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Does breeding environment affect eggshell bacteria load and female antibacterial defence investment?

Ovplyvňuje hniezdne prostredie baktérie vaječnej škrupiny a investície samíc do antimikrobiálnej obrany?

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Abstract. *Eggshell surface of birds constitutes a suitable environment for bacteria which may have an important impact on the embryo. One important determinant for bacteria development is humidity. We predict bacterial loads on eggshells to be higher in birds breeding in wet environments (e.g. marsh habitats) in comparison to species breeding in other (dry) habitats, assuming that eggs of wetland birds are more likely faced a higher degree of humidity due to increased evaporation and water contact. To minimize damage through bacteria female birds are known to develop several defence strategies including allocation of antimicrobial substances into the eggs. Here we aim to show whether habitat dependent differences in eggshell bacteria loads do exist, more specifically, whether bird species breeding in wetland habitats have to cope with higher bacterial loads on their eggs and whether maternal investment into egg immune defence may have evolved as a counterstrategy to protect embryos from bacteria penetrating the egg shell. Our results reveal eggshell bacteria loads in wetlands to be a multiply higher than in dry habitats. There is no obvious difference in parameters related to maternal egg immune defence investment which seems to be even lower in wetland habitats. In this context alternative possibilities of factors influencing studied parameters are discussed.*

Key words: *birds, bacteria, egg, nesting habitat, lysozyme, maternal investment*

Introduction

Pathogen transmission partly depends on interactions between individuals (Kulkarni & Heep 2006) but also environmental factors, which seems to be e.g. especially important for bacteria (Zhang et al. 2005). It is known that for bacteria, beneficial or potentially harmful, the ability to survive and grow can be influenced by environmental factors (Lewis et al. 2016, Lee

et al. 2017, Rothenburger et al. 2017), which furthermore greatly vary between bacterial strains (Portal-Celhay et al. 2012). A potentially interesting model to investigate the influence of environmental factors and environmental health on bacteria development and transmission could be the eggshell surface of birds. Eggshell bacteria mostly originate from the female-urogenital tract (Ruiz-de-Castañeda et al. 2011) and from the surrounding environment (Singleton and Harper 1998) and can have an important influence on embryonic health, development and survival (Pinowski et al. 1994, Houston et al. 1997, Stewart & Rambo 2000, Cook et al. 2003, 2005b, D'Alba et al. 2010, Hansen et al. 2015) or later cognitive skills (Soler et al. 2012).

Moisture has been identified as one of the most important environmental factors influencing bacteria development and their eggshell colonization (Maier et al. 2000, Madigan et al. 2005). A humid environment has been shown to provide favourable conditions for bacteria development on the eggshell surface (Cook et al. 2005a, D'Alba et al. 2010, Ruiz-de-Castañeda et al. 2011). In birds, humidity might be influenced by the nesting habitat since the environment surrounding the nest either directly or indirectly influence the condition on the eggshell surface (Godard et al. 2007). Eggs of wetland birds are more likely faced to a higher degree of moisture, because of increased evaporation or/and water contact, either direct (eggs laying in the water like in the Great Crested Grebe *Podicepcs cristatus*) or indirect, due to water transport to the nest via parent´s body or nest material. Consequently, we predict that bacterial loads on egg shells should be higher in birds breeding in wet environments (e.g. marsh habitats) in comparison to species breeding in dry habitats (scrublands, woodlands etc.).

To minimize bacteria damage on reproductive success female birds developed several defence strategies including the production a proper barrier (e.g. eggshell structure and cuticle deposition, Bain et al. 2013), allocation of antimicrobial substances into the egg (e.g. lysozyme, ovotransferrin, Shawkey et al. 2008, Wellman-Labadie et al. 2008; Wu & Acero-Lopez 2012), antimicrobial secretion of the uropygial gland and brood patch (Menon & Menon 2000, Shawkey et al. 2003), the use of antimicrobial nest material (Clayton & Wolfe 1993, Brouwer & Komdeur 2004, De Roode et al. 2013), and increased incubation temperature (Cook et al. 2005a, Shawkey et al. 2009, Mainwaring et al. 2014) since at least some bacteria are temperature sensitive (Mennerat et al. 2009).

In this study we would like to show whether habitat dependent differences in eggshell bacteria loads do exist and whether females adapt their maternal investment into eggs, in particular antimicrobial substances, accordingly (Fox & Mousseau 1998, Saino et al. 2002). Given that also egg yolk carotenoids have shown to have an immune stimulating and antioxidative function influencing embryo, in particular their immune system development (Blount et al. 2003, Tanvez et al. 2009, Jacob et al. 2015) we also include egg colour measurements as a proxy for carotenoid content in this study.

Assuming that the production of antimicrobials is costly (Van de Crommenacker et al. 2010) and carotenoid availability is limited (Partali et al. 1987, Olson & Owens 1998, Von Schantz et al. 1999, Tschirren et al. 2003), one might predict that females invest more into antimicrobial substances and carotenoids when the infection risk for embryos and nestlings is higher namely when breeding in wetland habitats.

However, environmental differences between wet and dry habitats may have a general impact on maternal egg investment. Thus, to identify a potential antimicrobial role in relation to eggshell bacteria, one needs to inspect other compounds of female egg investment as well. If female invests according to bacterial risk, one might predict a specific investment, mainly into antimicrobial substances (lysozyme) but not necessarily into a general investment into egg resources.

Maternal investment into eggs involves, besides immune defence substances other compounds, which are necessary for the embryonic development, like nutrients, vitamins

and hormones. Therefore, in the present study, we examined habitat dependent differences in bacteria load and their association with female egg investment in terms of egg white lysozyme concentrations and yolk carotenoid colour. Furthermore, we include yolk testosterone levels and egg nutrional state in terms of the proportion of yolk mass in relation to female body size and yolk mass in relation to egg mass, both measurements reflecting female general investment into egg resources. Finally, we also include eggshell pH level, given that pH could enhance a bacterial defence of immune compounds (Pooart et al. 2005, Wellmann-Labadie et al. 2010, Grizard et al. 2015).

In this study we are more interested in more general rules and therefore we examined a habitat dependent effect in eggshell bacterial loads and maternal investment on the species level. As mentioned earlier the microcliamte provided by the nest environment may also influence their bacteria development. Therefore, we selected species with a relatively diverse spectrum of nest types and constructions (for details see methods) for this comparison.

Methods

Study species and sample sites

Regarding species breeding in dry habitats we gathered samples (one egg per nest) from six species with different nest types including domed nests: Long-tailed Tit *Aegithalos caudatus* (n = 30), cavity nests: Great Tit *Parus major* (n = 27) and Eurasian Blue Tit *Cyanistes caeruleus* (n = 5) and open cup nests: Blackcap *Sylvia atricapilla* (n = 34), Common Blackbird *Turdus merula* (n = 32) and Song Thrush *Turdus philomelos* (n =33). Regarding wetland breeding species we selected six species with open nests but differed in respect to the nest material and the degree of moisture, including wet nests with eggs being in direct water contact: Great Crested Grebe *Podiceps cristatus* (n = 29) and Little Grebe *Tachybaptus ruficollis* (n = 1), nests close above the water level including nests mainly built of reed and reed mace material: Eurasian Coot *Fulica atra* (n = 28), and nests with the inner part with a thick feather layer: Mallard *Anas platyrhynchos* (n = 5), Common Pochard *Aythya ferina* (n = 2), and Tufted Duck *Aythya fuligula* (n = 3).

Moisture in some nests is additionally increased by water transferred into the nest by the wet body of the parents or by parents covering the eggs with wet submerse vegetation (Bauer & Glutz von Blotzheim 1966).

Bacterial samples from the egg surface and eggs for the egg content analyses were taken from wet and dry habitat breeding species in 2016 in the drainage basin along the March-Danube river system (Western Slovakia, Eastern Czech Republic, 49º33′06.18″ N–48º03′18.19″ N, 16º58′32.06″ E–18º17′34.42″ E, 115–219 m a.s.l.).

Bacteria sampling and cultivation procedure

We collected the second non-incubated egg of a nest and immediately sampled its eggshell bacteria. After that egg measurements were taken including egg- and yolk mass to the nearest of 0.0001 g, using an electronic balance. On the same day eggs were frozen to -20° C and stored until the end of the breeding season.

Since egg size influences the bacteria load found on the egg shell we used bacteria density. We removed the egg from the nest with sterile gloves and swabbed the egg surface for exactly 20 sec with a sterile cotton swab, soaked in sterile water. The same method was appllied to all species and in this way the surface sampled was standardized and we compare bacteria densities of different species independent of egg size. The swab was then stored in a transport medium (transport viscose swab with Amies transport medium, Sarstedt, Germany). Within 48 h the swab was transferred to 2.5 ml of PBS buffer in the lab. The 15 ml tube, containing 2.5 ml of PBS and the swab, was mixed by vortex agitating for 120 s. The bacterial suspension was then serially diluted. After dilutions were completed, 0.1 ml of all samples and dilutions were spread-plated on a non-selective (Columbia agar with sheep blood 7%, company Oxoid,

Czech Republic) and selective (Brilliance UTI, company Oxoid, Czech Republic) media that indicated total bacterial counts. Plates were then incubated for 24 h at 37°C. After the incubation period plates were removed and colony enumeration of culturable bacteria was performed. Given the importance of bacteria originating from the female urinary tract (see earlier) we used Brilliance UTI Agar as a chromogenic medium for the presumptive identification and differentiation of all the main micro-organisms that cause urinary tract infections including *Enterococcus* spp., *Escherichia coli*, *Proteus*, *Morganella*, *Providencia*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, coliforms. Additionally, Columbia blood agar was used with sheep blood as the medium which can isolate and cultivate fastidious microorganisms with clearly visible haemolytic reactions including Staphylococci and Streptococci. Thermo Scientific™ Columbia Agar with Sheep Blood promotes haemolysis typical of *Staphylococcus aureus*, and it gives typical growth for *Streptococcus pneumoniae*.

Thus, for the analyses and comparability we used specific and important cultivable bacteria. Finally, we used abundance measurements from three bacteria categories (see above), i) "all bacteria" includes all bacteria growing on Columbia agar with sheep blood, ii) "haemolytic bacteria" is a subset of "all bacteria" and includes those bacteria causing haemolyses on Columbia Agar plates, and iii) "UTI bacteria" which are bacteria growing on Brilliance UTI Agar reflecting bacteria originating from the urinary tract.

Egg compound analyses

After the breeding season (August) all collected eggs have been partly defrosted and the egg shell removed. The egg content was then divided into egg white for further lysozyme and pH analyses and egg yolk for hormone analyses and egg colour determination. Frozen yolk and egg white were weighed and both stored at -80 °C:

Lysozyme analyses

For the lysozyme analyses we prepared the albumen samples and then we determined the antibacterial activity. The albumen samples were collected into pre-weighed 2.0 ml reagent tubes. Each tube with albumen sample was weighed and albumen was lyophilised. Lyophilised powder was subsequently dissolved in 200 µl of distilled water and the obtained albumen solution was used for determination of antibacterial activity. Radial diffusion assay was used in order to evaluate antibacterial activity of albumen samples. In brief, one bacterial colony from an overnight agar plate culture of *Micrococcus luteus* was suspended in phosphate buffered saline (PBS) and the suspension turbidity was adjusted to 108 CFU (colony forming units/ml). One-hundred µl aliquot of suspension was inoculated into 10 ml of melted Luria broth (LB) containing 0.9 % (w/v) agar pre-heated at 48 °C and poured into 90 mm Petri dishes. After solidification, 5 mm-diameter wells were punched into LB agar and 5 µl of sample was added to each well. The antibacterial activity of examined samples was compared on the basis of the radius of clear inhibition zone around the well against standard solutions of a chicken egg white lysozyme (Sigma-Aldrich, UK) after 24 hours of incubation at 37 °C. Antibacterial activity of albumen samples were expressed as concentration of egg-white lysozyme of equivalent activity. Results given represent mean values from duplicate measurements of each independent sample.

pH analyses

Albumen was thawed at room temperature and pH was measured with the pH meter Sentrom (Leek, Netherland) with the ISFET pH electrode for 2 decimal numbers.

Yolk colour determination as a proxy for carotenoid content

Carotenoid content was indirectly determined by yolk colour. Therefore, we used the RGB colour space provided by the DSM yolk colour fan TM (LeVaux 2013). It offers a simple, accurate and consistent means of measuring egg yolk colour and consequently provides an indirect measure for carotenoid content. To ensure optimal accuracy during measurement

the evaluation was carried out under i) indirect daylight, which prevents distracting reflections from the glossy surface of the yolk, ii) against a white non-reflective surface to eliminate the influence of adjacent colours. The blades of the yolk fan have been held immediately above the egg yolk and are viewed vertically from above, with the blade numbers facing down and the yolk positioned between the tips of the blade. To ensure consistency the evaluation was carried out by the same person (AD).

Testosterone analyses

Yolk testosterone concentrations were measured by radioimmunoassay after yolk steroid extraction following the previously published protocol (Okuliarova et al. 2011). Briefly, yolk samples were homogenized in 10 volumes of deionized water and 1 g of homogenate was extracted twice with a mixture of diethyl and petroleum ether (7:3). The extracts were re-dissolved in 2 mL of 70% methanol and left to precipitate at -20°C for two days. Samples were centrifuged, decanted, dried under nitrogen and finally reconstituted in 300 µL of phosphate buffer saline with 0.1% gelatine and 0.1% sodium aside. The recoveries (80.1% ± 0.4%; mean ± SE) were estimated by adding of [3H]-testosterone into each sample before the extraction. Radioimmunoassay was performed using [1,2,6,7-3H]-testosterone (PerkinElmer; specific activity 63.47 Ci/mmoL) and a specific antibody generated in rabbits against testosterone-3-(carboxy-methyl) oxime bovine serum albumin conjugate (Zeman et al. 1986). All samples were run in three assays. The intra-assay coefficients of variation were lower than 2% and the inter-assay coefficient of variation was 9.2%. The assay sensitivity was 1.6 pg per tube.

Yolk mass investment

As another general measure for female egg investment we determined yolk in relation to egg mass (%) and egg mass in relation to female body size (%). Since females were not trapped, we used average values of female body mass offered in the literature (Hudec & Černý 1977, Hudec 1983, 1994).

Statistical analyses

We used linear mixed models within the R package 3.0.3 (R Core Team 2013) in order to analyse the effect of habitat and clutch size on all bacteria, haemolytic bacteria, and UTI bacteria loads in different models. All three response variables were ln (x+1)-transformed to achieve normally distributed residuals. Sample size differed between and being very low in some species, therefore species was used as random effect to account for different sample sizes per species.

In order to evaluate and compare different models, we used the Akaike Information Criterion corrected for small sample sizes (AICc, Anderson & Burnham 2002). If there was not one clearly best model, we used methods of model averaging and multimodel inference (Anderson & Burnham 2002). These methods allow inference over all models considered, but this was weighted according to model support by the data. Additionally, these methods provide the probability for single variables being in the unknown "true" model (the so-called relative variable importance – RVI). We assumed a RVI > 0.7 as relevant. We conducted these calculations in R using the package MuMIn (Barton 2013). Additionally, we inspected the residuals for normality visually using histograms and QQ-plots.

To determine maternal investment, we used six different parameters. We used i) egg white lysozyme concentration as the antimicrobial substance. Furthermore, we used egg white pH, egg yolk testosterone and egg yolk colouration as a proxy for carotenoid content. As nutrional parameters we included relative egg mass (%), which was determined as the proportion of egg mass (measured individually immediately after egg was collected) to average female body mass according to the literature; and vi) relative yolk mass (%), which was for each collected egg determined as the proportion of egg yolk mass to total egg mass.

In a fourth model we than used a multivariate response (lysozyme + pH + testosterone + carotenoids + relative egg mass + relative yolk mass) to study the effect of habitat and clutch

size on the bird investment. Since the multivariate model was not much different from an intercept-only-model (Δ AICc = 1.02), we did not follow up with single response regressions.

Results

When considering all bacteria – and UTI bacteria loads on eggshells prior to incubation between dry and wet nesting habitats our results revealed a highly significant difference (Fig. 1a, c; all bacteria RVI = 0.737, P < 0.001, UTI bacteria: RVI = 0.735, P < 0.001). All six species investigated under wet breeding conditions have eggshell bacteria loads a multitude higher than in dry habitats (Fig. 1a–c).

For haemolytic bacteria our results indicate an even stronger habitat effect (Fig. 1b, RVI = 0.925, P < 0.001). This holds for all but one species, namely the Common Pochard seems to have eggshell bacterial loads lower and similar to dry habitat species (Fig. 1b). Aditionally clutch size seems to have a minor effect on haemolytic bacteria. Bacterial load was negatively related to clutch size (P = 0.028). Only one wet habitat species, namely the Common Pochard seems to have eggshell bacterial loads lower and similar to dry habitat species (Fig. 1b).

Regarding the relationship between breeding habitat and specific egg content characteristics (lysozyme, pH, egg yolk testosterone, yolk colour, relative egg and yolk mass, Fig. 2–5), our results revealed no meaningful model (ΔAICc to intercept-only-model 1.02), which suggests that there is no obvious habitat specific variation in egg compound investment by the female.

Discussion

Our results revealed a very strong effect of breeding habitat on cultivable bacteria loads. We predicted higher eggshell bacteria loads in wet habitats but the huge difference found is surprising. Eggshell bacteria loads are in fact several hundred up to more than 4000 times higher for species breeding in wet than for those breeding in dry habitats (Fig. 1a–c). Our data are restricted to cultivable bacteria which make up about 10 % of the bacteria fauna, but they include bacteria like *Enterococcus*

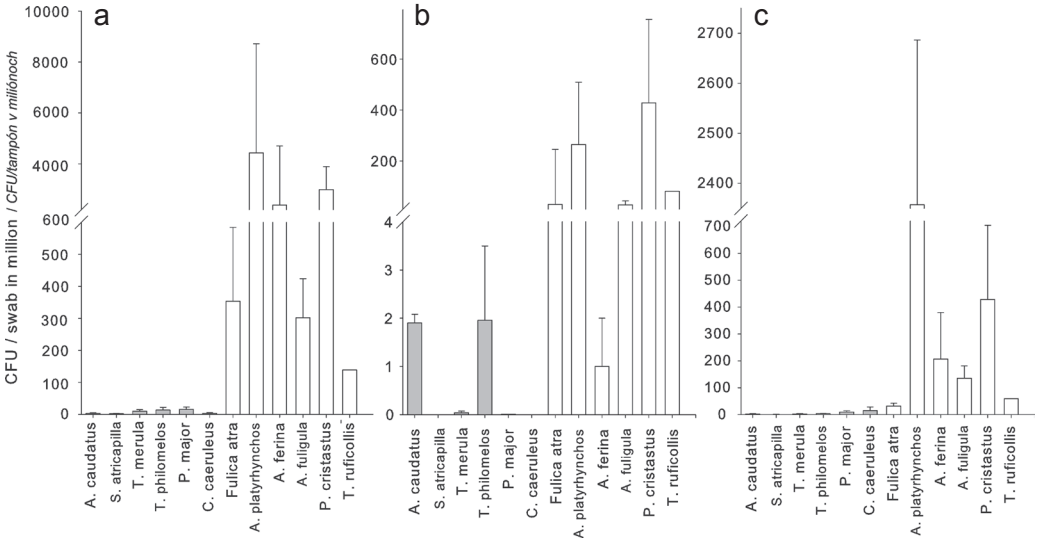

Fig. 1. Bacteria load (in Million) for all bacteria („all bacteria", a) and haemolytic bacteria (b) cultivated on blood agar, and bacteria load (in Million) cultivated on UTI agar (c) from egg shell of bird species breeding in dry (black) and wet (white) habitat. Mean ± SE (standard error) are given.

Obr. 1. *Počet baktérii (v miliónch) pre "všetky baktérie"(a) a hemolytické baktérie (b) kultivované na krvnom agare a baktérie (v miliónoch) kultivované na UTI agare (c) z povrchu vajec druhov vtákov hniezdiacich v suchom (čierny stĺpec) a mokrom (biely stĺpec) mokrom habitate. Graf udáva priemer ± štandardnú chybu.*

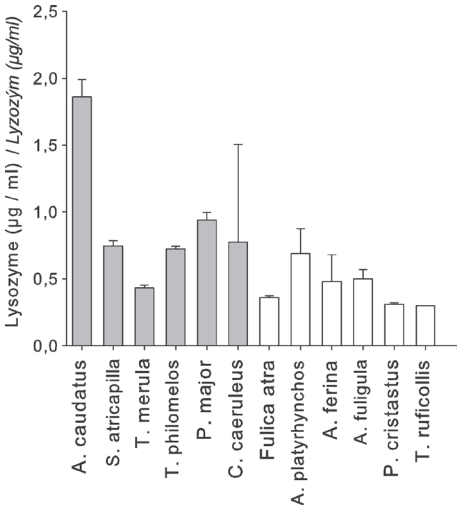

Fig. 2. Lysozyme concentration in egg white (µg/mg of egg white) for bird species breeding in dry (black) and wet (white) habitat. Mean ± SE (standard error) are given.
Obr. 2. *Koncentrácia lyzozýmu vo vaječnom bielku (µg/mg vaječného bielka) vtákov hniezdiacich v suchom (čierny stĺpec) a mokrom (biely stĺpec) habitate. Graf udáva priemer ± štandardnú chybu.*

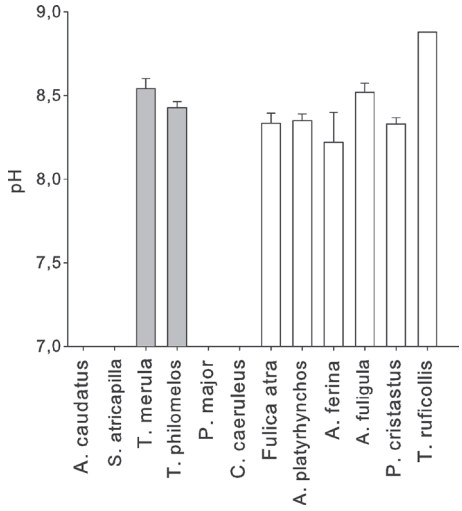

Fig. 3. pH of egg white in bird species breeding in dry (black) and wet (white) habitat. Mean ± SE (standard error) are given.
Obr. 3. *pH vaječného bielka vtákov hniezdiacich v suchom (čierny stĺpec) a mokrom (biely stĺpec) habitate. Graf udáva priemer ± štandardnú chybu.*

spp., *Escherichia coli*, *Proteus*, *Morganella*, *Providencia*, *Pseudomonas*, *Staphylococcus*, *Streptococsccus* are known to cause e.g. urinary tract infections or to have a significant impact on the developing embryo. Given that different bacteria prefer different ambient conditions e.g. haemolytic bacteria are sensitive to high temperature (Nester et al. 1978, Tortora et al. 2004, Fuchs 2007). Thus, separating bacteria e.g. into haemolytic and bacteria originating from the female genital tract (UTI bacteria, see methods), did not reveal a change in the results. Differences between wet and dry habitats are still very obvious for both bacterial strains (Fig. 1b–c).

There are several factors, which could additionally influence our results like i) the microclimate influenced by nest type or material, ii) species-specific or phylogenetic differences and, iii) the available bacteria environment (e.g. egg and clutch size or female body size). Regarding the immediate egg environment it has been demonstrated that the nest type (e.g. cavity or open cup nest) or nest material used can have a significant effect on bacterial load (Godard et al. 2007, Mennerat et al. 2009, Peralta-Sánchez et al. 2012, Ruiz-Castellano et al. 2016). Due to the antimicrobial effect of preen gland oil on feathers bacterial loads should be lower, in particular when feathers are incorporated (Peralta-Sánchez et al. 2012, Giraudeau et al. 2013, Moreno-Rueda 2017). Controlling for nest type was not possible in our study since open cup nests are the predominant nest type for wetlands birds. Variation in nest type could only be observed in dry nesting species where differences exist regarding nest type, and material. However, the variation in bacteria loads in particular within dry habitat species was minimal in comparison to the one found between the two habitats. The importance of aromatic plants and feathers seems to be also less significant in our study because there is no consistent difference in bacteria loads depending on the nest material used by species breeding in wet and dry habitat. Thus, a nest environment effect in relation to nest type or different material can not be excluded but seems only minimal in comparison to the difference found between habitats (Fig. 1a–c).

In relation to species-specific differences Peralta-Sánchez et al. (2012) found a species-

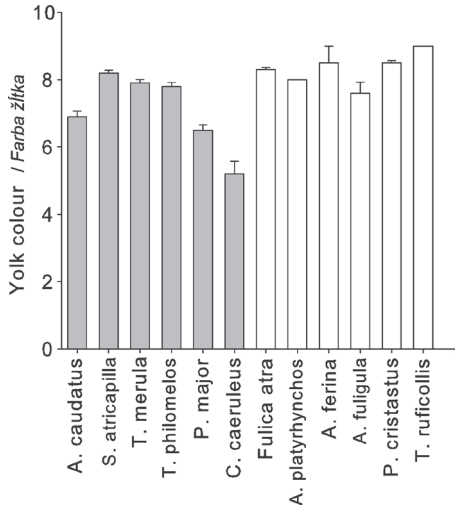

Fig. 4. Egg yolk colour indicating carotenoids in bird species breeding in dry (black) and wet (white) habitat. Mean ± SE (standard error) are given.
Obr. 4. *Farba vaječného žĺtka indikujúca karotenoidy vtákov hniezdiacich v suchom (čierny stĺpec) a mokrom (biely stĺpec) habitate. Graf udáva priemer ± štandardnú chybu.*

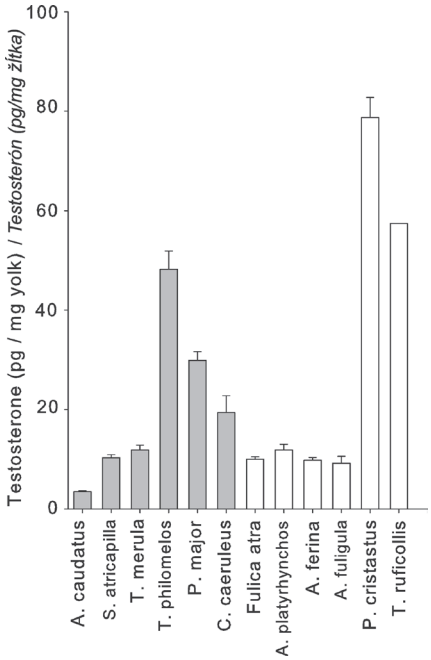

Fig. 5. Testosterone (pg/mg yolk) in yolk of bird species breeding in dry (black) and wet (white) habitat. Mean ± SE (standard error) are given.
Obr. 5. *Testosterón (pg/mg žĺtka) vaječného žĺtka vtákov hniezdiacich v suchom (čierny stĺpec) a mokrom (biely stĺpec) habitate. Graf udáva priemer ± štandardnú chybu.*

specific differrences in eggshell bacteria loads but not of phylogeny. We tried to control for species in the analysis but could not for a phylogeny because our dry habitat species have all been passerines, whereas all wet habitat species have been non-passerines. Thus, including phylogenetic differences in the statistical analyses would not be helpful. Additionally, phylogenetic differences are mediated by size, which would further complicate the interpretation of a phylogenetic effect. Thus, in support of a habitat rather than a phylogenetic effect, own unpublished results indicate that also reed passerines, like the Eurasian Reed Warbler (*Acrocephalus scirpaceus*) do have elevated bacteria loads.

Furthermore, we also found no differences in any maternal compound investigated between the two habitats and therefore also not between passerines and non-passerines. Thus, a phylogenetic impact is not supported examining maternal investment and therefore a strong phylogenetic impact is in general rather unlikely.

Finally, bacteria development could be influenced by habitat size represented by egg- and clutch size. We would predict that an increase in the available habitat e.g. clutch size could allow more bacteria to colonize. Our results suggest a clutch size dependent differences in bacterial load but opposite to the expectation we found a negative relationship between egg size and bacteria load (density). Thus, the available resource (clutch size) does not seem to be a determinant for bacteria to develop.

In conclusion species breeding in a wet habitat have dramatically higher bacterial loads.

To develop and to cope with the environment post hatching, in birds females provide all necessary substances already to the embryo inside the egg. This seems to include defence against bacteria via immune substances like lysozyme with the intention to protect the embryo against penetrating bacteria (Hincke et al. 2000). Lysozyme is a major antimicrobial protein and can mainly be found in the perivitelline layer, albumen and cuticle (Adil 2016).

Thus, because females seem to be able to allocate investment according to the needs e.g. varying risk of bacterial infections, we predicted a positive relationship between the amount of dangerous bacteria and lysozyme concentration. Surprisingly we found no evidence that these huge habitat differences in bacteria loads are mediated by the deposition of antimicrobial substance in terms of lysozyme in the egg white. In fact, egg lysozyme concentrations seem to be even lower in wet habitat birds. The enzymatic activity of lysozyme can also vary among species and depend on the pH level (Wellman-Labadie et al. 2010). Thus, the interaction between pH and lysozyme could influence the effectiveness of this antimicrobial defence as well. However, in this context our results did not reveal any difference in the pH level in egg white between the two habitats that may support such an effect.

Regarding maternal investment there seems to be a series of other antimicrobial egg proteins and some not even identified yet, which either cause direct degradation of microbes or binds vitamins, minerals necessary for microbial development and/or prevent bacterial proteases important for the invasion of pathogens (Shawkey et al. 2009, D'Alba et al. 2017). Ovotransferrin is one alternative antimicrobial substance maybe more efficient as a defensive protein than e.g. lysozyme. Grizard et al. (2015) e.g. used both antimicrobial substances, lysozyme as well as ovotransferrin, included all bacteria and used a quantitative approach in the Red-capped Lark *Callandrella cinera,* but they also did not find a relationship between bacteria abundance and these substances.

In conclusion our results do not suggests female birds performing risk-dependent adjustment nor habitat dependent allocation of maternal resources.

Alternatively, overall bacteria load may not necessarily reflect the risk of an infection, which may mainly depend on the presence of pathogenic bacteria. However, when examining haemolytic bacteria which are known to destroy red blood cells, we did not find any difference in lysozyme concentrations either. Grizard et al. (2015) did not provide an explanation for the missing relationship between all bacteria load and antibacterial substances (lysozyme and ovotransferrin).

One explanation for the unexpected result could be, that due to the huge difference in bacterial loads between wet and dry habitat species, a chemical defence would simply be inefficient or antimicrobials impossible or to costly to produce in such quantities. Beside a chemical also a physical bacteria defence is known (Adil 2016) due to a less permeable eggshell (Chavez et al. 2002), but also the cuticle could take part to physically prevent bacteria contamination (De Reu et al. 2006, Bain et al. 2013). Increasing incubation temperature (Cook et al 2005a, Grizard et al. 2014, Lee et al. 2014), to use antibacterial nesting substrate (aromatic plants, feathers, Gwinner 1997, Møller et al. 2013, Peralta-Sánchez et al. 2014, Ruiz-Castellano et al. 2016) or to keep the egg environment dry (Ruiz-de-Castañeda et al. 2011) could be alternative defence methods.

Beside investment into lysozyme we further did not find a habitat dependent variation in other maternal investment parameters. Our results do not indicate that there is a general difference in maternal resources provided to the embryo when comparing wet and dry habitat species. Testosterone e.g. shows some variation between species but independent of habitat (Fig. 5). Similarly, lysozyme and egg yolk carotenoids as well as pH seem to vary, but independent of habitat and phylogeny (Fig. 2–4). Thus, on the species level there is no indication for differential allocation strategies according to habitat and at the same time maternal investment also does not seem to differ among phylogeny (passerines versus non-passerines). There is also no difference in maternal investment parameters other than those linked to immune defence. Measures indicative for a more general maternal investment like yolk in relation to egg mass (%) or egg mass in relation to female body size (%) did not differ between dry and wet habitat species. Thus, overall ecological differences between wet and dry habitats seems to be small as species of both habitats did not differ in their maternal

performance and also follow similar strategies independent whether they are passerines or non-passerine species.

Future studies would be necessary to investigate whether and which other antimicrobial substances may play a role in bacteria defence and whether any or several of the other possibilities mentioned above may substitute a chemical defence.

Furthermore, it would be interesting to know the interactions between habitat and bacteria. One pathway could be that the water body provides the source for an increased "bacteria to eggshell" transfer. Another pathway could be that, the increased water transfer, related to parents living in that wet environment, creates favourable conditions for the bacteria to develop (reproduce). In this contact the degree of water contact of the parents could be the key variable determining bacteria loads. Finally, also the increased evaporation may contribute to create an environment in favour of egg shell bacteria to develop.

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Súhrn

Povrch vajec vtákov predstavuje vhodné prostredie pre baktérie, z ktorých viaceré môžu mať významný vplyv na embryo. Jedným z dôležitých faktorov pre vývoj baktérii je vlhkosť. Predpokladáme, že množstvo baktérii na vaječných škrupinách je vyššie u vtákov hniezdiacich vo vlhkom prostredí (napr. v mokradiach) v porovnaní s druhmi hniezdiacimi v iných (suchých) habitatoch, teda usudzujeme, že vajcia vtákov mokradí pravdepodobne čelia vyššiemu stupňu vlhkosti vplyvnom vyššieho výparu vody a kontaktu s vodou. Aby boli minimalizované škody spôsobené baktériami, samice si vyvinuli viaceré obranné mechanizmy, medzi ktoré patrí aj ukladanie antibakteriálnych látok do vajec. Naším cieľom je ukázať, či existujú rozdiely v množstvách baktérii v závisloti od typu habitatu, presnejšie, či sa druhy vtákov hniezdiace v mokradnom habitate musia vyrovnať s vyšším množstvom baktérii na vajciach a či maternálne investície do imunitnej obrany boli vyvinuté ako obranná stratégia pre ochranu embrya voči baktériám prenikajúcim cez škrupinu. Naše výsledky ukázali mnohonásobné vyššie množstvo baktérii v mokradných habitatoch v porovnaní s terestrickými. Nezistili sme zjavné rozdiely v parametroch maternálnych investícii do imunitnej obrany vajcec, ktoré sa zdajú byť nižšie v mokradnom prostredí. V tomto kontexte sú diskutované alternatívne vysvetlenia vpyvu rôznych faktorov na študované parametre.

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