*Current Zoology*, 2019, 65(5), 509–516
doi: 10.1093/cz/zoy084
Advance Access Publication Date: 14 November 2018
Article



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# Perinatal variation and covariation of oxidative status and telomere length in yellow-legged gull chicks

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*Handling editor: David Swanson*

Received on 22 June 2018; accepted on 10 November 2018

## Abstract

The perinatal period is critical to survival and performance of many organisms. In birds, rapid postnatal growth and sudden exposure to aerial oxygen around hatching markedly affect the chick redox status, with potentially negative consequences on physiology mediated by oxidative stress. In addition, telomere length (TL) undergoes reduction during birds' early life, partly depending on oxidative status. However, relatively few studies have focused specifically on the changes in oxidative status and TL that occur immediately after hatching. In this study of the yellow-legged gull *Larus michahellis*, we found that chicks undergo a marked increase in plasma total antioxidant capacity and a marked decrease in the concentration of pro-oxidant molecules during the first days after hatching. In addition, TL in erythrocytes decreased by 1 standard deviation over the 4 days post-hatching. Body mass and tarsus length covaried with total antioxidant capacity and concentration of pro-oxidants in a complex way, that partly depended on sex and laying order, suggesting that oxidative status can affect growth. Moreover, TL positively covaried with the concentration of pro-oxidant molecules, possibly because retention of high concentrations of pro-oxidant molecules results from mechanisms of prevention of their negative effects, including reduction in TL. Thus, this study shows that chicks undergo marked variation in oxidative status, which predicts growth and subsequent TL, prompting for more studies of the perinatal changes in the critical post-hatching stages.

**Key words**: early-life period, oxidative status, telomeres, yellow-legged gull

In many organisms, early postnatal stages are highly critical to survival and subsequent performance, with priming effects that can be expressed later in life (Lindstrom 1999; Metcalfe and Monaghan 2001, 2003; Saino et al. 2018 and references therein). Embryo development and early postnatal stages are typically accompanied by intense oxidative metabolism (Costantini 2014; Panda and Cherian 2014). Massive production of pro-oxidant compounds, which is part of normal physiological processes, must be accompanied by effective antioxidant mechanisms of prevention and repair of oxidative damage to biological molecules (Surai et al. 2016). Developmental and growth homeostasis is, therefore, dependent on maintenance of a balance between production of pro-oxidant molecules and antioxidant defense, and any imbalance in favor of the former can result in oxidative stress, with potentially severe consequences for physiological, immunological, and behavioral processes (Finkel and Holbrook 2000). Avian embryos develop in cleidoic eggs that contain all the nutrients that are required to pre-hatching life, with only gas and water exchanges occurring between

the egg and the external environment (Panda and Cherian 2014). Besides major components like lipids, proteins, and water the mother provides the egg with a suite of compounds, such as fat soluble vitamin E and carotenoids, which have a major role in regulating the oxidative status of the developing embryos and the newly hatched chicks (Surai et al. 2016). Redox homeostasis is challenged particularly around hatching when the embryo is suddenly exposed to atmospheric oxygen and undergoes a marked increase in metabolic rate (Hohtola 2002; Rollo 2002). Although the importance of oxidative status particularly for the hatchlings is well established, surprisingly few studies have focused on the changes in physiological variables that reflect oxidative status, including total antioxidant capacity and concentration of pro-oxidant molecules, during the early postnatal stages (e.g., Parolini et al. 2017a, 2017b; Possenti et al. 2018).

Oxidative status has also been functionally linked to telomere dynamics (Beaulieu et al. 2011). Telomeres are nucleoproteins located at the termini of eukaryotic chromosomes, which are involved in the maintenance of chromosome integrity (Haussmann et al. 2003; Monaghan 2010). Under normal physiological conditions, telomeres undergo shortening at each cell division due to the inability of DNA polymerase to completely replicate linear strand DNA. When telomeres reach a critical minimum length, cells enter a replicative senescence and stop dividing, and telomere shortening is thus believed to be one of the major mechanisms causing organismal loss of function (Monaghan and Haussmann 2006). The rate of telomere shortening has been shown to vary with age and some studies of birds have suggested that shortening may be faster in early life stages (e.g., Parolini et al. 2015). A growing number of studies has demonstrated that telomere shortening occurs at faster pace among relatively young as compared with older individuals (e.g., Bhattacharyya and Lustig 2006; Pauliny et al. 2006; Foote et al. 2011; Heidinger et al. 2012; Pauliny et al. 2012). The process of telomere shortening, however, also depends on a number of extrinsic as well as endogenous factors (Angelier et al. 2013; Hall et al. 2004; Monaghan and Haussmann 2006; Watson et al. 2015). For example, an imbalance between antioxidant defenses and the normal or pathological production of pro-oxidant molecules has been shown to affect the process of telomere shortening (Epel et al. 2004; Houben et al. 2008). Because rapid postnatal growth and sudden exposure to atmospheric oxygen after hatching can affect oxidative status (Hohtola 2002), any relationship between oxidative status and telomere dynamics is expected to be particularly expressed soon after hatching in birds. However, fine-grained information on the relationship between oxidative status and telomere dynamics in perinatal stages is scanty.

In the present correlational study of free-living yellow-legged gulls *Larus michahellis*, we first aimed at describing the change in 2 fundamental facets of oxidative status of the chicks, that is, the total non-enzymatic antioxidant capacity (TAC) and the concentration of circulating pro-oxidant molecules (TOS), from hatching until Day 4 after hatching. In addition, we investigated the change in telomere length (TL) in red blood cells during the early post-hatching stages. Finally, we tested if somatic growth, namely body mass and tarsus length, and TL in the early postnatal stages covaried with oxidative status variables. In the analyses, we accounted for the effect of sex and laying order because growth rates and general physiological traits may differ between the sexes and egg composition and developmental trajectories can vary according to laying order. Because of the exploratory nature of our study, we refrained from making predictions on the relationships among the variables that we investigated, except for the expectation of a decrease in TL with age.

We limited our analyses to the first 4 days after hatching because we were interested in early postnatal temporal physiological variation and also because large mortality and dispersal of the chicks from the natal nest prevents longer-term longitudinal sampling in our study population.

## Materials and Methods

### Study species

The yellow-legged gull is a monogamous, semi-colonial charadriiform bird (Cramp 1998). Females lay 1–3 eggs (modal clutch: 3 eggs) at 1–3 days intervals. Hatching is asynchronous (hatching spread: 1–4 days) and occurs after 27–31 days of incubation. Hatch order parallels laying order. The chicks are semi-precocial and fed by both parents that regurgitate food boluses (see Cramp 1998).

### Field procedures

The study was performed in a colony breeding in the Comacchio lagoon (44° 20' N–12° 11' E, NE Italy) during spring 2016. The colony was visited every second day to monitor the progress of laying and mark the new nests and eggs. Each egg was weighted (to the nearest 0.1 g) on the day when it was found and again when the eggshell started to show signs of hatching, consisting of fractures in the obtuse egg hemisphere (i.e., cracking stage). When the eggshell had been fractured by the chick ("pipping" stage), the egg was injected with a drop of blue or green food dye to associate the hatchling to its original egg (Bonisoli-Alquati et al. 2007). The chick was then made individually recognizable by banding it with a colored elastic rubber ring on either tarsus, which was removed at age 4 days. On the day of hatching, we measured body mass (to the nearest 0.1 g) and tarsus length (to the nearest 0.01 mm) and collected a blood sample in heparinized capillary tubes and quickly centrifuged (within 4 h from sampling) at 11 500 rpm for 10 min to separate red blood cells from plasma, which were stored at −80°C until molecular sexing (Saino et al. 2008), biochemical, and TL analyses. On Day 4 after hatching, we searched for the chicks. Those that were not dead and could be found in the dense herbaceous vegetation were measured again and a new blood sample was collected for oxidative status and TL analyses.

### Total antioxidant capacity and concentration of pro-oxidant molecules

We measured total antioxidant non-enzymatic capacity (TAC) and the concentration of pro-oxidant molecules (TOS) in the plasma of the chicks because these are considered as comprehensive measures of oxidative status, commonly used in studies of birds (e.g., Beaulieu et al. 2011; Haussmann et al. 2011; Boonekamp et al. 2017). TAC was measured according to a colorimetric method developed by Erel (2004), exploiting the bleaching of 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{*+}$) that reacts with antioxidants in the sample. The reaction is monitored spectrophotometrically and the absorbance is inversely related to the antioxidant capacity of the sample. The assay was calibrated with a standard curve of Trolox and the results were expressed as μM Trolox equivalent. Mean TAC intra-assay coefficient of variation (CV) was 3.4 ± 2.2% ($n = 5$ replicates), whereas the mean inter-assay CV was 4.8 ± 2.6% ($n = 3$ assay plates).

The concentration of pro-oxidant molecules (i.e., TOS) was measured according to the colorimetric method developed by Erel (2005). The pro-oxidants in the plasma oxidize the ferrous ion-*o*-dianisidine complex to the ferric ion, which reacting with xylenol orange returns a blue complex. Change in coloration was monitored spectrophotometrically and

it is proportional to the concentration of pro-oxidants in the plasma. The assay was calibrated using a standard curve of hydrogen peroxide ($H_2O_2$) and the results were expressed as nM $H_2O_2$ equivalent. The mean intra-assay CV was 4.2 ± 2.1% ($n = 5$ replicates) and the inter-assay CV was 6.3 ± 4.5% ($n = 3$ assay plates).

### TL analysis

TL analysis was performed according to the method described by Parolini et al. (2017a). TL measurement was performed in peripheral blood because it provides a good evaluation of TL and dynamics in the entire organism (Daniali et al. 2013; see also Reichert et al. 2013). Genomic DNA was extracted from 10 to 20 μL of red blood cells using Wizard® Genomic DNA Purification Kit (Promega). The quantity and the purity of genomic DNA were checked with a NanoPhotometer (IMPLEN). TL was measured by the monochrome multiplex quantitative PCR (MMQPCR) method using an iQ5 real-time PCR detection systems (BioRad). PCR reactions were prepared using 20 ng of genomic DNA as template, Quantitative Master Mix 2X SYBR Green (Genespin), telomere (T) and CTCF (S) primers at a final concentration of 1, 000 nM and 500 nM each, respectively. The sequence of telomere primers was: telg 5′-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3′ and telc 5′-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3′. The single copy sequence used as a control was a fragment from the 12th exon of the swallow CTCF gene (CCCTC-binding factor zinc finger protein). The sequence of CTCF primers was: forward (5′-CCCGCGGCGGGCGGCGCGGGCTGGGCGGCTCCCAATGGAGACCTCAC-3′) and reverse (5′-CGCCGCGGCCCGCCGCGCCCGTCCCGCCCATCACCGGTCCATCATGC-3′). The CTCF primers are composed of a swallow genomic sequence and a GC-clamp at the 5′ end (underlined) to increase the melting temperature of the PCR product. As the melting temperature of PCR products of telomeres and CTCF were different, both primer pairs were simultaneously included in the same reaction. Cycling parameters for the PCR reactions were previously described by Parolini et al. (2017a) and were: Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and Stage 3: 35 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition, 10 s at 84°C, 15 s at 88°C with signal acquisition. Four-fold serial dilutions (from 10 to 100 ng) of a yellow-legged gull hatchling considered as a reference sample (genomic DNA was extracted by red blood cells of a coeval chick not included in the experiment) were included in each plate to produce a standard curve to check for the reaction efficiency and to quantify the amount of telomere and CTCF repeats in each sample. The same reference sample was included in each plate. All reactions were run in triplicate and 6 plates containing 20 samples each were performed. Samples were randomly assigned to the plates. Five samples were replicated in each plate to assess repeatability of telomere measures. TL was measured as the T/S ratio, corresponding to the ratio between the mean values of the amount of telomere repeats (T) and of the CTCF gene (S), which was then related to the T/S value of the reference sample. Thus, TL was expressed as relative telomere length (hereafter RTL) according to Cawthon (2009). The mean reaction efficiencies for both CTCF and telomere amplifications were greater than 92% (88–94%) and 86% (84–89%), respectively. The mean intra-and inter-plate coefficient of variation (±*SD*) of RTL measures was 5.79 ± 3.45% and 9.60 ± 5.44%, respectively.

### Statistical analyses

We relied on linear mixed models (LMM) of chick phenotypic traits where we included nest identity as a random effect. In all the models, we assumed a normal error distribution. To analyze variation in phenotypic traits with age we adopted a repeated-measures design where chicks were the subjects. In all analyses degrees of freedom were estimated using the between-within method (Littell et al. 1996). In the models of egg mass and of variation of phenotypic traits with age we always included the main effects of age, sex and laying order (factors) together with their 2-way interactions. The non-significant 2-way interactions were all removed in a single step in order to reduce the risk of incurring type I statistical errors with respect to a procedure of stepwise removal of non-significant terms. In the analyses of body mass and tarsus length and of RTL at Day 4 of age, we also included the effects of concentration of pro-oxidant molecules and TAC, together with their interactions with sex and laying order. Again, non-significant interactions were removed in a single step. Post hoc least squares difference tests were applied to analyze the pairwise differences between sexes or laying order positions. Chicks were not included in analyses unless they had both Day 0 and Day 4 time points. All analyses were run using SAS 9.3.

## Results

The sample included 92 eggs and chicks from 59 broods; although information on individual physiological variables was missing for 1–3 chicks (see below for sample sizes). The sample of chicks included 34, 38, and 20 individuals from a- (first), b- (second) and c- (third) eggs, respectively, 55 of which were male whereas 37 were female. The sex ratio did not significantly deviate from parity ($\chi^2_1 = 3.52$, $P = 0.061$).

Egg mass did not vary according to the sex of the embryo both at laying and at the cracking stage (Table 1). At both stages, c-eggs were significantly lighter than a- and b-eggs, which did not differ reciprocally (Table 1). Laying date in this sample did not predict egg mass both at laying and at the cracking stage (Table 1).

### Variation in phenotypic traits with age

In repeated-measures linear mixed models, all phenotypic traits changed with age. Besides an increase in body mass and tarsus

**Table 1.** Linear mixed models of egg mass variation at laying and at the eggshell cracking stage in relation to embryo sex and laying order

| | *F* | *df* | *P* | Coefficient/Marginal mean (SE) | |
|---|---|---|---|---|---|
| Laying stage | | | | | |
| Sex | 0.52 | 1, 87 | 0.474 | Males: | 88.78 (0.98) |
| | | | | Females: | 87.95 (1.08) |
| Laying order | 12.42 | 1, 87 | <0.001 | a-eggs: | 90.40 (1.06) |
| | | | | b-eggs: | 90.01 (0.94) |
| | | | | c-eggs: | 84.69 (1.24)* |
| Laying date | 0.18 | 1, 87 | 0.672 | | −0.085 (0.201) |
| Eggshell cracking stage | | | | | |
| Sex | 1.04 | 1, 86 | 0.310 | Males: | 78.36 (0.97) |
| | | | | Females: | 77.10 (1.09) |
| Laying order | 11.19 | 1, 86 | <0.001 | a-eggs: | 79.93 (1.06) |
| | | | | b-eggs: | 79.38 (0.95) |
| | | | | c-eggs: | 73.88 (1.27)* |
| Laying date | 0.15 | 1, 86 | 0.700 | | 0.075 (0.193) |

Nest identity was included as a random effect in the model. The sex by laying order interaction effect was removed as it was non-significant. Sample sizes for a-, b-, and c-eggs was 34, 38, and 20, respectively. Sample size for males and females was 55 and 37, respectively. Information on mass at cracking stage was missing for 1 egg., *c-eggs were significantly smaller than a- and b-eggs at post hoc LSD tests.

**Table 2.** Repeated-measures linear mixed model of body mass, tarsus length, plasma antioxidant capacity, concentration of pro-oxidant molecules and TL at hatching and at 4 days of age

| | *F* | *df* | *P* |
|---|---|---|---|
| Body mass | | | |
| Sex | 5.98 | 1, 88 | 0.017 |
| Laying order | 6.51 | 2, 88 | 0.002 |
| Age | 946.74 | 1, 90 | <0.001 |
| Sex × Age | 3.89 | 1, 90 | 0.051 |
| Tarsus length | | | |
| Sex | 3.73 | 1, 88 | 0.057 |
| Laying order | 9.00 | 2, 88 | <0.001 |
| Age | 717.48 | 1, 91 | <0.001 |
| TOS | | | |
| Sex | 0.04 | 1, 86 | 0.851 |
| Laying order | 0.22 | 2, 86 | 0.801 |
| Age | 42.72 | 1, 89 | <0.001 |
| TAC | | | |
| Sex | 1.51 | 1, 85 | 0.222 |
| Laying order | 2.02 | 2, 85 | 0.140 |
| Age | 90.08 | 1, 88 | <0.001 |
| RTL | | | |
| Sex | 0.36 | 1, 86 | 0.551 |
| Laying order | 1.06 | 2, 86 | 0.351 |
| Age | 51.31 | 1, 89 | <0.001 |

Nest identity was included as a random effect in the models. The interaction terms between sex, laying order and age that were non-significant were excluded from the models. Sample sizes for a-, b-, and c-eggs was 34, 38, 20, respectively. Sample size for males and females was 55 and 37, respectively. Information was missing for 2 chicks for pro-oxidant molecules (i.e., TOS) and RTL and for 3 chicks for TAC.

length between hatching and Day 4 after hatching, we found that males were slightly but significantly heavier than females, whereas chicks from c-eggs were significantly smaller than chicks from a- and b-eggs (Table 2; Figure 1). The increase in body mass with age was marginally non-significantly larger in males than in females (Table 2; Figure 1).

The concentration of pro-oxidant molecules and TAC showed marked, opposite variation between hatching and Age 4, with pro-oxidants decreasing and TAC increasing with age (Table 2; Figure 1; see also Supplementary Appendix Figures S1 and S2). These changes in oxidative status variables with age were independent of sex and laying order, as shown by the non-significant 2-way interaction effects (Table 2). In addition, oxidative status variables did not vary according to sex or laying order (Table 2).

Change in concentration of pro-oxidant molecules between hatching and Day 4 was not predicted by change in TAC ($F_{1, 82} = 0.58$, $P = 0.449$), in a linear mixed model where we controlled for sex, laying order, and egg mass.

Finally, RTL markedly declined by 9.10% (1.3 SE) with age (Table 2; Figure 1, see also Supplementary Appendix Figure S3), with the decrease over 4 days being 0.96 standard deviations of RTL recorded at hatching.

### Covariation between phenotypic traits around hatching and at Day 4

In a subsequent set of analyses, we tested if body size and TL variables at age 4 were predicted by phenotypic traits at hatching. Body mass was differentially predicted by the concentration of pro-oxidant molecules and TAC in either sex, as shown by the significant 2-way interactions (Table 3). Body mass of males marginally non-significantly ($P = 0.08$) increased with pro-oxidant amount in males, whereas the relationship was far from statistical significance ($P = 0.97$) in females (Figure 2). Body mass significantly declined with TAC in males, whereas it was not significantly related with TAC in females (Table 3; Figure 2). In addition, body mass differentially covaried with the concentration of pro-oxidant molecules in chicks of different laying order (Table 3): body mass significantly declined with pro-oxidant levels in b-chicks, whereas it non-significantly increased in a- and c-chicks. In addition, body mass at Day 4 was significantly, positively predicted by body mass at hatching (Table 3).

Tarsus length at Day 4 significantly declined with TAC at hatching, irrespective of sex and laying order (Table 3; Figure 3). In addition, tarsus length at Day 4 differentially covaried with the concentration of pro-oxidants in chicks of different laying positions: tarsus length significantly increased with pro-oxidant amount in a- and c-chicks, whereas it significantly declined in b-chicks (Table 3; Figure 3). Tarsus length at Day 4 also increased with tarsus length at hatching (Table 3).

RTL at Day 4 was significantly, positively predicted by the concentration of pro-oxidants at hatching (Table 3; Figure 4). In addition, RTL was significantly, predicted by RTL at hatching (Table 3; Figure 4). Most of the individuals showed a decline in TL with age (see also Table 2) but some (17; 19%) showed an apparent increase (Figure 4).

## Discussion

In the present, correlational study we first investigated the changes in plasma antioxidant capacity and in the concentration of pro-oxidant molecules during the first post-hatching days of yellow-legged gull chicks under natural conditions. We found that the chicks undergo a marked increase in plasma antioxidant capacity and a marked decrease in the concentrations of circulating pro-oxidant molecules after hatching. Increase in non-enzymatic plasma antioxidant capacity may reflect the mobilization of maternal antioxidants, which are stored by the developing embryo mainly in the liver and/or to the postnatal contribution of dietary antioxidants. Mobilization of antioxidants may in turn concur to the observed reduction in the concentration of circulating pro-oxidant molecules. Contrary to our expectation, however, there was no negative relationship between antioxidant capacity and concentration of pro-oxidants. This may indicate that TAC is not limiting the clearance of pro-oxidant molecules and/or that other mechanisms intervene in reducing the concentration of pro-oxidants. Postnatal development of the chick is associated with changes in the antioxidant defense strategy (see Surai and Fisinin 2014 and references therein). The main protection from oxidative stress in newly hatched chicks is afforded by maternally-transferred exogenous antioxidants, mainly vitamin E and carotenoids (Surai 2002). However, studies of diverse captive bird species have shown that during the first 10 postnatal days the concentration of antioxidant molecules (i.e., vitamin E and carotenoids) in the liver markedly decreases (Surai et al. 1998). To compensate for this decrease, antioxidant enzyme may contribute to antioxidant defenses.

As expected, we observed a reduction in TL post-hatching. A number of studies of birds has shown telomere shortening during early postnatal periods (e.g., Hall et al. 2004; Salomons et al. 2009; Herborn et al. 2014; Parolini et al. 2015). It should be noted that the reduction found in this study over just 4 days was large (9.1%)

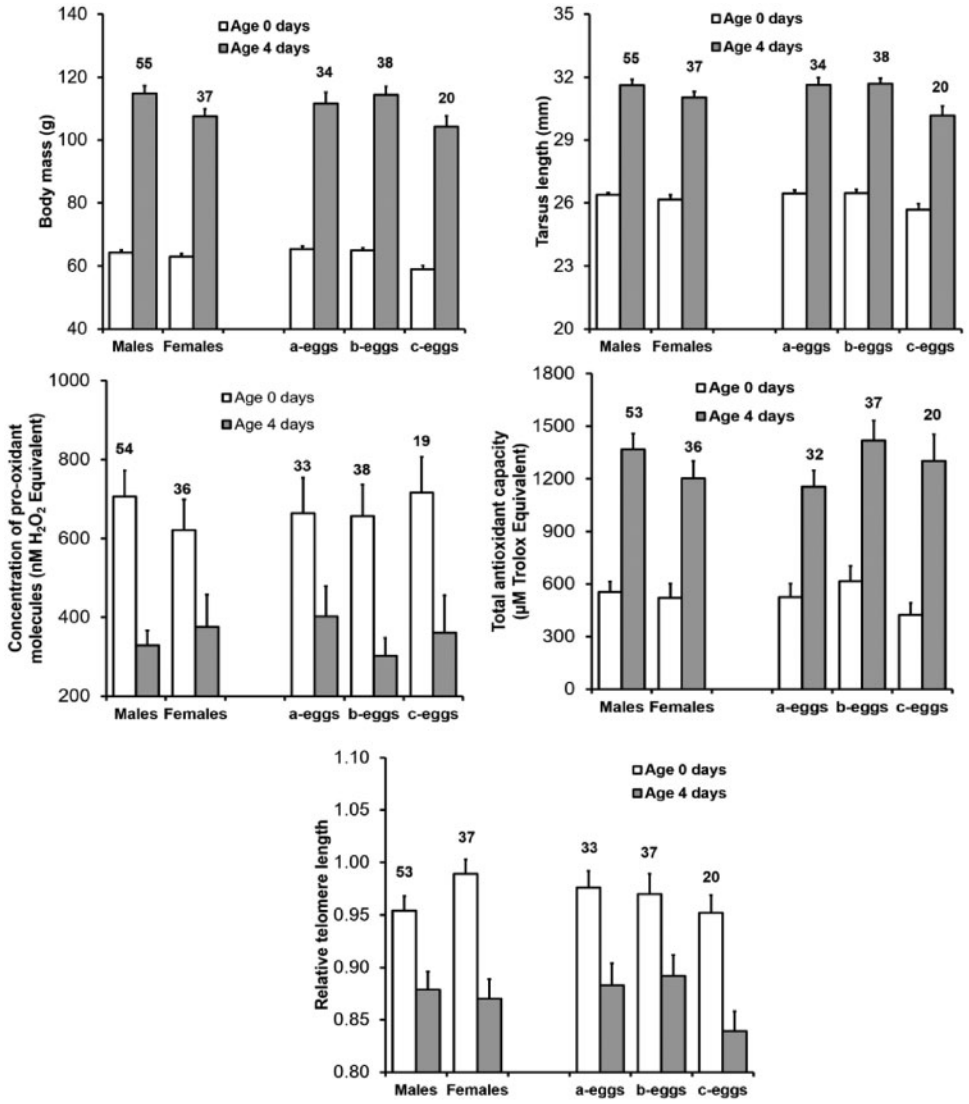


**Figure 1.** Mean (+ *SE* bar) body mass (g), tarsus length (mm), plasma concentration of pro-oxidant molecules (nM $H_2O_2$ Equivalent), plasma total antioxidant capacity (μM Trolox Equivalent) and RTL measured on chicks at age 0 and at age 4 days after hatching, according to sex and laying order. The number of chicks in each class is shown. See Results and Table 2 for the statistics of the effects of sex and age.

and similar to that observed (10%) over a larger (9 days) post-hatching growth period in the barn swallow, which has considerably shorter duration of the growth period (ca. 15 days) than the yellow-legged gull (ca. 30 days) (Parolini et al. 2015). However, in this study TL variation with age was measured immediately after hatching whereas in the barn swallow it was measured from Day 7 to Day 16, that is, in the last part of the pre-fledging growth period. Thus, a first main finding of our study is that during the 4 days post-hatching, RTL considerably decreased, on average by ca. 1 standard deviation of RTL at hatching. Our study thus shows that the perinatal stage is a period of rapid reduction of TL in circulating erythrocytes. The changes in oxidative status variables and in RTL were independent of sex and laying order, as shown by the non-significant interactions with sex. In addition, it is interesting to note that TL increased, rather than decreased, in some individuals. Our results agree some previous studies showing that a variable proportion of individuals appear to undergo an increase, rather than a decrease, in TL with age (Bize et al. 2009; Foote et al. 2011; Beaulieu et al. 2011; Parolini et al. 2015). Unfortunately, the factors that can predict the increase of TL in some individuals remain unclear and deserve further investigation. We may speculate that such differential variation depends on individual genetic differences in telomere dynamics and/or variation in early maternal effects as those mediated, for example, by the biochemical quality of the egg. Alternatively, we might evoke the role of telomerase, although no data concerning telomere elongation or telomerase activity in blood cells from the yellow-legged gull are currently available. However, a previous work by Haussmann et al. (2007) detected telomerase activity in different tissues from the tree swallow, and associated telomerase activity with the rate of telomere shortening in erythrocytes. Moreover, in blood cells from some long-lived bird species, an increase in TL with age, rather than a decrease, was observed in some individuals (Monaghan and Haussmann 2006; Pauliny et al. 2012; Young et al. 2013; Mizutani et al. 2013).

Body mass and size growth were also independent of laying order and sex, although males showed a marginally non-significant larger increase in body mass compared with females. Egg mass at laying and around hatching did not significantly differ between the sexes,

**Table 3.** Linear mixed models of body mass, tarsus length, and TL at age 4 days in relation to phenotypic traits at age 0, that is, at hatching

| | *F* | *df* | *P* | Coefficients (*SE*) |
|---|---|---|---|---|
| Body mass at Day 4 ($n = 88$) | | | | |
| Sex | 0.38 | 1, 77 | 0.540 | |
| Laying order | 4.05 | 2, 77 | 0.021 | |
| Body mass at hatching | 21.81 | 1, 77 | <0.001 | 1.318 (0.282)* |
| TAC at age 0 | 14.20 | 1, 77 | <0.001 | |
| TOS at age 0 | 0.37 | 1, 77 | 0.545 | |
| Sex × TOS at age 0 | 4.63 | 1, 77 | 0.035 | males: 0.012 (0.007); females: $3.0 \times 10^{-4}$ (0.009) |
| Sex × TAC at age 0 | 5.08 | 1, 77 | 0.027 | males: −0.019 (0.004)*; females: −0.005 (0.005) |
| Laying order × TOS at age 0 | 3.20 | 2, 77 | 0.046 | a-chicks: 0.001 (0.005); b-chicks: −0.013 (0.005)*; c-chicks: 0.012 (0.006) |
| Tarsus length at Day 4 ($n = 88$) | | | | |
| Sex | 0.36 | 1, 79 | 0.548 | |
| Laying order | 14.43 | 2, 79 | <0.001 | |
| Tarsus length at hatching | 11.21 | 1, 79 | 0.001 | 0.604 (0.180)* |
| TAC at age 0 | 6.96 | 1, 79 | 0.010 | −0.010 (0.004)* |
| TOS at age 0 | 1.47 | 1, 79 | 0.229 | |
| Laying order × TOS at age 0 | 8.88 | 2, 79 | <0.001 | a-chicks: 0.011 (0.005)*; b-chicks: −0.014 (0.005)*; c-chicks: 0.017(0.009)* |
| TL at Day 4 ($n = 87$) | | | | |
| Sex | 1.12 | 1, 80 | 0.292 | |
| Laying order | 1.75 | 2, 80 | 0.181 | |
| TL at hatching | 10.27 | 1, 80 | 0.002 | 0.383 (0.120)* |
| TAC at age 0 | 1.16 | 1, 80 | 0.284 | |
| TOS at age 0 | 6.70 | 1, 80 | 0.012 | $6 \times 10^{-5}$ ($2 \times 10^{-5}$)* |

Nest identity was included as a random effect. Only main effects and significant 2-way interaction effects are retained in the models (see statistical analyses), *Indicate that the coefficient significantly differed from 0.

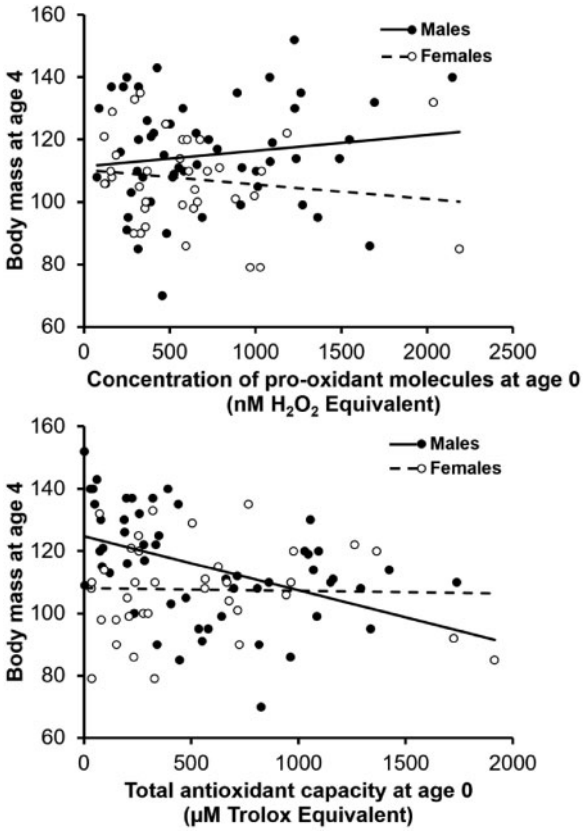


**Figure 2.** Body mass at age 4 days in relation to plasma concentration of pro-oxidant molecules (nM $H_2O_2$ Equivalent; upper panel) or plasma total antioxidant capacity (μM Trolox Equivalent; lower panel) in male and female chicks at age 0. The slopes of the relationships for either sex significantly differed (see also Results). Simple linear regression lines are fitted to better illustrate the sex-specific trends.

while being smaller in c- compared with a- and b-eggs. Thus, no sex-differences exists in egg and perinatal egg mass, whereas egg size variation according to laying order at least partly caused variation in body mass and tarsus length according to laying order, as expected.

The second main finding of our study was that body size and RTL at age 4 days were predicted by oxidative status variables around hatching (i.e., at age 0) in a complex way, that partly depended on sex and laying order. Specifically, body mass differentially covaried with both the concentration of pro-oxidant molecules and TAC around hatching in either sex. In males, body mass was significantly negatively related with TAC at hatching. In addition, body mass differentially covaried with plasma pro-oxidant levels according to laying order, with the relationship being significantly negative for b-chicks. Tarsus length at age 4 significantly declined with TAC and was also differentially related to pro-oxidant levels at hatching depending on laying order. The negative relationships between body mass (males only) and tarsus length (all chicks) and TAC suggest that chicks using antioxidants to counter the oxidative effects of increased metabolism associated with growth can afford higher growth rate. A partly different interpretation is that faster-growing chicks are those with relatively low TAC levels but these traits are not functionally related. These results also indicate that the relationship between body mass and TAC depends on sex. The interpretation of the significant negative relationship between body size measures and the concentration of pro-oxidants for b-chicks and the opposite trends for a- and c-chicks is open to speculation. In a previous manipulative study of testosterone levels in the eggs of yellow-legged gull, we have found a differential pattern of variation of brain size and the amount of pro-oxidants in the brain according to laying order in embryos from untreated eggs (Parolini et al. 2017b). However, in contrast to the present findings, pro-oxidant levels measured in the brain from embryos developed in b-eggs were lower compared with siblings from a- or c-eggs. These findings suggest that chicks from b-eggs differ in physiology from their siblings from

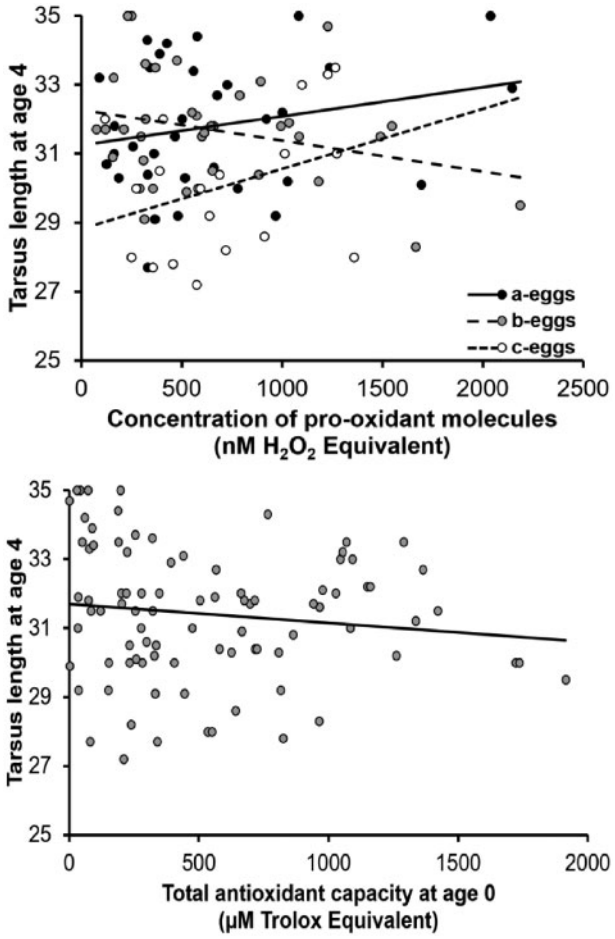


**Figure 3.** Tarsus length at age 4 days in relation to plasma concentration of pro-oxidant molecules in chicks of different laying order (nM $H_2O_2$ Equivalent; upper panel) and in relation to plasma total antioxidant capacity (μM Trolox Equivalent; lower panel) at age 0. The relationship with plasma total antioxidant capacity was negative. The relationship with the concentration of pro-oxidant molecules significantly varied according to laying order. Simple linear regression lines are fitted to better illustrate the trends.

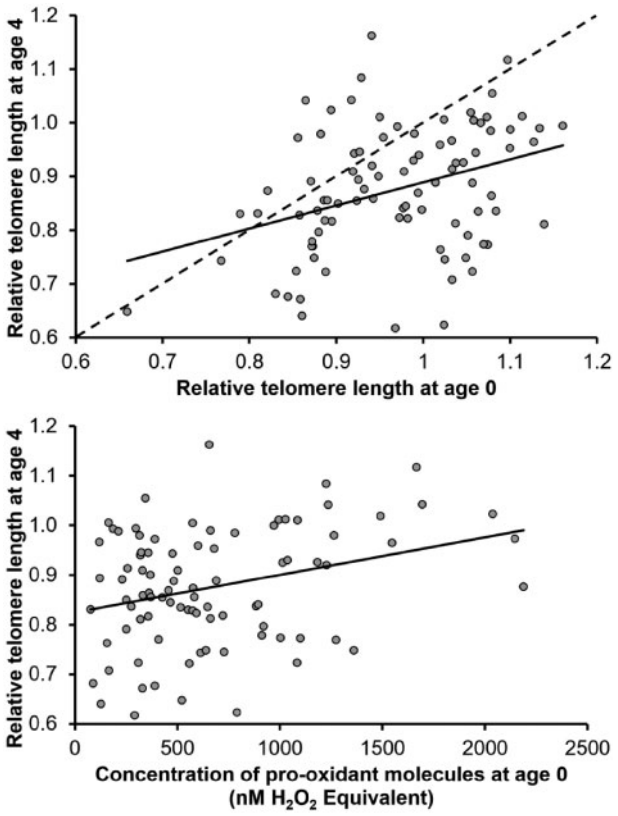


**Figure 4.** RTL at age 4 days in relation to RTL (upper panel; $N = 90$) and in relation to plasma concentration of pro-oxidant molecules (nM $H_2O_2$ Equivalent; lower panel; $N = 89$) at hatching. In both panels, the simple linear regression lines (continuous) are fitted to better illustrate the trends. In the upper panel, the dashed line represents equivalence of RTL at either age. In the graphs, we included all the bivariate data available, justifying the slight discrepancy in sample sizes between the model in Table 3 and the figure.

a- and c-eggs in a number of ways, although the causes and the function, if any, of these differences remain obscure.

RTL at age 4 was significantly, positively predicted by RTL at hatching. Hence, RTL considerably declined with age (see above), but chicks that had long telomeres at hatching also had relatively long telomeres 4 days after hatching. Similar results have been obtained in a previous study of the barn swallow, showing that RTL declined on average over the period of nestling maximal growth rate (between 7 and 16 days of age) (Parolini et al. 2015). RTL at age 4 was not predicted by TAC at hatching but positively covaried with the concentration of pro-oxidant molecules at hatching, independently of sex and laying order. These results suggest that some individuals can afford having high concentration of pro-oxidant molecules because they have longer telomeres, possibly because some mechanisms prevent high pro-oxidant potential from being translated into oxidative damage in terms of reduction of TL. For instance, early life stress experienced by offspring may prime the antioxidant machinery to withstand oxidative stress (Costantini et al. 2012), preventing telomere shortening in chicks experiencing high production of pro-oxidants.

In conclusion, this study shows that yellow-legged gull chicks undergo marked changes in physiological traits in the post-hatching period, including increase in plasma total antioxidant capacity, reduction in the concentration of circulating pro-oxidant molecules and reduction in TL. Post-hatching growth was related to antioxidant status in a complex way, depending on sex and position in the laying sequence of chicks. Interestingly, TL was positively predicted by the concentration of pro-oxidant molecules, suggesting that individuals where the pro-oxidants do not express their oxidative potential retain longer telomeres. The present results prompts for more studies of the marked changes in major physiological traits that occur around hatching in birds.

## Acknowledgements

The authors thank the Parco Regionale del Delta del Po that allowed the execution of the work. The authors also thank their colleagues of the University of Bologna, Prof. Elena Fabbri and Prof. Silvia Franzellitti, for storage of blood samples at −80°C in their freezers located at the Laboratori Sartori, Ravenna.

## Authors' contributions

M.P. participated in the field activity, performed biochemical and telomere analyses and contributed to write the article; C.D.P. and M.C. performed the field activity, biochemical, and molecular analyses; D.R. and A.R. participated in the field activity; N.S. designed the experiment, performed statistical analyses, contribute to write the article, and supervised the work.

## Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz

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