Partial incubation-induced changes in concentrations of egg white antimicrobials do not influence trans-shell infection but affect hatchling phenotype

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Abstract

Host-microbiome interactions during embryonal and early phase of life is critical point in microbiome formation and assemblage in neonates. In birds, transmission of microbes from the outer environment into the egg interior was found to shape embryo viability and hatchlings phenotype. Microbes' transmission may be modulated by egg white antimicrobial proteins (AMPs) whose concentration and antimicrobial action are temperature-modulated. As partial incubation and clutch covering with nest-lining feathers during pre-incubation period may both significantly alter temperature conditions acting on eggs, we experimentally investigated effects of these behavioural mechanisms on the concentrations of primary egg white AMPs - lysozyme and avidin using Mallard (*Anas platyrhychos*) eggs. Moreover, we studied *in vivo* if concentrations of egg white AMPs reduced probability and intensity of bacterial trans-shell infection and hatchlings phenotype. We found significantly higher egg white lysozyme concentration, while avidin concentration tended to be higher in partially incubated eggs. Clutch covering with nest-lining feathers had no effect on egg white AMPs concentrations. Neither probability nor intensity of bacterial trans-shell infection was associated with concentrations of egg white AMPs. Finally, increased egg white lysozyme was associated with decreased scaled body mass index of hatchlings. These outcomes demonstrate that incubation prior to clutch completion in precocial birds may modulate concentrations of particular egg white AMPs, yet without any effect on transmission of bacteria into the egg *in vivo*. Furthermore, increased egg white lysozyme may compromise body condition of hatchlings supporting growth-regulating role of lysozyme during embryogenesis in precocial birds.

Introduction

Microbiome interactions and assembly in neonates seems to be determining for prosperity and overall success of progeny[1-4]. The most recent concept suggests so called "nidobiome" as interconnected system shaping the microbial colonization of neonates and thus a new unit of host-microbiome interactions[5]. This concept integrates parents, nest and neonates per se as the main modifiers of microbiome assembly in progeny[5]. Based on this concept, eggshell microbiome and penetration of the eggshell microbes into the egg content (i.e. microbial trans-shell infection) could be considered as one of the critical sources of nidobiome.

Avian eggshell microbiome has been found to be diverse[6,7] and primarily shaped by the nest material and environment and parents' skin and feathers[8-11]. Microorganisms have, however, also ability to enter avian egg interior, as was documented under both natural[12] and experimental conditions[13,14]. Unlike broadly documented associations between eggshell microbiota and hatching success[15,16], studies investigating proximate effects of penetrated microorganisms on avian embryo and hatchlings are scarce with only a few documenting suppressed embryo viability[12,17-19] or decreased residual body weights of hatchlings[13]. Considering diversified eggshell microbiome and its ability to enter egg interior, numerous egg-related and behavioural mechanisms protecting against uncontrolled proliferation of microbes outside and inside the eggs have been evolved in birds.

Eggshell pigmentation[20], eggshell microstructure characteristics[21-23], cuticle nanostructuring[24], deposition of antimicrobial proteins into the eggshell structures[25-27] and nest material[8,10,28] have been found to significantly shape eggshell microbiota. Nevertheless, mechanisms reducing microbial trans-shell infection and proliferation of microbes inside the eggs are primarily linked with egg incubation[29,30] and the concentrations of abundant egg white proteins[31,32], especially those having antimicrobial potential[30,33,34].

76 Among the most investigated and abundant egg white antimicrobial proteins (AMPs)
77 are lysozyme , ovotransferrin and avidin[32,35]. While lysozyme has strong bactericidal activity
78 against both G+ and G- bacteria[33,36], avidin and ovotransferrin are rather bacteriostatic[33,37,38]
79 due to their ability to reversibly bind biotin and iron thus making them unavailable for bacterial
80 growth[39-41]. Howerver, despite documented antibacterial action of egg white AMPs, egg white
81 proteomic profile may be significantly changed during incubation[42,43] leading to alterations in
82 egg white antimicrobial potential[29,30,44,45]. For example, changes of egg white lysozyme and
83 ovotransferrin concentrations were found to enhance proliferation of beneficial probiotic
84 microorganism in egg white[30]. Therefore, while incubation may significnatly shift eggshell
85 microbiota[6,45-48], its selective antimicrobial effect inside the egg is most probably inherent in
86 mediation of changes in chemical and proteomic profile of the egg white. Furthermore, as
87 evidence exists for physiological role of lysozyme and avidin on developing embryo resulting
88 in alterations of hatchlings phenotypic traits[49,50], incubation-mediated changes in egg white
89 AMPs profile might also significantly shape hatchlings´ phenotype. Despite these facts,
90 experimenal evidence for the interactive effects of egg white AMPs profile and incubation
91 under natural conditions are lacking.
92 Another behavioural mechanism with potential to reduce risk of microbial trans-shell
93 infection is clutch covering with nest lining material during pre-incubation period. Recent
94 studies revealed the role of nest material and nest-lining feathers on eggshell microbial
95 assemblage most probably related to antimicrobial agents produced by microorganisms in preen
96 gland[8,10,28]. Excepting such direct antimicrobial action of nest-lining feathers, clutch covering
97 protects exposed clutch against temperature fluctuations caused by ambient temperatures
98 during pre-incubation period[51,52]. Although in our previous experimental study clutch covering
99 with the nest lining had no effect on bacterial trans-shell infection[13], it may affect temperature
100 properties on exposed eggs and induced temperature-mediated changes in egg white AMPs

concentrations. Nevertheless, proximate role of clutch covering with feather nest lining on egg white AMPs concentration has not yet been evaluated to date.

Partial incubation is behaviour preceding full incubation of complete clutch in many bird species[53] with documented function to keep eggs dry[54], modulate eggshell microbiota[18,48], or have antipredator role[55,56]. Yet, if partial incubation may affect or stabilize antimicrobial properties of egg content is unknown. In this study, we experimentally tested if partial incubation and clutch covering with feather nest lining during pre-incubation period affect concentrations of two principal egg white AMPs - lysozyme and avidin using precocial mallard eggs exposed in natural breeding habitat. Furthermore, as in our previous study[30] experimental increase of egg white AMPs concentrations enhanced *in vitro* antimicrobial activity of egg white against selected bacterial strains, we hypothesized that different concentrations of egg white AMPs will affect probability and intensity of bacterial trans-shell infection *in vivo*. Finally, due to documented phenotype modulating roles of egg white lysozyme and avidin in other precocial birds[49,50,57], we predicted ducklings´ phenotype will be modified owing to variability in egg white lysozyme and avidin concentrations.

Material and Methods

Ethical statement

All experiments and analyses were performed in accordance with relevant institutional guidelines and regulations. The field experiment was carried out under permission no. 162 (15/2/2006), issued by the Ministry of Environment, on behalf of the Government of the Czech Republic.

Experimental procedures

We applied experimental approach with freshly laid Mallard eggs (n = 160) obtained from a commercial hatchery (Mokřiny Duck Farm, Třeboň Fisheries Ltd, Czech Republic) in June 2010. Experimental eggs were randomly selected over a single day, ensuring that they came from different females. To compute egg volume (Rohwer 1988) eggs length and width were measured with digital callipers (0.01 mm accuracy; Kinex, Prague, Czech Republic). Subsequently, each egg was cleaned with 70% ethanol to eliminate the initial eggshell bacterial assemblage, and placed into sterile portable boxes.

Four randomly selected mallard eggs were placed into each experimental nest distributed in typical breeding habitat of Mallards (Dívčice, Czech Republic (49°6′ N, 14°18′ E). Eggs in each nest were sorted based on a balanced 2 × 2 factorial design (see Fig. 1 in[13] and for all experiment details); two eggs were covered with a mixture of feather nest-lining collected from active mallard nests and two eggs remained uncovered. In addition, two eggs (one covered and one uncovered) were incubated daily in an incubator (OvaEasy 190 Advance, Brinsea Products Inc., Titusville, FL, USA) for periods that mimicked the pattern of partial incubation observed for Mallards during the egg-laying period[13,58] (see Table S1 in[13] , for details). The two remaining eggs were not incubated. Incubated eggs were transferred from experimental nests to the incubator and back each day in portable sterilized boxes. Total exposure of incubated eggs was for 45 h at 37.6 °C with a relative humidity of 60%. Eggs were turned 180° twice daily to maintain optimal egg hatchability[59]. All experimental procedures were described in[13], in detail.

Egg white sampling and assessment of egg hatchability

Egg white sampling procedures are identical with those described in[13]. Particularly, all experimental eggs were cleaned with 70% EtOH and their shell was gently perforated with a 22 G (0.7 × 40 mm) sterile needle (Terumo®, Germany) at the blunt end. Thereafter, 300 μL

of egg white removed with a 0.5-mL sterile syringe (B Braun, Germany) and in sterile cryotubes stored at -20 °C. Needle perforations in the eggshell were sealed using a gel-based adhesive (Loctite-Super Attack, Henkel, USA). Based on previous studies[60,61], such a procedure does not significantly affect the hatchability of eggs. To assess eggs hatchability, the eggs were placed back in the incubator with temperature and relative humidity at 37.6 °C and 60%, respectively, and relative humidity was increased to 80% during the egg-hatching period[62]. The weight (± 0.1 g) and tarsus-length (± 0.1 mm) of each duckling was measured immediately after hatching. An egg was assigned as fertile in the case of its successful hatching or in the presence of a cicatricule or dead embryo inside an un-hatched egg[63].

AMPs concentrations

Concentration of egg white lysozyme

Lysozyme concentration was measured using an agar well-diffusion assay[64] with 50mg of lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich, ATTC 4698, M3770) and 10µL of egg white samples that were transferred in duplicates into the holes on agar plates. Standard solutions (10µL) of known concentrations (20, 15, 7, 4, 2, 0.5 mg/mL) of lyophilized hen egg white lysozyme (62971, Fluka) was added into the holes of each agar plate. The plates were incubated for 24 hours at 21°C and 50-60% humidity. Photographs of plates with clearance zones around the holes were analysed using ImageJ. Lysozyme concentrations (mg/mL) for each egg white sample were interpolated from a calibration curve using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego California USA). Methods for measuring of lysozyme concentration is described in detail in[30] .

Concentration of egg white avidin

Avidin concentration (μg/mL) was based on a slightly modified version of the 96-well plate method of[65]. We diluted each egg white sample 10-fold in carbonate-bicarbonate buffer (made from Sigma-Aldrich C3041 capsules, following the manufacturer's instructions). We then added 100 μL of carbonate-bicarbonate buffer to each well (except the first, fifth and ninth well in each row) along rows one to 11 of a Nunc MaxiSorp® flat-bottom 96-well plate. The wells in the bottom row 12 contained 100 μL of avidin standard solution (2.5-0.002 μg/mL; Sigma Aldrich; A9275) diluted in carbonate-bicarbonate buffer. In order to ensure accurate pipetting of undiluted and diluted egg white samples, we used GENO-DNA S pipette tips (CS960 9405120, Thermo Fisher Scientific), specially designed for viscous liquids. The 96-well plate was then sealed with parafilm and incubated at 4 ºC overnight. The next day, the content of the wells was poured out and the plate rinsed three times by adding 200 μL of 0.05% Tween washing buffer (Tween 20/PBS) to each well and shaking for five minutes on an IKA KS 260 basic lab shaker. Non-specific protein sites were blocked by adding 200 μL of blocking buffer (1% solution of bovine serum albumin (Sigma Aldrich) in PBS) to each well three times for 30 sec. Then, 100 μL of a 1:4000 dilution of Superblock buffer (0.05% Tween 20/blocking buffer) with HRP (Invitrogen, Thermo Fisher Scientific) was added to each well and incubated at room temperature for 25 minutes. The wells were then washed five times with 200 μL of washing buffer, followed by 30 sec. shaking on the lab shaker. We then added 100 μL of TMB Substrate blocking buffer (Sigma Aldrich) to each well and incubated the plate at room temperature for 30 minutes. The reaction was stopped by adding 100 μL of TMB Substrate Stop Reagent (Sigma Aldrich) to each well and mixing it. Samples absorbances were measured at 450 nm using a TECAN Infinite® 200 PRO UV/Vis microplate reader (Tecan Group, Männedorf, Switzerland). Each sample was analysed in duplicates. Avidin concentrations (considering four egg white serial dilutions) were determined by interpolating from a standard curve for each

plate using GraphPad Prism 5 Software (inter-assay and intra-assay coefficients of variability were 12.6% and 3.2%, respectively).

Analysis of bacterial trans-shell infection

Bacterial genomic DNA was extracted from egg white using the EliGene MTB Isolation Kit (Elisabeth Pharmacon, Brno, Czech Republic). Incidence and intensity of bacterial trans-shell infection (BTSI) were analysed using RT-PCR based entirely on the targeting of 16S rRNA using an RT-PCR LightCycler® 480 system (Roche, Mannheim, Germany). The LightCycler 480 SYBR Green I Master (Roche) and the universal Eubacteria primer set, including forward primer Uni331 (5′-TCCTACGGGAGGCAGCAGT-3′) and reverse primer Uni797 (5′-GGACTACCAGGGTATCTAATCCTGTT-3′), were used for RT-PCR amplification[66].. Serial dilutions (10^1 to 10^9) of purified genomic *Streptococcus bovis* DNA from known number of bacterial cells were used to construct calibration curves for quantification of BTSI intensity, expressed as number of bacterial cells per 1 mL of egg white. Details on conditions for DNA amplification are in detail described in[13].

Statistics

As AMPs measurements were highly repeatable (interclass correlation coefficient = 0.86 for avidin and 0.95 for lysozyme), we used average avidin and lysozyme concentration values of each biological sample for all later analyses. Moreover, concentrations of avidin and lysozyme were not correlated (Spearman correlation, rho = 0.01, p = 0.869). We therefore built separate models predicting concentrations of these two AMPs. Eggs were clustered into quadruplets during the experimental phase of our study, which may affect probability of BTSI as shown in our previous study[13]. To account for this source of data non-independence, quadruplet´s identities were included as random intercepts into all models, if not otherwise stated. Using Generalized Linear Mixed Models (GLMMs) with Gaussian distributed errors, we tested if egg

225 volume is related to AMPs concentrations and if AMPs concentrations were affected by partial
226 incubation, clutch covering and interactions between these two variables. The effect of protein
227 concentration along with effects of the above mentioned incubation treatments on incidence of
228 BTSI and hatching success were analysed using logistic GLMMs (binomial error distribution,
229 logit link). Next, using a subset of eggs with positive BTSI (i.e. number of bacterial cells
230 estimates per 1ml of albumen > 1) and GLMMs with Gaussian error distribution, we asked if
231 the intensities of bacterial infection (log^{10} scaled) were predicted by concentrations of the two
232 AMPs. Finally, we searched for an association between AMPs concentrations and hatchling's
233 phenotype. To do so, we used (i) body mass adjusted for the effect of egg volume (i.e. residuals
234 from a linear regression on body mass vs. egg volume) and (ii) scaled body mass index[67] as
235 response variables. AMPs concentrations, along with the effect of partial incubation (that
236 considerably affects hatchling's phenotype[13]) were used as predictors. Effect of AMPs on
237 phenotype traits was modelled using linear regression since mixed models exhibited poor
238 convergence on this data subset. Moreover, there were only two quadruplets with more than a
239 single egg successfully hatched, suggesting negligible effect of data non-independence on the
240 outcomes of these analyses.

241 As data on avidin concentrations exhibited skewed distribution, we used its log^{10}
242 transformed values in all statistical calculations. Models were fitted using R software[68] running
243 in Rstudio (version 1.1.453)[69] and package lme4[70]. Backward elimination of non-significant
244 terms in the GLMM was used to select the best minimal adequate model (MAM), i.e. the most
245 parsimonious model with all effects significant[71], first eliminating non-significant interactions
246 and subsequent non-significant main effects. The significance of a particular explanatory
247 variable was derived from the change in deviance between the model containing this term and
248 the reduced model assuming χ^2 or F distribution of difference in deviances, with degrees of
249 freedom equal to the difference in degrees of freedom between the models with and without the

term of question[71].

Results

Effect of partial incubation and clutch covering with nest-lining feathers on egg white AMPs concentrations

There was no association between egg volume and lysozyme or avidin concentrations (Δd.f.= 1, $\chi^2 = 0.05$, $p = 0.816$ and $\chi^2 = 0.33$, $p = 0.566$, respectively; Table 1). Lysozyme concentrations increased in partially incubated eggs (Δd.f.= 1, $\chi^2 = 25.72$, $p < 0.001$; Table 1 and Figure 1). Similar, yet nonsignificant trend was observed for avidin (Δd.f.= 1, $\chi^2 = 3.28$, $p = 0.070$; Table 1 and Figure 1). There was, however, no effect of clutch covering or the interaction between clutch covering and partial incubation on avidin and lysozyme concentrations ($p > 0.300$ in all cases, see Table 1).

Effect of egg white AMPs concentrations on bacterial trans-shell infection (BTSI)

Concentrations of lysozyme and avidin did not affect incidence of BTSI (Δd.f. = 1, $\chi^2 = 0.05$, $p = 0.82$ and Δd.f. = 1, $\chi^2 = 0.01$, $p = 0.999$, respectively; Table 2) and at the same time, incidence of BTSI was not affected by interaction between AMPs concentrations and experimental treatments ($p > 0.2$ in all cases; Table 2). There was also no correlation between intensities of BTSI and egg white lysozyme (Δd.f. = 1, $\chi^2 = 0.42$, $p = 0.517$; Table 2) or avidin (Δd.f. = 1, $\chi^2 = 2.64$, $p = 0.104$; Table 2) concentrations in a subset of infected eggs.

Effect of egg white AMPs concentrations on hatching success

In our previous study, hatching success increased in partially incubated eggs[13], yet here we demonstrated that it was not affected by lysozyme or avidin concentrations (Δd.f.= 1, $\chi^2 = 1.26$, $p = 0.262$ and Δd.f.= 1, $\chi^2 = 1.58$, $p = 0.209$, respectively; Table 3). Similarly, we found no support for any interaction between both AMPs concentrations and experimental treatments on

hatching success ($p > 0.1$ in all cases, Table 3).

Effect of egg white AMPs concentrations on hatchling phenotype

Residual body mass and scaled BMIs of hatchlings were decreased in partially incubated eggs ($F_{(1,25)} = 23.98$, $p < 0.001$ and $F_{(1,24)} = 10.97$, $p = 0.002$, respectively; Table 4). Accounting for this source of variation, there was no effect of AMPs concentrations on residual body mass ($F_{(1,24)} = 0.02$, $p = 0.903$ for lysozyme and $F_{(1,24)} = 0.01$, $p = 0.935$ for avidin; Table 4). At the same time, avidin failed to predict variation in scaled BMI ($F_{(1,23)} = 0.14$, $p = 0.711$; Table 4). However, scaled BMI decreased with increasing lysozyme concentration ($F_{(1,24)} = 7.23$, $p = 0.013$) after statistical control for the variation induced by partial incubation (Table 4 and Figure 2).

Discussion

We found partial incubation affected concentrations of egg white AMPs in our study. Particularly, lysozyme concentration significantly increased and avidin shown only non-significant trend to increase in partially incubated eggs. In our previous experimental study, we have shown non-significant effect of partial incubation on changes in egg white lysozyme concentration in quail and pigeon eggs[30]. On the other side, previous studies documented decrease in egg white lysozyme concentration owing to full incubation in precocial chicken eggs[72] or in altricial red-capped lark eggs[45], or slight decrease of egg white lysozyme in chicken eggs in the early phase of full incubation[29,44]. Based on these facts, it is apparent that inconsistency exists in temperature-induced changes of egg white AMPs under different incubation modes when various embryonic developmental stages might play a role[37]. In any case, previous studies documented that lysozyme decrease in egg white owing to incubation is a result of proteins aggregation[42,43], binding of lysozyme to other proteins[73], or lysozyme

301 degradation early after incubation[29]. Yet, most recent works revealed that thermal aggregation
302 of proteins and resulted changes in protein abundances were highly dependent on the content
303 of particular amino acids such as arginine, lysine or aspartic acid acting as protein stabilizer
304 and/or destabilizers[74,75]. Moreover, temperature-induced level of proteins aggregation was
305 found to be linked with the concentrations of other heat-sensitive egg white proteins such as
306 ovotransferrin[76]. As proteomic and amino acids profile including arginine and ovotransferrin
307 significantly vary among species[32,65], it is highly probable that increase of egg white lysozyme
308 owing to partial incubation in in mallard eggs might be related to various ratio and
309 concentrations of aggregation preventing arginine and/or ovotransferrin. In our study, we were
310 failed to analyse egg white ovotransferrin concentration due to high inconsistency in replicate
311 measurements and thus to test these associations. Yet, although above mentioned relationships
312 with aggregation preventing egg white substances require further experimental testing, it is
313 necessary to take them into account in future research focused on thermal properties of egg
314 white in birds.

315 In our study, we did not find support for the role of egg white AMPs concentrations
316 preventing risk of bacterial trans-shell infection *in vivo*. Even if we revealed increased egg white
317 lysozyme in partially incubated eggs, neither lysozyme nor avidin egg white concentrations
318 affected incidence or intensity of bacterial trans-shell infection. These outcomes however do
319 not explicitly imply costs for studied species or birds in general as in our previous experimental
320 study, egg whites enriched *in ovo* with hen egg white lysozyme significantly increased *in vitro*
321 antimicrobial action against indicator strains[30]. On the other side, there is no study evaluating
322 *in vivo* antimicrobial potential of naturally varied egg white AMPs and investigating associated
323 microbial trans-shell infection. Besides, we have found in our previous study selective *in vitro*
324 antimicrobial activity of egg whites originated from precocial eggs treated with partial
325 incubation enhancing proliferation of a beneficial probiotic bacterial strain[30]. Similarly,

326 incubation was documented to shift diversity of the eggshell microbiota from initial highly
327 diverse communities including opportunistic pathogens toward less diverse communities with
328 dominance of less harmful or even beneficial microorganisms[6,77]. It seems therefore that partial
329 incubation might be the mechanisms acting outside the eggs to modulate eggshell microbial
330 communities toward harmless or beneficial microorganisms and inside to maintain beneficial
331 bacterial invaders. Moreover, coupled protective role of incubation with egg white AMPs
332 against pathogenic microorganisms seems to be most effective only in early phase of embryonic
333 development, while developing extraembryonic structures seem to play a crucial protective role
334 later during incubation[37,78].

335 Furthermore, we documented increased egg white LSM compromised body condition
336 of hatchlings expressed as scaled BMI. This is in accordance with our previous study
337 documenting growth-regulating role of egg white lysozyme as experimental increase in egg
338 white lysozyme in precocial quail (*Coturnix japonica*) eggs resulted in reduced tarsus length of
339 hatchlings[49]. Mechanisms of action could be inherent in the fact that LSM was documented to
340 have growth-regulating role in development of embryonic cartilage and skeletal structures[79]
341 including inhibition of mouse bone collagenase activity which may significantly affect
342 development of particular skeletal elements[80]. Effect of egg white avidin concentrations on
343 hatchlings phenotype was not apparent in this study. Although we documented egg white avidin
344 to alter chicks' phenotype in quail[50], growth-inhibition effect of egg white avidin on chicks was
345 documented to be strongly dependent on egg weight since only chicks originated from lighter
346 eggs enriched *in ovo* with avidin hatched with reduced tarsus length. It follows, that although
347 egg white AMPs may fulfil protective antimicrobial role for the embryo during early phase of
348 embryo development, their increase in egg white may significantly compromised embryo
349 growth and negatively affect resulted phenotype of hatchlings in precocial birds.

As in the case of partial incubation, we were failed to find any effect of clutch covering with feather nest lining on egg white AMPs and related incidence and intensity of bacterial trans-shell infection. Although clutch covering with nest lining was found to provide insulation of eggs against ambient temperatures[51,52], it seems that it rather helps to maintain eggs in optimal temperatures around physiological zero to sustain eggs viability and improved hatchability and hatchlings growth performance[81-83], than to change temperature conditions to level leading to temperature-induced changes in egg white AMPs profile. Nevertheless, only two studies have shown that nest material and feather nest-lining have strong antimicrobial potential with ability to shift especially eggshell microbiota in hoopoe (*Upupa epops*)[10,28]. As evidence for such antimicrobial action of feather nest-lining in other bird species is lacking and we did not find any effect on bacterial trans-shell infection via alterations of egg white AMPs, it is highly possible that nest lining feather in our study species may provide antimicrobial action only on the eggshells without effect on egg white AMPs and their antimicrobial potential, or its primary function is clutch insulation and/or clutch protection against visually oriented predators[56].

To conclude, we have shown in our study that partial incubation, as behavioural mechanisms documented to have various functions ranging from antipredator nest protection to maintaining egg viability, has also function to alter concentrations of egg white antimicrobials during pre-incubation phase. Furthermore, we found that even if concentrations of egg white AMPs were not associated with reduced intensity and incidence of bacterial trans-shell infection in mallard eggs *in vivo*, increased concentration of particular egg white AMPs had growth regulating role during embryogenesis at least in our precocial model species.

Data availability

The datasets generated during and/or analyzed during the current study are available from

the corresponding author on reasonable request.

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Authors' contributions

V.G.J. and J.K. conceptualized research and proposed experimental design. V.G.J. acquired funding and managed the project. J.K. conducted statistics, graphical visualization and wrote results. J.S and V.G.J. wrote the main manuscript text. All authors reviewed the manuscript.

Additional information

Competing interests

The authors declare no competing interests.

Table 1. Egg white AMP concentration - A) Avidin and B) Lysozyme as a response of partial incubation, clutch covering with feather nest lining and their interactions. Step-wise elimination of non-significant terms was used to select the best minimal adequate model (MAM). Predictors retained in the minimal adequate model (MAM) after step-wise elimination of nonsignificant variables are in bold. Significance (p) was assessed based on deviance change ($\chi 2$) and corresponding degrees of freedom (Δ D.f.)

Response	Predictor	Δ D.f.	χ^2	p
A) Avidin	Partial incubation	1	3.281	0.070
	Clutch covering	1	0.000	0.987
	Partial incubation x Clutch covering	1	0.005	0.946
B) Lysozyme	**Partial Incubation**	**1**	**25.716**	**< 0.001**
	Clutch covering	1	0.755	0.385
	Partial incubation x Clutch covering	1	0.951	0.330

645 **Table 2**. Variation in bacterial trans-shell infection (BTSI) - A) prevalence and B) intensity due
646 to the effect of egg white AMPs concentrations, partial incubation, clutch covering, and their
647 interactions. Predictors retained in the minimal adequate model after step-wise elimination of
648 nonsignificant variables are bold. Shown are probability values (p), values of $\chi 2$ statistics and
649 associated degrees of freedom
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Response	Predictor	Δ D.f.	χ^2	p
A) BTSI Prevalence	Lysozyme	1	1.619	0.203
	Avidine	1	0.001	0.981
	Partial incubation	1	0.052	0.820
	Clutch covering	1	0.052	0.820
	Lysozyme x Partial incubation	1	0.209	0.648
	Lysozyme x Clutch covering	1	0.488	0.485
	Avidin x Partial incubation	1	0.660	0.417
	Avidin x Clutch covering	1	0.328	0.567
B) BTSI Intensity	Incubation	1	3.071	0.080
	Avidin	1	2.642	0.104
	Lysozyme	1	0.420	0.517
	Clutch covering	1	0.420	0.517
	Lysozyme x Partial incubation	1	1.480	0.224
	Lysozyme x Clutch covering	1	0.772	0.379
	Avidin x Partial incubation	1	0.037	0.847
	Avidin x Clutch covering	1	0.305	0.581

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Table 3. Variation in hatching success due to the effect of egg white AMPs concentrations, partial incubation, clutch covering with feather nest lining and their interactions. Predictors retained in the minimal adequate model (MAM) after step-wise elimination of nonsignificant variables are in bold. Shown are probability values (p), values of $\chi 2$ statistics and associated degrees of freedom

Predictor	Δ D.f.	χ^2	p
Partial incubation	**1**	**8.796**	**0.003**
Lysozyme	1	1.257	0.262
Avidin	1	1.575	0.209
Clutch covering	1	1.575	0.209
Lysozyme x Partial incubation	1	0.645	0.422
Lysozyme x Clutch covering	1	2.579	0.108
Avidin x Partial incubation	1	0.080	0.778
Avidin x Clutch covering	1	0.844	0.358

Table 4. Variation in morphometric parameters - A) body mass adjusted for egg volume and B) scaled body mass index (BMI) due to the effect of egg white AMPs concentrations and partial incubation. Data were analyzes using GLMM assuming Gaussian distribution of residuals. Predictors retained in the minimal adequate model after step-wise elimination of nonsignificant variables are in bold. Shown are probability values (p), values of F statistics and associated degrees of freedom

Response	Predictor	D.f.	F	p
A) residual body mass	**Partial incubation**	(1,26)	**23.982**	**0.000**
	Lysozyme	(1,25)	0.015	0.903
	Avidin	(1,24)	0.007	0.935
B) scaled BMI	**Lysozyme**	(1,25)	**10.965**	**0.003**
	Partial incubation	(1,25)	**7.227**	**0.013**
	Avidin	(1,24)	0.140	0.711

Figure legends:

Figure 1. Variation of A) lysozyme and B) avidin concentrations in mallard eggs treated with partial incubation (incub.) vs. control un-incubated eggs (unincub.). GLMM-based probability values are shown

Figure 2. Effect of lysozyme concentrations on scaled BMI of mallard hatchlings (n = 27). Regression were adjusted for the effect of partial incubation treatment. Predictions and 95% confidence intervals are shown