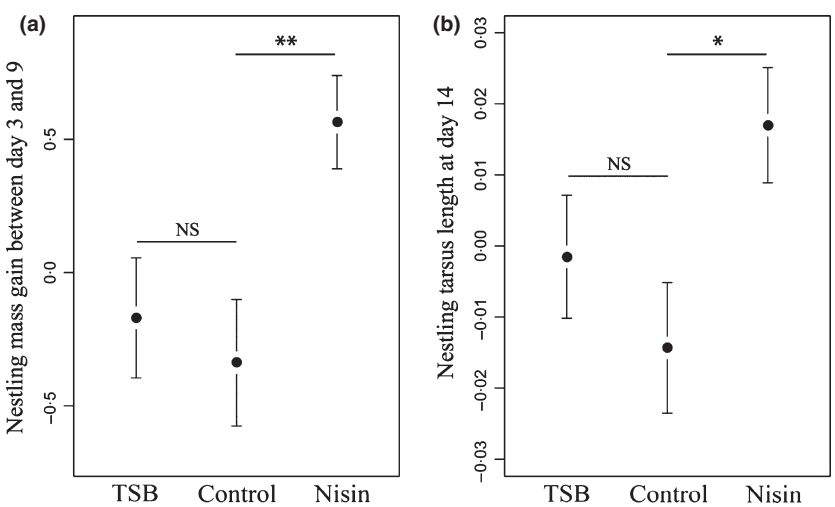

Fig. 2. Effects of microbiome modifications on (a) nestling early growth and (b) tarsus length expressed as partial residuals (see Materials and methods).

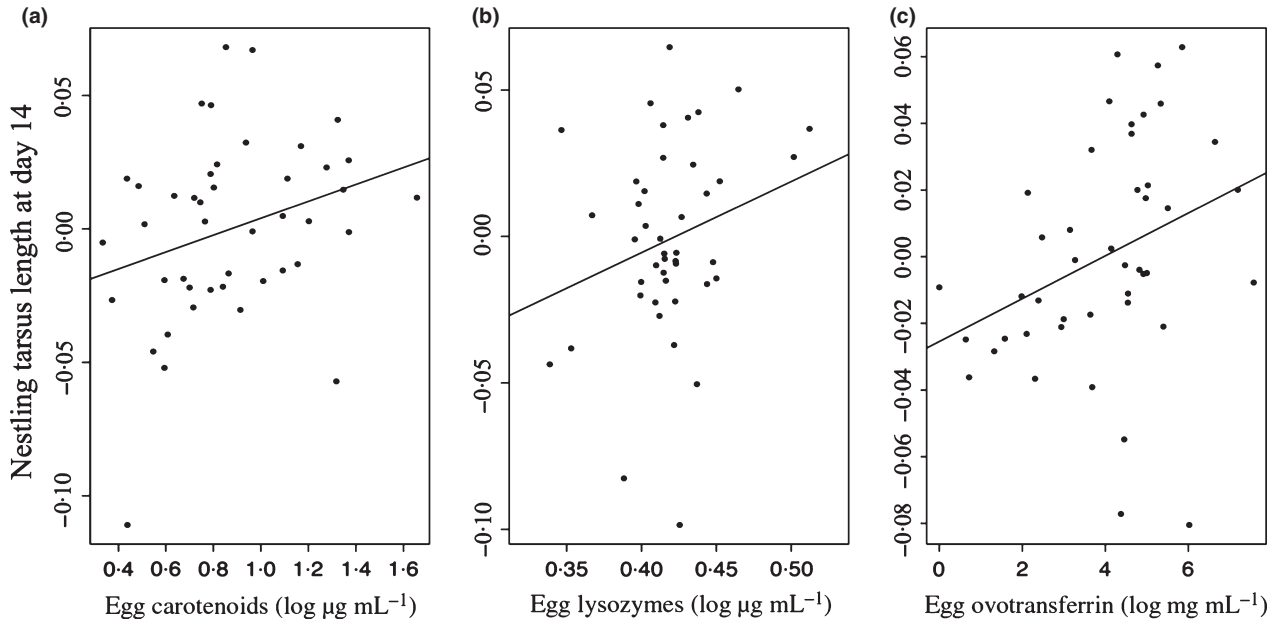

Fig. 3. Correlations between nestling tarsus length at fledging and egg (a) carotenoids, (b) lysozymes and (c) ovotransferrin. Figures presented the mean nestling tarsus length values per nest, as used in the analyses, expressed as residuals (see Materials and methods).

Table 4. Analysis of factors affecting oxidative damage of adult Great tits. Oxidative damage is expressed as the amount of malondialdehyde (nmol mL^{-1} of plasma; see Data S1). Final models after backward selection are shown.

	Estimate ± SE	d.f.	*F*	*P*
Adult oxidative damage				
Treatment				
Nisin	4·24 ± 1·52	2, 45	5·2	0·009
TSB	0·47 ± 1·55			
Clutch size	0·09 ± 0·13	1, 45	0·5	0·507
Year	−0·95 ± 0·13	1, 45	53·2	<0·001
*Clutch size*treatment*				
Nisin	−0·44 ± 0·16	2, 45	5·32	0·008
TSB	−0·06 ± 0·16			

TSB, tryptic soy broth.

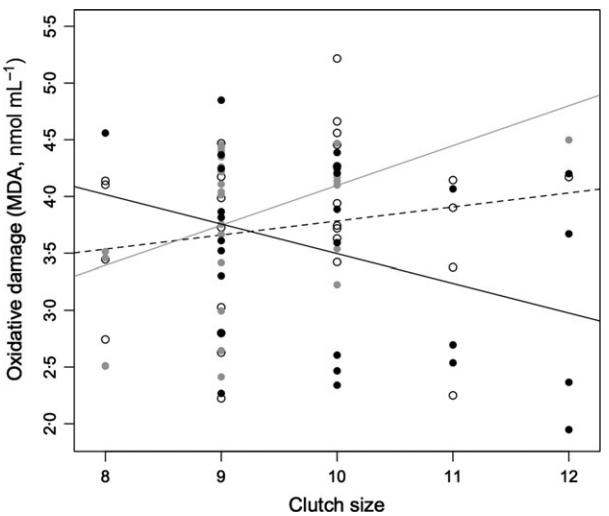

Fig. 4. Modifications of nest microbiome affect the trade-off between clutch size and oxidative damage. Symbols represent the experimental treatments: tryptic soy broth (TSB; *dotted line* and *empty circles*), control (*grey line* and *grey circles*) and nisin (*black line* and *black circles*; interaction treatment by clutch size: $F_{2,45} = 5{\cdot}32$; $P = 0{\cdot}008$, see Table 1). Oxidative damage of adult Great tits significantly increased with clutch size in the control treatment (slope: 0·37 ± 0·14; $F_{1,17} = 2{\cdot}58$; $P = 0{\cdot}02$), whereas this trade-off was non-significant when we experimentally modified the microbiome (TSB slope: 0·13 ± 0·16; $F_{1,14} = 0{\cdot}83$; $P = 0{\cdot}42$; nisin slope: −0·26 ± 0·16; $F_{1,14} = -1{\cdot}65$; $P = 0{\cdot}12$).

used here allowed us to increase the bacterial densities and modified the bacterial community composition compared to the control group. In contrast, the nisin treatment decreased nest bacterial densities, increased feather bacterial loads and had no significant effect on bacterial community composition (Jacob *et al*. 2014a). These modifications of Great tit microbiome led to modifications in the levels of carotenoids females allocated to their eggs, and further impacted nestling growth and tarsus length at fledging. We also found that adults in the control group endured more oxidative damage as they cared for more offspring, whereas such positive relationship between oxidative damage and reproductive effort did not exist in birds exposed to an experimentally modified microbiome.

Egg carotenoids have been found to be beneficial to nestlings by promoting immunity or increasing fledging success (Saino *et al*. 2003; Marri & Richner 2014). Additionally, supplementing mothers with carotenoids during laying increase female carotenoid investment in eggs and have positive effects on nestlings (Berthouly, Helfenstein & Richner 2007; Biard, Surai & Møller 2007; Berthouly, Cassier & Richner 2008). These studies are in line with the common hypothesis that females balance carotenoid allocation between reproduction and self-maintenance

(Lozano 1994; Blount, Houston & Møller 2000). Under this hypothesis, we would expect females exposed to decreased bacterial densities to have more carotenoids available to invest in eggs. In contrast, we found that eggs laid by females exposed to decreased bacterial densities in nests contained carotenoids at lower concentrations (Fig. 1). In Great tits, egg yolk size is positively correlated with egg volume (Ojanen 1983; Isaksson, Johansson & Andersson 2008). In this study, we found no significant differences of egg volume between the treatments ($F_{2,38} = 0{\cdot}87$; $P = 0{\cdot}43$), but found a positive correlation between egg volume (length $\times$ width^2 $\times$ 0·51; Hargitai *et al.* 2013) and carotenoid concentration in eggs (estimate $\pm$ SE = 0·51 $\pm$ 0·14; d.f. = 38; $t = 3{\cdot}79$; $P < 0{\cdot}001$), showing that eggs with greater yolk carotenoid concentration probably also contained more carotenoids. These results thus indicate that females exposed to decreased bacterial densities invested less carotenoids in their eggs, suggesting that female carotenoid allocation to eggs could depend mainly on nestling need of carotenoids. Further experiments should be devoted to investigate the relative importance of nestling needs of carotenoids and potential female allocation trade-off between self-maintenance (immunity) and reproduction (investment into the eggs; Saino *et al.* 2002a) in governing carotenoid investment in eggs.

We found no evidence that female Great tits adjust their investment in ovotransferrin and lysozymes into the eggs according to their microbiome (Table 1). Ovotransferrin and lysozymes are known to be involved in antimicrobial defences (Li-Chan, Powrie & Nakai 1995; Shawkey *et al.* 2008), and differential investment according to mate quality has been shown in previous studies (Saino *et al.* 2007; D'Alba *et al.* 2010; Giraudeau *et al.* 2011). It is thus surprising that females do not appear to adjust egg ovotransferrin and lysozyme according to their exposure to micro-organisms, thus pointing out the need for further studies investigating the role of these substances regarding to embryo and nestling microbial exposure and the factors determining their allocation in eggs.

In Great tits, nestlings fledge <3 weeks after hatching (Gosler 1993), and their survival and future reproduction are greatly affected by body size and mass at fledging (Tinbergen & Boerlijst 1990; Lindén, Gustafsson & Pärt 1992; Both, Visser & Verboven 1999; Heeb *et al.* 1999). Interestingly, our study shows experimentally that a reduction in the nest microbial density positively affects nestling growth and size at fledging. This is in agreement with studies on Corsican blue tits and colonial terns in which the use of aromatic plants as nest material reduces bacterial loads and favours nestling growth, hence suggesting a negative effect of nest micro-organisms on nestling growth (Mennerat *et al.* 2009a,b; Møller *et al.* 2013). Since immune responses are traded off against growth in altricial bird species (Sheldon & Verhulst 1996; Soler *et al.* 2003; Brommer 2004), we might hypothesize that nestlings exposed to less bacteria in the nest might may be able to redirect resources from immunity to growth with potential fitness benefits in terms of post-fledging survival (Mennerat *et al.* 2009b). Here, we found that reducing bacterial densities in nests led to faster early growth. However, since the treatments had no significant effect on nestling mass at fledging, nestlings from control and TSB treatments appeared to have compensated for these early negative effects of bacterial exposure. Further experimental studies should investigate whether this compensation is associated with costs that will express later in life (Metcalfe & Monaghan 2001).

Ovotransferrin, lysozymes and carotenoids in eggs were positively correlated with nestling tarsus length at fledging (Fig. 3), suggesting positive effects of these maternal immune factors on nestling development. However, such effects appeared independent of the treatment (non-significant treatment by egg compounds interactions). This suggests that the effects of the microbiome on nestlings were not mediated by the concentrations of egg compounds. Yolk carotenoids have been found to promote nestling growth and immunity in several species, although recent evidence suggests that the effects of maternally derived carotenoids might be sex and hatching-rank specific (Saino *et al.* 2003, 2011; Romano *et al.* 2008; Newbrey & Reed 2009; Tanvez *et al.* 2009).

Contrary to our expectations, we found no effect of our experimental modifications of the nest microbiome on nestling immunity as measured by PHA response and no significant correlation between PHA response and egg concentrations of ovotransferrin, lysozymes and carotenoids (Table 3). Multiple aspects of both the innate and acquired immunity are involved in the response to PHA (Kennedy & Nager 2006; Martin *et al.* 2006). Therefore, nestling response to PHA may be unable to reflect whether and how exposure to micro-organisms may alter various aspects of nestling immunity, which highlights the need for more specific measures of both innate and acquired immunity to understand how the microbiome shapes nestling immunocompetence.

As expected under the oxidative costs of reproduction hypothesis, we found that oxidative damages of breeding Great tits were positively correlated with investment in reproduction in the control treatment (Fig. 4). However, this increase in oxidative damage with increasing reproductive investment (oxidative cost of parental investment) was not detected when we experimentally modified the nest microbiome (Fig. 4). Our results thus suggested that the microbiome might play a role in shaping the trade-off between reproduction and self-maintenance in Great tits. However, although the relationship between clutch size and oxidative damages was affected by treatments (i.e. clutch size*treatment interaction), we found no significant difference in mean oxidative damages between treatments ($F_{2,47} = 0{\cdot}22$; $P = 0{\cdot}80$). Furthermore, we found no significant correlation between female oxidative damages and egg carotenoids ($F_{1,42} = 1{\cdot}79$; $P = 0{\cdot}19$), suggesting that the effects of microbial treatments on adult oxidative costs of reproduction are not the outcome of female investing

more or less carotenoids into their eggs in relation to the treatments. Why adults in the TSB and nisin groups did not accumulate more oxidative damage when increasing the number of offspring raised remains to be investigated and stresses our need to investigate how the microbiome affects animal homeostasis and physiological trade-offs between reproduction and self-maintenance. Potential mechanisms span from differences in exposure to microbes, modifications in gut microbial communities and/or skin infections (Potti *et al.* 2002; Cani *et al.* 2008), to modifications in the costs of raising nestlings that were themselves affected by the microbiome (reproduction self-maintenance trade-off; Stearns 1992; Oppliger, Christe & Richner 1996; Harshman & Zera 2007; Royle, Smiseth & Kölliker 2012). Noticeably, we reduced the birds' reproductive effort by sampling two eggs in all nests and still found a positive correlation between oxidative damage and number of offspring raised in control birds, making our analyses conservative.

The hosts and their microbiome are involved in complex reciprocal interactions (Faust *et al.* 2012; Morgan, Segata & Huttenhower 2013; Jacob *et al.* 2014a,b), and our understanding of the relative effects of the various components of the microbiome on the birds is particularly limited. Here, we found that increasing nest bacterial densities and modifying the bacterial community composition, as produced in the TSB treatment, had no overall significant effect on egg compounds and nestling development. In contrast, a decrease in bacterial densities in nests but no significant modification of bacterial communities composition, as produced in the nisin treatment, favoured nestling growth and condition at fledging and affected carotenoid investment in eggs. It thus seems reasonable that nestling development and investment of carotenoids in eggs could have been affected by the quantity of bacteria present in the nests, and not by the composition of the nest microbiome. Furthermore, while we found a positive correlation between oxidative damages and reproductive investment in control birds, this trade-off was not detected in the TSB and nisin treatment, possibly because of positive effects on nestlings that reduced required parental investment. Alternatively, since birds are able to regulate feather microbial communities in different ways (Gunderson 2008; Jacob *et al.* 2014a), future studies should investigate whether beneficial bacteria that might have been favoured on feathers could have led to this positive effect on adults. Moreover, our study pointed out that the relationships between nest and feather bacterial communities might be more complex than previously thought. We indeed found that the reduction in nest bacterial densities induced by the nisin treatment led to an increase in feather bacterial loads. Investigating whether competitive interactions between microbes led to this intriguing result and to what extent feather microbiome regulation by birds might take part in it is required to develop our understanding of bird–microbiome interactions (Shawkey, Pillai & Hill 2003; Jacob *et al.* 2014a,b).

One might hypothesize that the observed effects of the nisin treatment on nestlings and adults resulted from a direct effect of spraying a nisin solution in the nests instead of an effect of modification of bird microbiome. nisin is widely used as a food additive (Harris, Fleming & Klaenhammer 1992), and ingested by mice at higher concentrations than the ones used here can be irritant and immune-stimulant (Pablo *et al.* 1999). Here, we found no significant effect of the nisin treatment on nestling PHA response. Moreover, a potential negative, irritating effect of nisin is incompatible with the positive effect that nisin had on nestling development in our study, and such effect thus most likely arose from a modification in the exposure to micro-organisms.

Reproduction is a highly demanding activity that can lead to reduced survival and future opportunities of reproduction (Stearns 1992; Oppliger, Christe & Richner 1996). Here, we experimentally demonstrated for the first time that the microbiome influences the concentration of carotenoids deposited in eggs, early growth and size of nestlings at fledging and play a role in the trade-off between self-maintenance (oxidative damage) and reproduction in adult Great tits. Whereas maternally transferred compounds in the eggs have been found to affect nestling immune system development, growth and fledging success (Saino *et al.* 2003, 2011; Marri & Richner 2014), nestling growth and size at fledging can influence survival and future reproductive opportunities (Tinbergen & Boerlijst 1990; Lindén, Gustafsson & Pärt 1992; Both, Visser & Verboven 1999; Heeb *et al.* 1999). Moreover, our study suggests for the first time a role of the microbiome in the oxidative costs of reproduction in birds, pointed out to be a major component of the costs of reproduction (Monaghan, Metcalfe & Torres 2009; Metcalfe & Monaghan 2013). It follows that the bird microbiome during reproduction is likely to have long-lasting effects that could influence the evolution of reproductive strategies and other life-history traits. By illustrating the various consequences that the microbiome can have on host reproduction in a wild bird, our study emphasizes the importance of experimental approaches to study the role of the microbiome in the evolution of metazoan life (Ezenwa *et al.* 2012; McFall-Ngai *et al.* 2013).

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Data accessibility

Data for this paper are deposited in the Dryad Digital Repository. http://dx.doi.org/10.5061/dryad.9n741 (Jacob *et al.* 2014c).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Data S1. Methods of oxidative stress analysis.

SUPPLEMENTARY MATERIALS

Methods of oxidative stress analysis

Plasmatic concentrations of malondialdehyde (MDA), formed by the β-scission of peroxidized fatty acids, were assessed using HPLC with fluorescence detection (Losdat *et al.* 2011), and samples were processed blindly with respect to the treatments and in random order. All chemicals were HPLC grade, and chemical solutions were prepared using ultra pure water (Milli-Q Synthesis; Millipore Corporation, Billerica, MA, USA). Sample derivatization was done in 2 ml capacity conical-bottom screw-top microcentrifuge tubes. To a 5 µl aliquot of sample or standard (1,1,3,3-tetraethoxypropane, TEP; see below) 5 µl butylated hydroxytoluene (BHT) solution (0.05% w/v in 95 % ethanol), 40 µl phosphoric acid solution (0.44 M), and 10 µl thiobarbituric acid (TBA) solution (42 mM) were added. Samples were capped, vortex mixed for 5 seconds, then heated at 100°C for exactly 1 hour in a dry bath incubator to allow formation of MDA-TBA adducts. Samples were then cooled on ice for 5 min, before 100 µl n-butanol was added and tubes were vortex mixed for 20 seconds. Tubes were then centrifuged at 12000 rpm and 4 °C for 3 min. A 70 µl aliquot of the epiphase was collected and transferred to an HPLC vial for analysis. Samples (5 µl) were injected into a Dionex Ultimate 3000 Rapid Separation LC system (Dionex Corporation, California, USA) fitted with a GL Sciences Inc. (Tokyo, Japan) Inerstil 2µ ODS-4 2.1 x 100 mm column maintained at 37°C. The mobile phase was methanol-buffer (30:70, v/v), the buffer being a 50mM anhydrous solution of potassium monobasic phosphate at pH 6.8 (adjusted using 5M potassium hydroxide solution), running isocratically over 6 min at a flow rate of 0.3 ml/min. Data were collected using a fluorescence detector set at 515 nm (excitation) and 553 nm (emission). For calibration, a standard curve was prepared using a TEP stock solution (5 µM in 40% ethanol) serially diluted using 40% ethanol. TEP standards were assayed in

quintuplicate and showed very high repeatability ($r = 0.996$, $P < 0.0001$, $n = 13$). Plasma volumes were too small to estimate repeatability using samples, but repeatability assessed using plasma samples from another study and bird species was found to be very high ($r = 0.90$, $P < 0.0001$, $n = 12$). FH and AVM did all analyses blindly with respect to treatments.

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