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# Cognitive skills and bacterial load: comparative evidence of costs of cognitive proficiency in birds

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**Abstract** Parasite-mediated selection may affect the evolution of cognitive abilities because parasites may influence development of the brain, but also learning capacity. Here, we tested some predictions of this hypothesis by analyzing the relationship between complex behaviours (feeding innovations (as a measure of behavioural flexibility) and ability to detect foreign eggs in their nests (i.e. a measure of discriminatory ability)) and abundance of microorganisms in different species of birds. A positive relationship would be predicted if these cognitive abilities implied a larger number of visited environments, while if these skills favoured detection and avoidance of risky environments, a negative relationship would be the prediction. Bacterial loads of eggshells, estimated for mesophilic and potentially pathogenic bacteria (i.e. *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*), were used as a surrogate of probability of contact with pathogenic bacteria. We found that bird species with higher feeding innovation rates and rejection

rates of experimental brood parasitic eggs had higher density of bacteria on their eggshells than the average species. Since the analysed groups of microorganisms include pathogenic bacteria, these results suggest that both feeding innovation and ability to recognize foreign eggs are costly and highlight the importance of parasite-mediated selection in explaining the evolution of cognitive abilities in animals.

**Keywords** Behavioural flexibility · Bacterial infection · Brain size · Brood parasitism · Egg rejection · Eggshell bacterial load · Feeding innovation

## Introduction

Cognition, which refers to information-processing abilities including perception, learning, memory, judgment and

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Feeding innovation reliably reflects an important component of cognitive ability of animals (Sol et al. 2005). In accordance with the hypothesis that risk of parasitism could constrain the evolution of cognitive abilities of animals, Garamszegi et al. (2007) have shown that bird species with higher level of feeding innovation have larger immune defence organs and higher prevalence of Haematozoa. Moreover, Vas et al. (2011) have recently shown that more innovative species are parasitized by a more diverse lice fauna (Amblycera). These two results suggest that the evolution of feeding innovation has implications for parasite-mediated natural selection.

Here, we tested this hypothesis in a comparative framework using both feeding innovation and ability to detect foreign eggs in their nests as proxies of cognitive abilities of birds. Eggshell bacterial loads were used as a proxy of probability of contact with pathogenic bacteria that should affect the risk of infection of embryos that are particularly susceptible to parasitism. Feeding innovation may represent cognitive abilities related to behavioural flexibility, technical innovation and/or ability to exploit novel environments, while rejection rates of parasitic egg indicate the ability of birds to locate deviant objects within their particular nest environment (Rothstein 1975a, 1975b; Lyon 2003; Shizuka and Lyon 2010). The two estimates of cognitive abilities reflect important aspects of cognition in birds. The bacterial environment of bird nests can be estimated by quantifying eggshell bacterial loads. Eggshell bacterial density is positively related to probability of embryo infection (Bruce and Drysdale 1994; Cook et al. 2003, 2005b) that is more likely to occur before or at the beginning of incubation (Cook et al. 2003), and thus, our estimations of eggshell bacterial loads are likely to reflect the probability of embryo infection. Since cognitive skills are related to the activity and the diversity of habitats encountered by adult birds, we predicted a positive relationship between cognitive skills and eggshell bacterial loads. Cognitive abilities are known to be associated with brain size (Sol et al. 2005; Avilés and Garamszegi 2007), and consequently, we included brain size as an additional independent variable in the models. Hypothetical background supporting the predicted relationships between cognitive skills of birds and probability of bacterial infections is shown in Fig. 1. Support for the hypothesis would suggest unexplored costs associated with cognitive skills and would thus have important consequences for understanding the evolution of cognition in animals.

## Materials and methods

### Study sites and nest locations

Bacterial communities on eggshells of nests were sampled during the breeding seasons 2007–2008 and 2006–2008 at

Kraghede (Denmark, 57° 12' N, 10° 00' E) and Guadix (Spain, 37°18'N, 3°11'W), respectively. For a detailed description of the Danish and Spanish study area, see, respectively, Møller (1987) and Martín-Vivaldi et al. (2006) and Soler and Avilés (2010).

JMP, EFJ and APM in Denmark and JMP, JJS and MMV in Spain made extensive systematic searches for nests in suitable habitats throughout the breeding season. We relied on extensive help from amateurs with a good knowledge of birds in locating nests of all common breeding species. Adult birds carrying nest material in their beak were particularly used as a means of locating nests with fresh eggs. Most nests were therefore located during nest building by intensively searching suitable habitat in the study area and by checking nest boxes. We deliberately attempted not to touch nests or disturb the surrounding vegetation to avoid increasing the risk of nest predation. When a nest was detected during egg laying, on the basis of the typical clutch size of the species, we estimated the date of clutch completion and visited the nests the following day to sample eggshell bacteria. Nests were re-visited at hatching. The number of nest checks was, in this way, minimized to reduce any unnecessary predation due to investigators.

### Estimation of eggshell bacterial loads

We sampled eggs at the beginning of incubation (i.e. 2–3 days after clutch completion), which assured that independent of the species, all sampled eggs were incubated, and therefore, possible interspecific differences in eggshell bacterial loads due to variation in the onset of incubation (Cook et al. 2003) were partially mitigated.

While sampling eggshells and mainly to prevent between nest contamination, we wore latex gloves sterilized with ethanol and took bacterial samples by cleaning eggshells with a sterile swab slightly wet with sterile sodium phosphate buffer (0.2 M; pH 7.2). The entire clutch was cleaned with the same swab, which was preserved in an eppendorf tube at 4°C containing the sterile buffer until lab analyses during the following 30 days. The duration of the storage period did not affect rank position of different species as shown by a comparison of ranked values of heterotrophic bacterial loads of 21 species from which we, in 2006, collected samples that were stored less than 3 days ( $N=120$ ) and resulting values of adding other samples that were stored up to one month ( $N=156$ ) (Kendall Coefficient of Concordance=0.95; average Spearman Rank Correlation=0.91, Friedmann ANOVA,  $\chi^2=38.13$ ,  $P=0.009$ ). Estimates of bacterial load were standardized for total eggshell surface sampled by taking into account the number and the surface of eggs in the nests. Eggshell surface was estimated according to the formula:

$$S = (3.155 - 0.0136 \times L + 0.0115 \times W) \times L \times W$$

(Narushin 2005) where  $S$  is the surface in square centimetres,  $L$  is the length of the egg and  $W$  is the width of the egg. Length and width of all eggs were measured with a calliper (accuracy: 0.02 mm).

In the lab, samples were collected from eppendorf tubes after vigorously shaking the eppendorf in vortex for at least three periods of 5 s. Serial decimal dilutions up to  $10^{-6}$  were cultivated by spreading homogeneously 100  $\mu$ l of sample (measured with a micropipette) in plates containing four different sterile solid growth media (Scharlau Chemie S.A. Barcelona). We used tryptic soy agar (TSA), a broadly used general medium to grow mesophilic bacteria, and three specific media: Kenner Fecal Agar (KF) for growing bacteria belonging to the genus *Enterococcus*, Vogel–Johnsson Agar (VJ) for bacteria of the genus *Staphylococcus*, and Hecktoen Enteric Agar (HK) for Gram-negative bacteria of the family *Enterobacteriaceae*. Plates were incubated at 32°C for 72 h, and afterwards, the number of colonies on each plate was counted. Bacterial density was estimated as colony forming units (CFU) per square centimetre. See Peralta-Sánchez et al. (2010) for repeatability estimates of intraspecific variation. Permission for egg manipulations were granted from the Junta de Andalucía (Spanish Regional Government).

*Enterobacteriaceae* and *Staphylococcus* sp. include saprophytic and opportunistic bacteria (Singleton and Harper 1998; Houston et al. 1997; Cook et al. 2005a) that live on skin, hair and feathers of mammals and birds (Krieg and Holt 1984). They commonly appear on avian eggshells and are known to include pathogenic strains for avian embryos (Bruce and Drysdale 1994). Enterococci, the third analysed group of bacteria, are also frequently found inside unhatched eggs (Bruce and Drysdale 1994) and are opportunistic pathogens (Franz et al. 1999), although some species might also have beneficial effects (Moreno et al. 2003; Soler et al. 2008, 2010; Martín-Vivaldi et al. 2010). Most of these bacteria are able to penetrate eggshells, and therefore, their density predicts probability of embryonic infections (Board et al. 1994; Cook et al. 2003). In addition, eggshell density of bacteria able to grow in aerobic mesophilic medium is positively related to the probability of embryo infection (Bruce and Drysdale 1994; Cook et al. 2003, 2005b). Therefore, there are good reasons for considering the estimated eggshell bacterial loads as proxies for probability of trans-shell bacterial infection of embryos.

#### Feeding innovation and rejection rates

Feeding innovations can be quantified from the ornithological literature, using descriptions of novel kinds of feeding behaviour (Lefebvre et al. 1997). This data set was collated from an exhaustive survey of 30 years (1970–2000) of the short note sections of 65 generalist ornithology journals covering six geographical areas of the world (Lefebvre et al.

2004). For a detailed description of the systematic data collection, see Lefebvre et al. (1997, 2001), Nicolakakis and Lefebvre (2000), Sol et al. (2002) and Nicolakakis et al. (2003). These estimates are species-specific attributes, as shown by comparisons of estimates for the same species in different continents (Garamszegi et al. 2007).

For rejection rates of potential hosts of the European cuckoo (*Cuculus canorus*), we used the weighted mean values calculated for the different experiments with non-mimetic models performed by different scientists throughout Europe (von Haartman 1981; Järvinen 1984; Davies and Brooke 1989; Moksnes et al. 1991; Moksnes et al. 1994; Palomino 1997; Brooke et al. 1998; Alvarez 1999; Moskat and Fuisz 1999; Stokke et al. 1999; Lindholm and Thomas 2000; Grim and Honza 2001; Amundsen et al. 2002; Rutila et al. 2002, 2006; Moskat et al. 2003a; Prochazka and Honza 2003, 2004; Honza et al. 2004; Lovaszi and Moskat 2004; Antonov et al. 2006b; 2006a; 2009; 2010; Stokke et al. 2008), including our data from Sierra Nevada, Spain (Martín-Vivaldi et al. 2012). We only considered studies providing data on rejection rates of artificially introduced non-mimetic models or eggs that did not use additional stimuli reflecting risk of parasitism (cuckoo dummies). Experiments using conspecific eggs with their natural coloration or models or eggs painted to be similar to the specific gens (host race) of the host species being tested are not included. Rejection rates of non-mimetic model eggs have been broadly used in the literature as a surrogate of recognition ability of foreign eggs by potential hosts of brood parasites (Avilés and Garamszegi 2007; Soler and Møller 1996; Soler et al. 1999b; Soler 1999). However, the single study that has attempted to relate egg-rejection rates to variables reflecting cognitive abilities (i.e. relative brain size) found that species with relatively larger brains had lower egg-rejection rates (Avilés and Garamszegi 2007). However, total brain size may not appropriately reflect the ability of birds to discriminate against foreign eggs in their nests because particular tasks may be more closely related to particular areas of the brain (Timmermans et al. 2000). In any case, we statistically controlled the predicted relationship between egg-rejection rates and bacterial loads of eggshells by inclusion of relative brain size of birds as an additional predictor variable (see the following discussion).

Information on feeding innovation and rejection rate is shown in Online Resource 1.

#### Brain size

Information on brain size was obtained from data on brain mass reported by Mlikovsky (1989), Iwaniuk and Nelson (2003) and J. Erritzøe (personal communication). Highly significant repeatabilities among studies indicate that information on brain mass can be combined across sources

(Garamszegi et al. 2005). Relative brain mass was obtained from the residuals of the log–log regression of brain mass against body mass. Brain mass and body mass values are given in Online Resource 1. This relationship between brain mass and body mass had a strong phylogenetic signal ( $\lambda=0.93$ ), and thus, residuals were estimated from the phylogenetic generalized least square regression model (Pagel 1997, 1999) as implemented in R with the appropriate libraries (“ape”, “MASS” and “mvtnorm”) and additional unpublished functions by R. Freckleton (University of Sheffield) (pglm3.3.r, available on request) as implemented in the package “caic” and using the composite molecular phylogeny described in the following discussion and shown in Online Resource 1.

### Sample sizes and statistical analyses

We successfully collected information on eggshell bacterial loads for 1,012 nests (346 from Denmark and 666 from Spain) of 58 species of birds (49 from Denmark and 25 from Spain) (see Online Resource 1). For 55 of these species, information on feeding innovation was available, while information on rejection rates of non-mimetic model eggs was available for 25 species. For 22 species with estimates of eggshell bacterial loads, we obtained information on both feeding innovation and rejection rates (see Online Resource 1).

We know from previous analyses that interspecific variation in eggshell bacterial load is significantly larger than intraspecific and interyear variation (Peralta-Sánchez 2011) (results from GLM's testing for effects of year (random factor) and species identity (fixed factor) on rank-transformed eggshell bacterial density estimated at the beginning of incubation in four different growth media;  $F_{18, 538} > 6.5$ ,  $P < 0.001$ ), and thus, we estimated geometric means of  $\log_{10}$ -transformed eggshell bacterial load for each species sampled in each country. Frequency distributions of  $\log_{10}$ -bacterial density did not differ from normality for mesophilic bacteria or Enterobacteria (Kolmogorov–Smirnov tests for continuous variables,  $P > 0.2$ ), but did so for Enterococci and *Staphylococcus* (Kolmogorov–Smirnov tests for continuous variables,  $P < 0.05$ ). Thus, because we were interested in considering all estimations of eggshell bacterial load in the same analyses, we ranked values of eggshell bacterial loads and used these in subsequent analyses. Moreover, because we were not interested in differences among locations, but in maximizing the number of species with information on eggshell bacterial loads, we used residuals of such values after controlling for the effect of country. For species sampled in both countries, we used mean values of these residuals. Residuals from the statistical models did not differ from normal distributions (Kolmogorov–Smirnov test for continuous variables,  $p > 0.2$ ), which justify the use of parametric statistical tests.

The probability of finding a feeding innovation in different species may depend on several methodological factors, and therefore, absolute counts of innovation events should be corrected (Lefebvre et al. 1997; Sol et al. 2002). First, there may be more reports available for intensely studied species. We estimated research effort by using the number of studies published since 1972 on each species as cited in the ISI Web of Science (<http://www.isiknowledge.com/>). Second, we assessed the importance of population size that may affect the probability of detection of feeding innovations. We used the minimum estimates of breeding population size (in number of pairs) given in Tucker and Heath (1994), rounding population size to the nearest million if more than a million pairs were found for a species. Third, we assessed the potential confounding effect of body mass on feeding innovations because larger species may be more likely observed when feeding. We used our own data for body mass (see Garamszegi et al. 2007). Trying to correct our analyses for these potential confounding factors, we included them in the MANOVA models explaining eggshell bacterial loads and removed those with the largest (non-significant)  $P$ -values one by one. After removing population size (Wilks=0.90,  $F_{4,46}=1.34$ ,  $P=0.27$ ) and body mass (Wilks=0.87,  $F_{4,47}=1.70$ ,  $P=0.17$ ), final models included estimates of research effort and feeding innovation. Thus, we present results from these final models. The most common and abundant species may be those with higher research effort and more frequent feeding innovation, and thus, we repeated the analyses without including research effort in the models. Brain size relative to body size of birds is known to be positively (Sol et al. 2005) and negatively (Avilés and Garamszegi 2007) related to rates of feeding innovation and foreign egg rejection, respectively, and thus, models including residual brain size after controlling for body mass were also run.

Interspecific statistical relationships could be affected by phylogenetic non-independence (Harvey and Pagel 1991). Therefore, we tested residuals of the regressions between estimated eggshell bacterial loads and feeding innovation and rejection rates using the lambda statistic of Pagel (1999; Freckleton et al. 2002), relying on a composite molecular phylogeny derived from Jönsson and Fjeldså (2006), Hackett et al. (2008) and Pons et al. (2005) with branch length arbitrarily assigned to one (Online Resource 1). We found that the estimated values of  $\lambda$  were either not statistically significantly different from zero (relationships between rejection rates and eggshell density of *Staphylococcus* and between eggshell density of *Enterobacteriaceae* and feeding innovation and research effort) or equal to zero (all other analysed relationships) (see Online Resource 1), indicating that there was no phylogenetic signal, and therefore, the analyses did not need to be corrected for phylogenetic effects (see, Sæther et al. 2011).

The predicted relationships between eggshell bacterial loads and avian cognitive abilities were tested by means of

MANOVAs with eggshell bacterial loads (i.e. mesophilic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*) as dependent variables and rejection rate and feeding innovation rate (and research effort for correcting feeding innovation) as independent continuous variables in the models. These MANOVAs were weighted by the number of nests of each species that were sampled for eggshell bacterial load estimations which should adjust our comparative analyses for heterogeneity in data quality due to the large variation in sample sizes among species (Garamszegi and Møller 2010).

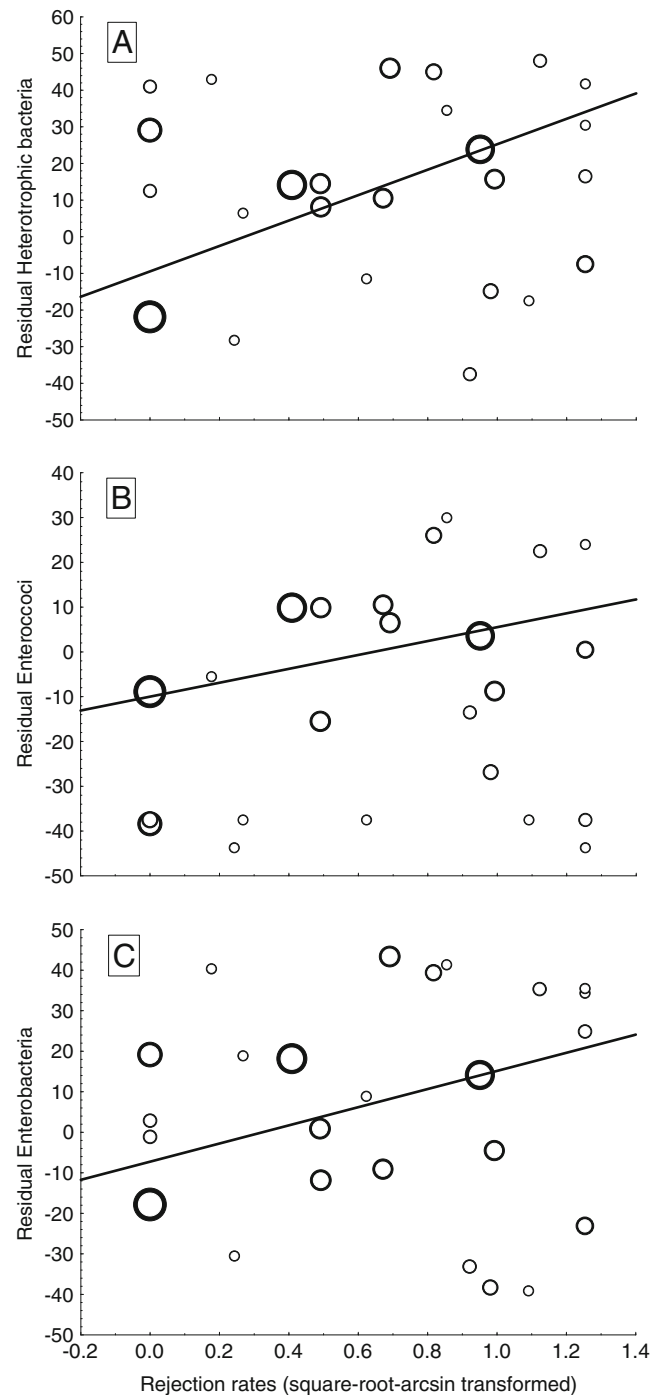
## Results

Estimates of rejection and feeding innovation rates were not significantly related ( $Beta (SE)=0.04 (0.26)$ ,  $F_{1,20}=0.04$ ,  $P=0.85$ ), and thus, they represent two different measures of cognitive abilities.

Potential host species of the European cuckoo showed higher rejection rates of non-mimetic model eggs if they had higher bacterial loads on their eggshells (weighted MANOVA,  $Wilks=0.355$ ,  $F_{4,20}=9.09$ ,  $P=0.0002$ ). Univariate results indicate that this association was mainly due to the positive association with mesophilic bacteria ( $Beta (SE)=0.63 (0.16)$ ,  $P=0.0007$ ), Enterococci ( $Beta (SE)=0.43 (0.19)$ ,  $P=0.030$ ) and Enterobacteria ( $Beta (SE)=0.47 (0.18)$ ,  $P=0.017$ ) (Fig. 2), while no significant association was detected for Staphylococci ( $Beta (SE)=-0.21 (0.20)$ ,  $P=0.31$ ). Inclusion of residual brain mass in the model after correction for body mass and phylogeny did not affect the main conclusions (weighted MANOVA, effect of rejection rates:  $Wilks=0.413$ ,  $F_{4,19}=6.74$ ,  $P=0.0014$ ; effect of brain mass:  $Wilks=0.700$ ,  $F_{4,19}=2.08$ ,  $P=0.124$ ). Univariate results of this model confirmed the relationship between rejection rates and abundance of mesophilic bacteria ( $Beta (SE)=0.46 (0.15)$ ,  $P=0.006$ ), but not that with Enterococci ( $Beta (SE)=0.40 (0.21)$ ,  $P=0.062$ ) and Enterobacteriaceae ( $Beta (SE)=0.30 (0.18)$ ,  $P=0.110$ ), while brain mass was positively associated with abundance of mesophilic bacteria ( $Beta (SE)=0.45 (0.15)$ ,  $P=0.001$ ) and Enterobacteriaceae ( $Beta (SE)=0.45 (0.18)$ ,  $P=0.022$ ). No other bacterial counts were significantly related with rejection rates or brain mass ( $P>0.18$ ).

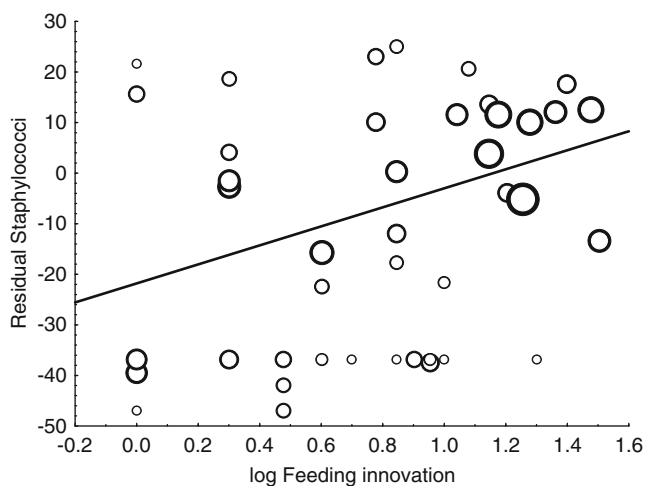
Species with higher rates of feeding innovation had larger eggshell bacterial loads (weighted MANOVA,  $Wilks=0.617$ ,  $F_{4,50}=7.76$ ,  $P=0.0009$ ). Univariate results showed that this relationship was due to the positive association between eggshell density of *Staphylococcus* and feeding innovation ( $Beta (SE)=0.51 (0.19)$ ,  $P=0.0001$ ; Fig. 3) because no other bacterial count showed a significant effect ( $P>0.45$ ). Inclusion in the model of the statistically significant effects of brain mass (weighted MANOVA,  $Wilks=0.685$ ,  $F_{4,48}=5.51$ ,

$P=0.001$ ) did not affect the strength of the detected effect of feeding innovation on eggshell bacterial loads (weighted MANOVA,  $Wilks=0.704$ ,  $F_{4,48}=5.06$ ,  $P=0.0018$ ). Univariate results of this model showed that eggshell density of



**Fig. 2** Relationship between eggshell bacterial loads of potential host species of the European cuckoo estimated for mesophilic bacteria (a), Enterococci (b) and Enterobacteriaceae (c), and rejection rates of non-mimetic model eggs. Lines are weighted regression lines. The areas of circles are proportional to log-transformed number of nests of each species sampled for eggshell bacterial loads





**Fig. 3** Relationship between eggshell bacterial loads of potential host species of the European cuckoo estimated for Staphylococci bacteria and feeding innovations. Lines are weighted regression lines. The areas of circles are proportional to log-transformed number of nests of each species sampled for eggshell bacterial loads

*Staphylococcus* was positively related to feeding innovation ( $Beta (SE)=0.65 (0.14), P=0.0001$ ) and negatively related to brain mass ( $Beta (SE)=-0.39 (0.14), P=0.01$ ). In addition, residual brain mass was negatively related to *Enterobacteriaceae* on eggshells ( $Beta (SE)=-0.47 (0.15), P=0.003$ ), while no other univariate results reached statistical significance ( $P>0.06$ ). When we included research effort (log-transformed) for estimating feeding innovation in the previous model, neither research effort (weighted MANOVA, Wilks=0.856,  $F_{4,47}=1.98, P=0.113$ ) nor feeding innovation did explain a significant proportion of interspecific variance in eggshell bacterial loads (weighted MANOVA, Wilks=0.851,  $F_{4,47}=2.05, P=0.102$ ), but residual brain mass did (weighted MANOVA, Wilks=0.763,  $F_{4,47}=3.65, P=0.011$ ). However, univariate results confirmed that feeding innovation tended to explain density of Staphylococci ( $Beta (SE)=0.44 (0.23), P=0.056$ ), while brain size was negatively related to density of both

Staphylococci ( $Beta (SE)=-0.33 (0.15), P=0.037$ ) and *Enterobacteriaceae* ( $Beta (SE)=-0.45 (0.17), P=0.009$ ). The relationships between research effort for estimating feeding innovation and eggshell bacterial loads were far from significant independently of the specific bacterial count ( $P>0.22$ ).

Finally, when including information on rejection rates and feeding innovation in the same model, thereby greatly reducing sample size, the conclusions remained unchanged. Both feeding innovation (weighted MANOVA, Wilks=0.374,  $F_{4,16}=6.70, P=0.002$ ) and rejection rates (weighted MANOVA, Wilks=0.287,  $F_{4,16}=9.93, P=0.0003$ ) were significantly positively associated with eggshell bacterial load. Univariate results indicated that two estimates of eggshell bacterial density were significantly related to rejection rates (mesophilic bacteria:  $Beta (SE)=0.62 (0.18), P=0.003$ ; Enterobacteria ( $Beta (SE)=0.46 (0.20), P=0.035$ )), while that was not the case for Enterococci ( $Beta (SE)=0.41 (0.20), P=0.052$ ) and Staphylococci ( $Beta (SE)=-0.21 (0.15), P=0.18$ ). The relationship with feeding innovation was mainly due to the positive association with *Staphylococcus* load ( $Beta (SE)=0.73 (0.15), P=0.0001$ ), with no other bacterial count being significantly related to feeding innovation rate (results not shown).

When residual brain mass and sampling effort for feeding innovation estimates were included as additional factors in the model, rejection rate but not feeding innovation was still significantly associated with eggshell bacterial load, and both research effort and residual brain mass entered significantly in the model (Table 1). Thus, it is possible that lack of statistical significance for feeding innovation was due to research effort and feeding innovation sharing a considerable amount of variance (Garamszegi et al. 2007). Univariate results confirmed the positive associations between variables related to cognitive skills and eggshell bacterial loads since the abundance of Staphylococci was positively related to feeding innovation and research effort, while rejection rate was related to Enterococci. Residual brain mass were positively related to density of mesophilic bacteria (Table 1).

**Table 1** Results from MANOVA testing the relationship between bacterial loads of eggshells estimated as density of mesophilic bacteria (TSA), Enterococci (KF), Staphylococci (VJ) and Enterobacteria (HK) as dependent factors, and feeding innovations (log-transformed), research effort (log-transformed) for estimates of feeding innovation,

residual brain size after controlling for body mass (Brain size) and rejection rates of non-mimetic model eggs (square-root arcsine-transformed) as predictors. Multivariate (Wilks and associated  $F$ -statistics) and univariate results (Beta, standard error (SE) and associated  $P$ -value) for each kind of quantified bacteria are shown

	Univariate results											
				TSA		KF		VJ		HK		
	Wilks	$F_{4,14}$	$P$	Beta (SE)	$P$	Beta (SE)	$P$	Beta (SE)	$P$	Beta (SE)	$P$	
Feeding innovation	0.623	2.121	0.132	0.06(0.21)	0.78	0.02(0.26)	0.95	0.43(0.19)	0.039	-0.03(0.25)	0.89	
Research effort	0.387	5.533	0.007	-0.21(0.24)	0.40	0.46(0.30)	0.14	0.48(0.22)	0.040	0.08(0.28)	0.77	
Brain size	0.427	4.689	0.013	0.53(0.18)	0.009	-0.12 (0.23)	0.60	0.01(0.17)	0.96	0.45(0.22)	0.053	
Rejection rate	0.492	3.610	0.033	0.62(0.21)	0.13	0.65(0.26)	0.023	0.01(0.19)	0.97	0.33(0.25)	0.20	

When relative brain size was the only independent variable included in the model, it explained a significant proportion of interspecific variance in eggshell bacterial load (weighted MANOVA, Wilks=0.619,  $F_{4,63}=9.69$ ,  $P=0.00004$ ). This association was mainly due to the significant negative associations with Enterococci ( $Beta (SE)=-0.38 (0.11)$ ,  $P=0.001$ ) and *Enterobacteriaceae* ( $Beta (SE)=-0.45 (0.11)$ ,  $P=0.0001$ ), while no other bacterial count was significantly associated with brain size ( $P>0.06$ ). These results indicate that, in general, birds with larger brain size enjoy reduced probability of eggshell bacterial infection.

## Discussion

Previously, Garamszegi et al. (2007) and Vas et al. (2011) found a positive interspecific relationship between feeding innovation and prevalence of haematozoan parasitism and diversity of parasitic Amblyceran lice, respectively. These results could be interpreted in a general scenario if feeding innovation reflected complex cognitive functions. Behavioural flexibility and opportunism may be associated with environmental variability, width of the ecological niche, social structure, morphological variability and population density (Lefebvre and Bolhuis 2003; Vas et al. 2011), which may all involve increased parasite-mediated selection. However, feeding innovation per se may increase the risk of parasitism because, independently of other cognitive skills, opportunistic feeding behaviour by exploiting novel food sources and/or acquiring novel feeding styles may imply higher probability of encountering pathogenic microorganisms. Therefore, Garamszegi et al. (2007) concluded that “further analyses are required to distinguish whether feeding innovation alone or its cognitive or ecological correlates enhance the risk of parasitism”. Here, we used two different independent estimates of cognitive proficiency of animals, feeding innovation and ability to recognize foreign eggs and found an association with intensity of bacterial parasitism, inferred from estimates of eggshell bacterial loads. Thus, our results lend further support to the existence of evolutionary costs of being innovative.

### Cognitive abilities and costs of microbial infection

Costs associated with cognitive abilities of animals are not yet well understood. Most hypothetical costs are mainly energetic (Aiello and Wheeler 1995; Sengupta et al. 2010). In accordance with the idea that cognitive abilities may trade against energy consumption (Aiello and Wheeler 1995), there is evidence suggesting that larger brain size is traded against the size of other energetically expensive tissues (reviewed in Niven and Laughlin 2008). Interestingly, resolution of this trade-off would vary depending on environmental conditions (i.e. habitat) and life history traits, which

therefore may explain extreme variation in cognitive proficiency among animals (Burns et al. 2011). The probability of parasitism also varies depending on environmental conditions (Møller et al. 2006; Merino and Potti 1996), and it strongly affects the evolution of life history traits of animals (Møller 1997; Martin et al. 2001). Consequently, parasites could also play a role in the resolution of trade-offs between cognitive skills and energy consuming activities. For example, it is known that honey bees *Apis mellifera* (Mallon et al. 2003) and bumblebees *Bombus terrestris* (Riddell and Mallon 2006) perform poorly in learning assays when their immune system has been challenged by a non-pathogenic elicitors of the immune system. Apart from a trade-off between energy consuming activities (Alghamdi et al. 2009), this association may also suggest a role of parasites driving the evolution of cognitive abilities if animals with enhanced cognitive proficiency experienced higher probability of parasitism, as is likely to be the case (i.e. larger number of flowers visited; Ruiz-Gonzalez and Brown 2006). In this situation, individuals or species with high probability of parasitism (higher cognitive abilities) should be those with stronger anti-parasite defences (Møller and Erritzøe 2002). Therefore, this scenario predicts a positive association between immune response and cognitive proficiency in birds (Garamszegi et al. 2007) and bumblebees (Alghamdi et al. 2009). We have found a positive association between two different components of cognitive abilities and probability of bacterial infection, which further suggests an important role of parasitism in driving the evolution of cognition in animals.

We used brain size in our analyses to statistically control the association between cognitive tasks (feeding innovation and foreign egg rejection) and bacterial infection. After correcting for rejection rates, species with larger brains had higher bacterial density. However, after correcting for rate of feeding innovations, species with larger brain size had lower bacterial density on their eggshells. Estimates of rejection and feeding innovation rates were not significantly related to each other, and the association between brain size and rejection rate was negative, while the association between brain size and feeding innovation rate was positive. Finally, relative brain size was negatively related to eggshell bacterial load when no other independent variables were included in the statistical models. Variation in the sign of the association between brain mass and eggshell bacterial load depending on the independent variables included in the models should obviously be interpreted as partial correlation coefficients. Consequently, these results suggest, first, that birds with relatively larger brains have higher capacity of eluding bacterial contamination of eggshells and, second, that for species with similar relative brain size, those with higher rates of feeding innovation and egg rejection had higher bacterial density on eggshells. Relative brain size has repeatedly been used as indicative of cognitive capacity of animals (Lefebvre et al.

1997, 2004; Sol et al. 2005), and thus, these results could be interpreted as brain size playing a role in diminishing bacterial infection, while bird species with higher cognitive activities relative to their brain size suffer higher risk of bacterial infection.

#### Recognition of brood parasitic eggs and costs of microbial infection

Particularly intriguing is the association between rejection ability of potential hosts of the common cuckoo and eggshell bacterial density since it would represent unexplored costs associated with recognition of foreign eggs that would help to explain the low or intermediate rates of egg rejection of most suitable hosts (Rothstein 1990). The study of costs of egg rejection has focused on the existence of recognition errors (Lotem et al. 1995; Davies et al. 1996), costs of mis-imprinting on parasitic eggs (Lotem 1993) or breakage of own eggs when trying to eject parasitic eggs (Rohwer et al. 1989; Lotem et al. 1995; Martín-Vivaldi et al. 2002). Our analyses show an increased probability of bacterial infections associated with superior egg-recognition skills, suggesting an important additional cost constraining the evolution of rejection ability. This relationship may suggest that cognitive skills of hosts of brood parasites when encountering a parasitic egg in their nests may be non-adaptive in other environments in which it increases the probability of bacterial infection. Egg recognition proficiency of birds may indicate neophilia and ability to find strange objects in particular environment (Rothstein 1975a, 1975b; Lyon 2003; Shizuka and Lyon 2010). It is believed that the ability to recognize foreign eggs evolved as a consequence of favourable cognitive skills related to nest sanitation (Bartol et al. 2003; Moskat et al. 2003b; Guigueno and Sealy 2009). Thus, it is unlikely that the positive relationship with eggshell bacterial loads were related to a lower effort maintaining hygienic conditions of nests by bird species with higher rejection rates. Rather, these cognitive skills may make birds more prone to explore environments, objects or locations with an associated increased probability of encountering bacteria. However, we did not find support for a positive relationship between foreign-egg recognition ability and feeding innovation. These suggestions should, in any case, be experimentally tested to reach further conclusions. An alternative explanation for the detected relationship between egg-rejection ability and eggshell bacterial load of potential host species is related to the historical associations with the European cuckoo. For instance, adults of hosts and parasitic cuckoos differ in their intestinal microbiota (see Ruiz-Rodríguez et al. 2009), and consequently, the addition of brood parasitic eggs to the host clutch may have resulted in a more diverse bacterial community of the eggs of the most commonly parasitized hosts. Moreover, some rejecter species puncture parasitic eggs or break some of their own eggs (Soler et al.

2002; Antonov et al. 2006c), which may deteriorate the hygienic conditions in host nests (Soler et al. 2011). Nest lining material and eggs could become smudged with yolk and egg white from damaged eggs, increasing nutrient availability for bacterial growth on the eggshell (Stadelman 1994). Although we cannot reject these alternative scenarios explaining the detected interspecific association between egg-rejection ability and eggshell bacterial loads, none of the sampled nests were parasitized by the European cuckoo, and thus, these alternatives are improbable.

In summary, we found a positive relationship between intensity of bacterial colonization of avian eggshells and two variables reflecting different cognitive abilities of birds, rates of feeding innovation and recognition of foreign eggs. These relationships suggest that parasite-mediated selection may constrain the evolution of cognitive proficiency in birds. Studies at the intraspecific level are needed to further examine the causes explaining the relationships detected here.

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**Cognitive skills and bacterial load: Comparative evidence of costs of cognitive proficiency in birds, *Naturwissenschaften*, Soler JJ, Peralta-Sánchez JM, Martín-Vivaldi M, Martín-Platero AM, Flensted-Jensen E and Møller AP**

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**Log-transformed values of body mass, brain mass, population size, feeding innovation and research effort, and arcsine transformed values of rejection rates of bird species with information on eggshell bacterial loads. Residual eggshell bacterial loads in ranks after controlling for the effect of country shown for mesophilic bacteria (TSA), *Enterococcus* (KF), *Staphylococcus* (VJ), and Gram negative *Enterobacteriaceae* (HK). N refers to number of nests sampled for estimates of eggshell bacterial load.**

	Body mass (log)	Brain mass (log)	Pop. Size (log)	Feed. innov. (log)	Res. Effort (log)	Rej. Rates (arcsin)	Res. TSA (ranks)	Res. KF (ranks)	Res. VJ (ranks)	Res. HK (ranks)	N
<i>Acrocephalus scirpaceus</i>	1.072	0.488	6.585			0.672	10.500	10.492	-36.872	-9.131	9
<i>Anas platyrhynchos</i>	3.049	0.599	6.623	0.903	3.272		-15.500	-37.508	-36.872	6.869	2
<i>Apus apus</i>	1.598	0.578	7.077	0.000	2.656		-35.500	4.492	15.628	-21.131	6
<i>Burhinus oedicephalus</i>	2.665	-0.263	4.792	0.000	2.274		25.724	-43.759	-46.979	40.000	1
<i>Buteo buteo</i>	2.907	0.250	5.980	1.301	3.006		42.000	-37.508	-36.872	16.869	1
<i>Carduelis cannabina</i>	1.278	0.747	7.279	0.301	1.903	0.000	12.500	-37.508	-36.872	-1.131	2
<i>Carduelis carduelis</i>	1.193	0.596	7.312	0.477	2.373		-16.276	-18.259	-46.979	-22.500	3
<i>Carduelis chloris</i>	1.442	0.442	7.362	0.845	2.465	0.492	8.112	9.866	-11.925	-11.815	10
<i>Circus aeruginosus</i>	2.767	0.846	5.066	0.000	2.662		-1.500	-37.508	-36.872	-41.131	1



<i>Columba palumbus</i>	2.694	-0.274	7.114	0.301	2.442		-12.888	-11.384	-2.675	-10.815	30
<i>Coracias garrulus</i>	2.167	-0.279	4.911	0.301	2.182		-4.276	23.241	-1.479	5.500	23
<i>Corvus corone</i>	2.736	-0.321	7.079	1.505	2.950		-4.888	-9.134	-13.425	-16.815	29
<i>Corvus monedula</i>	2.396	-0.164	7.004	0.845	2.640		6.112	-0.134	0.325	-1.815	24
<i>Cuculus canorus</i>	2.081		6.806	0.301	2.680		-3.500	14.492	4.128	-3.131	6
<i>Cyanistes caeruleus</i>	1.070	-0.245	7.505	1.041	2.932	0.000	29.112	-38.384	11.575	19.185	23
<i>Cygnus olor</i>	4.031	-0.071	5.013	0.000	2.900		-13.500	-37.508	-36.872	4.869	1
<i>Delichon urbicum</i>	1.291	-0.143	7.229	0.845	2.844		39.000	30.992	-36.872	29.869	1
<i>Emberiza cirrus</i>	1.376	-0.044	6.556			1.253	41.724	-43.759	-46.979	35.500	1
<i>Emberiza citrinella</i>	1.427	-0.120	7.389			1.123	48.000	22.492	-36.872	35.369	2
<i>Falco tinnunculus</i>	2.242	-0.307	5.618	1.362	3.015		8.112	-6.134	12.075	7.185	29
<i>Fringilla coelebs</i>	1.384	-0.441	8.267	0.845	2.763	0.981	-14.888	-26.884	-17.675	-38.315	3
<i>Fulica atra</i>	2.865	0.747	6.255	0.477	2.836		2.500	-37.508	-36.872	-37.131	3
<i>Garrulus glandarius</i>	2.209	0.635	6.978	1.000	2.330		25.112	-3.134	-21.675	24.935	2
<i>Haematopus ostralegus</i>	2.725	-0.167	5.574	0.301	2.916		-33.500	20.492	18.628	-11.131	4
<i>Hirundo rustica</i>	1.281	0.771	7.415	1.146	3.042	0.000	-21.888	-8.884	3.825	-17.815	132
<i>Lanius excubitor</i>	1.825	0.301	5.512	0.954	2.566	0.993	15.724	-8.759	-37.479	-4.500	9

<i>Larus argentatus</i>	2.952	-0.466	6.183	1.204	3.206		18.500	28.992	-3.872	42.369	10
<i>Larus canus</i>	2.587	-0.009	6.019	0.903	2.722		22.500	27.992	-36.872	32.869	2
<i>Larus ridibundus</i>	2.448	0.176	6.267	1.398	3.076		32.500	31.992	17.628	38.369	10
<i>Mergus merganser</i>	3.215	0.129	4.782	0.000	2.547		-42.000	-37.508	21.628	-5.131	1
<i>Motacilla alba</i>	1.317	0.232	7.290	0.954	2.571	0.921	-37.500	-13.508	-36.872	-33.131	2
<i>Motacilla cinerea</i>	1.239	-0.198	6.068	0.000	2.364	1.253	-7.500	0.492	-36.872	-23.131	5
<i>Muscicapa striata</i>	1.190	-0.287	7.255	0.903	2.286	0.854	34.500	29.992	-36.872	41.369	1
<i>Oenanthe leucura</i>	1.553	-0.332	4.002	0.000	1.531		-6.276	-10.259	-39.479	-18.500	19
<i>Oenanthe oenanthe</i>	1.379	0.287	6.944	0.477	2.465	0.243	-28.276	-43.759	-46.979	-30.500	1
<i>Parus ater</i>	0.966	0.200	7.312	0.602	2.491		29.112	-20.634	-22.425	15.185	4
<i>Parus major</i>	1.267	0.313	7.836	1.176	3.221	0.409	14.112	9.866	11.575	18.185	73
<i>Poecile palustris</i>	1.076	0.488	6.653	0.602	2.382	0.000	41.000	-37.508	-36.872	2.869	2
<i>Passer domesticus</i>	1.482	0.599	7.985	1.279	3.211		29.112	16.116	10.075	24.185	65
<i>Passer montanus</i>	1.336	0.578	7.568	0.602	2.671		26.862	10.366	-15.675	16.685	38
<i>Phoenicurus phoenicurus</i>	1.201	-0.263	7.057	0.000	2.358	0.623	-11.500	-37.508	-36.872	8.869	1
<i>Phylloscopus collybita</i>	0.886	0.250	7.657	0.903	2.545	1.253	30.500	23.992	-36.872	34.369	1
<i>Pica pica</i>	2.358	0.747	7.122	1.255	2.959		-16.888	-12.384	-5.175	-25.815	295

<i>Picus viridis</i>	2.287	0.596	5.975	0.845	2.121		39.724	13.241	25.021	31.500	3
<i>Prunella modularis</i>	1.278	0.442	7.279	0.699	2.318	0.177	43.000	-5.508	-36.872	40.369	1
<i>Pyrrhocorax pyrrhocorax</i>	2.519	0.846	4.884	0.778	2.356		21.724	-6.759	23.021	-14.500	6
<i>Recurvirostra avosetta</i>	2.450	-0.274	4.677	0.000	2.449		8.500	-1.508	-36.872	37.369	10
<i>Riparia riparia</i>	1.119	-0.279	6.872	0.778	2.731	0.691	46.000	6.492	10.128	43.369	10
<i>Sitta europaea</i>	1.378	-0.321	7.122	0.477	2.441		-27.500	-37.508	-36.872	-44.131	1
<i>Sterna paradisaea</i>	2.039	-0.164	5.845	0.301	2.603		47.000	8.492	-36.872	36.369	8
<i>Streptopelia decaocto</i>	2.304	NA	6.895	0.477	2.619		-16.888	-40.634	-41.925	-12.815	4
<i>Sturnus vulgaris</i>	1.906	-0.245	7.597	1.146	3.281	0.490	14.500	-15.508	13.628	0.869	10
<i>Sylvia atricapilla</i>	1.275	-0.071	7.568	1.000	2.715	1.091	-17.500	-37.508	-36.872	-39.131	1
<i>Sylvia communis</i>	1.161	-0.143	7.290	0.477	2.272	1.253	16.500	-37.508	-36.872	24.869	2
<i>Troglodytes troglodytes</i>	0.949	-0.044	7.498	0.301	2.515	0.267	6.500	-37.508	-36.872	18.869	1
<i>Turdus merula</i>	1.982	-0.120	7.785	1.477	2.936	0.950	23.862	3.616	12.575	14.185	62
<i>Turdus philomelos</i>	1.848	-0.307	7.447	1.079	2.540	0.817	45.000	25.992	20.628	39.369	4
<i>Vanellus vanellus</i>	2.339	-0.441	6.352	0.477	2.850		-39.000	-37.508	-36.872	-35.131	2

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### Phylogenetic tree in phylip style used in the analyses.

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**Phylogenetic signals (lambda statistics) of statistical models explaining interspecific variation in eggshell bacterial loads estimated for mesophilic bacteria, *Enterococcus*, *Staphylococcus* and *Enterobacteriaceae*. Results of probability of lambda values differing from zero and one are also shown (maximum likelihood Chi-square).**

	Mesophilic bacteria	<i>Enterococcus</i>	<i>Staphylococcus</i>	<i>Enterobacteriaceae</i>
<b>Models</b>				
<b>Bacteria = rejection rates</b>				
Lambda statistic	6.610696e-05	6.610696e-05	0.9999339	6.610696e-05
Test of Lambda = 0	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$	$\chi^2 = 2.20, P = 0.138$	$\chi^2 = -0.001, P = 1$
Test of Lambda = 1	$\chi^2 = 11.55, P < 0.001$	$\chi^2 = 16.45, P < 0.001$	$\chi^2 = -0.001, P = 1$	$\chi^2 = 13.66, P < 0.001$
<b>Bacteria = feeding innovation + research effort</b>				
Lambda statistic	6.610696e-05	6.610696e-05	6.610696e-05	0.1498980
Test of Lambda = 0	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$	$\chi^2 = 0.445, P = 0.50$
Test of Lambda = 1	$\chi^2 = 25.83, P < 0.001$	$\chi^2 = 16.36, P < 0.001$	$\chi^2 = 34.54, P < 0.001$	$\chi^2 = 18.85, P < 0.001$
<b>Bacteria = rejection rates + feeding innovation + research effort</b>				
Lambda statistic	6.610696e-05	6.610696e-05	6.610696e-05	6.610696e-05
Test of Lambda = 0	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$
Test of Lambda = 1	$\chi^2 = 11.46, P < 0.001$	$\chi^2 = 13.28, P < 0.001$	$\chi^2 = 7.55, P = 0.006$	$\chi^2 = 10.34, P = 0.001$

Bacteria = residuals of brain mass

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Lambda statistic	6.610696e-05	6.610696e-05	6.610696e-05	6.610696e-05
Test of Lambda = 0	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$	$\chi^2 = -0.001, P = 1$
Test of Lambda = 1	$\chi^2 = 26.98, P < 0.001$	$\chi^2 = 16.35, P < 0.001$	$\chi^2 = 22.39, P < 0.001$	$\chi^2 = 24.59, P < 0.001$

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