**Electronic supplementary material for**

**Pale and dark morphs of tawny owls show different patterns of telomere dynamics in relation to disease status**

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This document includes:

-Supplementary methods

-Supplementary results

-Supplementary references

-Supplementary figure S1

**Supplementary methods**

**Plumage colour determination**

Plumage colouration was determined by authors KA and PK on adults using a semi-continuous scale from 4 to 14. The colour scoring method focuses on the degree of reddish-brown pheomelanin pigmentation in the facial disc, the ventral- and dorsal side plumage and an overall appearance. The colour scoring method is highly repeatable (females r = 0.90; males r = 0.92, [1]). A low score implies less pigmentation and pale grey dominated plumage, whereas a higher score indicates higher degree of reddish-brown pheomelanin pigmentation. The distribution of colouration across individuals is bimodal, and the colour score can be used as a semi-continuous variable, or it can be used to classify individuals *a posteriori* to a grey morph (score < 10) and a brown morph (score ≥ 10, see [1] for details). Here we use colour score as a semi continuous variable in all statistical analyses.

**Age determination of adult tawny owls**

Breeding tawny owls which were not ringed as nestlings were aged as one-year-old, two-year-old, or older than two-year-olds by their plumage characteristics, where one-year-olds have retained their juvenile plumage, two-year old owls have moulted only part of their flight feathers while retaining some juvenile flight feathers, in contrast to birds older than two-years which have both old and new adult flight feathers [2, 3]. Juvenile feathers can be determined by their different shape and banding pattern as compared to adult feathers.

**Molecular analyses**

***Leucocytozoon* parasitemia and total DNA quantification: qPCR mix, thermal cycle protocol and standard curve analyses**

Each 25μl reaction contained 5μl DNA template (1 ng/μl), 12.5 μl Supermix (Platinum SYBR-green q-PCR SuperMix-UDG, Invitrogen), 0.1 μl ROX, 1 μl (10μM) of each primer and ddH2O. Thermal cycling condition after an initial incubation at 50°C for 2min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, (55°C for STAL and 58°C for *sfsr*3 for 45 s) and at 72°C for 45 s. The method included both melt curve (to check the specificity of the PCR products) and standard curve analysis (to check the efficiency of PCR). Standard curves were produced by diluting samples of known parasitemia of 0.8 % with uninfected tawny owl DNA in five step -5x dilutions (0.8, 0,16, 0.032, 0.0064, 0.0012) and for quantification of host DNA with ddH2O in five step -5x dilutions (25 ng, 5 ng, 1 ng, 0.2 ng, 0.04 ng). Each qPCR plate contained samples, serially diluted standards, two negative controls, all in duplicates. We discarded and reran experiments producing standard curves that were outside the 100 ± 15% qPCR efficiency range and relative parasitemia was recalculated after adjusting for the total DNA content in each reaction [4, 5].

**Telomere quantification: qPCR mix, thermal cycle protocol and calculation of T/S ratio**

For telomere quantification, each 25μl reaction contained 5 μl DNA template (1 ng/μl), 12.5 μl Supermix (Platinum SYBR-green q-PCR SuperMix-UDG, Invitrogen), 0.1 μl ROX, 0.3 μl (10μM) of the *Tel* primers and ddH2O. Samples were incubated at 50°C for 2 min and 95°C for 10 min, followed by 30 thermal cycles of (95°C for 15 s, 60°C for 30 s and 72°C for 30 s).

Each DNA sample was run in duplicate and a reference sample (one tawny owl sample of 1ng/μl) was also run in duplicate on each plate to control for inter-plate variability. Standard curves were produced by diluting one random tawny owl DNA sample with ddH2O in 5 steps dilution series (25, 5, 1, 0.2 and 0.04 ng). Each 96-well plate included samples, an “inter-plate control”, serially diluted standards along with two negative controls, all in duplicates. Each sample melt curve was analysed individually and we discarded and re-ran qPCR plates that produced standard curves that were outside the 100 ± 15 % qPCR efficiency range. We ran the telomere repeat and single copy nuclear sequence primers on separate plates due to differences in annealing temperatures. To control for between plates variation, we first adjusted both the telomere measurement and the total DNA content measurement by dividing them by the values obtained from the “inter-plate control” run on each plate. We then calculated a relative telomere length (T/S ratio) value, by dividing the (plate-adjusted) qPCR value for the telomere length Ct (T) with the (plate-adjusted) qPCR value for the single copy nuclear sequence Ct (S) [6, 7]. As the standard curves are expressed in ng / μl, this is the unit we use to calculate T/S ratio. Hence, this approach can easily be compared with ΔΔCT -values as –log2 of T/S is equal to ΔΔCT.

**qPCR repeatability**

To evaluate the repeatability of the qPCR for parasite quantification, telomere quantification and single host DNA quantification, we calculated within-plate as well as between-plate variability. To evaluate the within-plate repeatability (i.e., between technical replicates of the same sample) and between plate, we used three plates for each primer set (*sfsr*/3, tel and stal) with 12 random samples, run in duplicates on each plate. We analysed the data using linear mixed effect models (LMM) fitted with restricted estimate maximum likelihood (REML, Lme4 package [8] in R 3.3.1) and fitting the sample id as random factor. The within-plate repeatability was very high at all primer sets (*sfsr*/3, mean Intra Class Correlation (ICC) = 0.97; tel, mean ICC = 0.98 and stal, mean ICC = 0.80). Between plates repeatability (normalised for inter-plate control) was also very high (tel, mean ICC = 0.98; *sfsr*/3, mean ICC = 0.98). Finally, we calculated the repeatability of the T/S ratio (i.e., our estimate of ‘telomere length’ after normalization for both the ‘inter-plate control’ and the total DNA content in the sample) and final parasites intensity values (i.e., after normalization of total DNA contents) using the same statistical method as above. We found a high between plates repeatability for the final measurements (T/S, ICC = 0.98; Parasites intensity, ICC = 0.78). This method has also been shown to be highly repeatable in previous studies [6, 7].

**Statistical methods**

**LMM on variation in telomere length: explanatory variables**

In the telomere length model (N = 158 observations of 60 individuals) we tested the fixed effects laying date, colour score, infection prevalence (infected, not infected), age class (3 classes, 1-, 2- and 3-year-old and older) sex (0 = female, 1 = male), breeding experience (first breeder = 1, experienced breeder = 2). We also tested the two-way interactions colour score \* year, infection prevalence \* age class and infection prevalence \* colour score to explore whether colour morphs differed in telomere dynamics over time, and whether parasite infections had different effects on telomeres depending on age or plumage colour. The explanatory variable ‘laying date’ was transformed from date to a standardized continuous variable with the long-term median 31. March coded as ‘0’ and all observations calculated as deviations from that (range 14. February (-46) to 25. April (+25)).

Resistance to oxidative stress (which is predicted to be associated with telomere dynamics) differs between tawny owl males and females during breeding [9]. We therefore ran preliminary analyses where we also included sex-specific interactions (sex \* age class, sex \* infection prevalence, sex \* year) in the above described models, but these were all non-significant and are therefore not shown.

**LMM on variation in telomere length with age**

In order to present the general population-level trend in telomere length in different age classes we designed a model which only included as explanatory variables the four-level factor “age class” (fledgling, one-year-old, two-year-old, three-year-old and older) and the eight-level factor “year” (2006-13). The model included data on fledglings (N = 6) and all data on breeding individuals on which we had at least one estimate of telomere length (236 observations on 132 breeding individuals).

**LMM on variation in telomere length among parasitized individuals**

In addition to the telomere length model described in the main article we also constructed a similar model on the data on infected individuals (N = 128 observations of 59 individuals). In this linear mixed model we tested the fixed effects laying date, colour score, infection intensity score, age class (3 classes, 1-, 2- and 3-year-old and older) sex (0 = female, 1 = male), breeding experience (first breeder = 1, experienced breeder = 2) while individual ID was entered as a random effect. We tested the two-way interactions (fixed effects) colour score \* year, infection prevalence \* age class and infection prevalence \* colour score to explore whether colour morphs differed in telomere dynamics over time, and whether parasite infections had different effects on telomeres depending on age or plumage colour.

**Parasite intensity data**

The parasite intensity data were highly skewed with a negative binomial distribution. Log10- or square root transformation did not normalize the distribution. We therefore modelled parasitemia as a semi-continuous variable, where we assumed the data to follow a gaussian distribution (6 categories from zero to high intensity):

“0”: not infected (N = 30 obs); “1”: 0.0001 < x ≤ 0.01 % infected blood cells (N = 25 obs); ”2”: 0.01 < x ≤ 0.1 % infected ( N = 16 obs); “3”: 0.1 < x ≤ 1 % infected (N = 45 obs); “4”: 1 < x ≤ 3 % infected ( N = 32 obs); “5”: x > 3 % infected ( N = 10 obs).

The “0” not infected observations were discarded from the parasitemia analyses and used only in relation to infected individuals (prevalence: infected vs not infected, see main article). We separately analysed parasitemia among parasitized birds and parasite prevalence (infected vs not infected, see main text), because these are different processes: infection with a novel disease can cause systemic stress (infected vs not infected) and defence against a chronic disease (variation in parasitemia) cannot necessarily be directly compared with individuals not suffering from the disease.

In the *Leucocytozoon* infection intensity score model (N = 130 observations of 59 individuals, excluding uninfected individuals) we tested the effects of the continuous variables laying date and colour score, as well as the factors age class (3 classes, 1-, 2- and 3-year-old and older), sex (0 = female, 1 = male) and year (8 years), and additionally the two-way interactions colour score \* year, colour score \* laying date and colour score \* age class.

**Supplementary results**

**Variation in infection intensity score**

In the data set with infected individuals only (uninfected removed) there was large variation between years in infection intensity scores (score range 1-5) with lower scores in 2009 (t = -5.72, P < 0.0001), and 2012 (t = -2.13, P = 0.04) as compared to other years (LMM; Year: χ27 = 45.16, P < 0.001). The infection intensity score was not associated with plumage colouration (LMM, Colour score: χ21 = 0.53, P = 0.47) and did not differ between age classes (LMM, age class: χ22 = 0.52, P = 0.77). There were no differences in infection intensity score between sexes (LMM, Sex: χ21 = 2.16, P = 0.14) or any effects associated with timing of breeding (LMM, Laying date: χ21 = 0.001, P = 0.97), and no two-way interactions were significant (Colour score \* laying date: χ21 = 0.68, P = 0.41, Colour score \* year: χ27 = 5.11, P = 0.65, Colour score \* age class: χ22 = 1.38, P = 0.50). In the LMM both the marginal and conditional R2 = 0.38.

**Variation in telomere length with age**

Telomere length was shorter in older individuals (LMM, age class: χ23 = 13.30, P = 0.004, R2marginal = 0.17, R2conditional = 0.27). This effect of age was due to shorter telomere length between fledglings and adults and it was maintained on a more stable level in the different adult age classes (Figure S1). Telomere length also varied between years (LMM, year: χ27 = 35.46, P < 0.0001).

**Variation in telomere length among infected individuals**

In the data set with infected individuals only (uninfected removed) the infection intensity score was not associated with telomere length (Infection score: χ21 = 0.001, P = 0.97, Fig. 3A right panel). Darker pheomelanic brown individuals had shorter telomeres than paler grey ones (Colour score: χ21 = 7.42, P = 0.007) and telomere length varied between years (Year: χ27 = 32.22, P < 0.0001), but telomere length did not vary over time differently depending on colouration (Colour score \* Year: χ27 = 8.07, P = 0.33). All other variables and interactions were non-significant (Infection score \* age class: χ22 = 0.20, P = 0.91; Infection score \* colour score: χ21 = 1.63, P = 0.20; Sex: χ22 = 0.11, P = 0.74; Breeding experience: χ21 = 0.10, P = 0.75; Laying date: χ21 = 0.33, P = 0.57; Age class: χ22 = 2.03, P = 0.36; Model R2marginal = 0.26, R2conditional = 0.37; Between-individual variance: 14.43 %).

**Supplementary references**

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**Supplementary figure caption**

Supplementary figure S1: Variation in telomere length between different age classes. Shown are log10 transformed raw data mean values (± SE). Three-year-olds and older individuals are all pooled into the same age class (x-axis: Fledglings: ‘Fle’, one-year -olds (‘1-yr’), two-year-olds (‘2-yr’) and three-year-olds and older (‘3+yr’). Sample sizes are given above error bars.



Supplementary figure S1