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### ► To cite this version:

Charlotte Brun, Oscar Hernandez-Alba, Agnès Hovasse, François Criscuolo, Christine Schaeffer-Reiss, et al.. Resistance to glycation in the zebra finch: mass spectrometry based analysis and its perspectives for evolutionary studies of ageing. *Experimental Gerontology*, 2022, 164, pp.111811. 10.1016/j.exger.2022.111811 . hal-03650701

**HAL Id: hal-03650701**

**<https://hal.science/hal-03650701v1>**

Submitted on 25 Apr 2022

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Published in "Experimental Gerontology"

## **Resistance to glycation in the zebra finch: mass spectrometry-based analysis and its perspectives for evolutionary studies of ageing**

**Charlotte Brun<sup>1,2</sup>, Oscar Hernandez-Alba<sup>1,2</sup>, Agnès Hovasse<sup>1,2</sup>, François Criscuolo<sup>1\*</sup>, Christine Schaeffer-Reiss<sup>1,2†</sup>, Fabrice Bertile<sup>1,2†\*</sup>**

<sup>1</sup>Université de Strasbourg, CNRS, IPHC UMR7178, Strasbourg, France

<sup>2</sup>Infrastructure Nationale de Protéomique ProFi, FR2048, Strasbourg, France

† These authors share senior authorship

**\* Correspondence:**

Fabrice Bertile

[fbertile@unistra.fr](mailto:fbertile@unistra.fr)

François Criscuolo

[francois.criscuolo@iphc.cnrs.fr](mailto:francois.criscuolo@iphc.cnrs.fr)

**Keywords: glycation, glucose, hemoglobin, albumin, mass spectrometry, bird**

## **Abstract**

In humans, hyperglycemia is associated with protein glycation, which may contribute to ageing. Strikingly, birds usually outlive mammals of the same body mass, while exhibiting high plasma glucose levels. However, how birds succeed in escaping pro-ageing effects of glycation remains unknown. Using a specific mass spectrometry-based approach in captive zebra finches of known age, we recorded high glycaemia values but no glycated hemoglobin form was found. Still, we showed that zebra finch hemoglobin can be glycated in vitro, albeit only to a limited extent compared to its human homologue. This may be due to peculiar structural features, as supported by the unusual presence of three different tetramer populations with balanced proportions and a still bound cofactor that could be inositol pentaphosphate. High levels of the glycated forms of zebra finch plasma serotransferrin, carbonic anhydrase 2, and albumin were measured. Glucose, age or body mass correlations with either plasma glycated proteins or hemoglobin isoforms suggest that those variables may be future molecular tools of choice to monitor glycation and its link with individual fitness. Our molecular advance may help determine how evolution succeeded in associating flying ability, high blood glucose and long lifespan in birds.

## 1 Introduction

The survival of living systems is conditioned by the maintenance of many variables in the internal milieu within a limited range of values (Bernard, 1879), this steady state being called homeostasis (Calow, 1982; Cannon, 1929). One key aspect of homeostasis is the regulation at a stable level of blood glucose (Herman and Kahn, 2006), any drift from the species-specific set-points leading to deleterious consequences for health (Polakof et al., 2011). Glucose is indeed the main circulating energy fuel in organisms (Frayn, 2010) and the main energy source for key organs like the brain (Butterfield and Halliwell, 2019). Because dysregulation of glucose metabolism is more frequent as organisms age, glucose homeostasis has been highlighted as part of the deregulated nutrient-sensing, one of the hallmarks of ageing (Lopez-Otin et al., 2013). This physiological decline is related to a loss of insulin sensitivity and to an increase in circulating glucose that give rise to insulin resistance (Gluckman and Hanson, 2004). Metabolic inflexibility corresponds to a loss of organism's ability to adapt substrate oxidation rates to variations in fuel availability, *e.g.* to switch from carbohydrate to lipid metabolism in fasting *vs.* fed nutritional conditions (Rynders et al., 2018). In mammals, metabolic inflexibility is mainly involved in the development of insulin resistance (Bergouignan et al., 2011; Goodpaster and Sparks, 2017), leading to a reduction in life expectancy. Similarly in birds, baseline glucose level has been reported to be higher in response to adverse environmental conditions, and it was negatively associated with lifespan of zebra finches (*Taeniopygia guttata*) (Montoya et al., 2018). Therefore, understanding the mechanisms linking variations in glucose metabolism with individual fitness (*e.g.* lifespan) takes on a particular importance in evolutionary biology.

One astonishing biological observation is the remarkable slow ageing rate of many bird species in comparison to similar-sized mammals (Holmes and Harper, 2018), with as an example the 10 g hummingbirds living up to 10-15 years in the wild (Calder, 1990). Interestingly, birds are often presented as the animals with the highest blood glucose levels among vertebrates (Polakof et al., 2011). Despite this and their high metabolic rate (Prinzinger, 1993), their lifespans are proportionally 2-3 times greater than in comparable sized-mammals (Holmes et al., 2001). This paradoxical observation actually raises a large number of yet unresolved questions about the exact health-related impact of elevated plasma glucose concentrations in birds, and their possible mechanisms of resilience.

The ability of reducing sugars to chemically react with protein amino groups is a phenomenon called glycation (Tessier, 2010). It corresponds to the non-enzymatic covalent bonding of sugar molecules, *e.g.* glucose or fructose, to a protein molecule to form a Schiff base, which then undergoes an irreversible Amadori rearrangement in the case of glucose or Heyns rearrangement in the case of fructose. Further reactions yield a highly heterogeneous group of advanced glycation end products (AGEs, (Poulsen et al., 2013; Ramasamy et al., 2010)). Abnormal glycation levels in humans, *e.g.* when glycaemia is high, promotes oxidative stress and diabetes, cardiovascular and neurodegenerative diseases, and may ultimately contribute to ageing (Masoro et al., 1989; O'Brien and Timmins, 1994; Sell et al., 1996; Semba et al., 2010; Ulrich and Cerami, 2001). Recent hypotheses put forward to explain the bird paradox "high glucose – low glycation – long lifespan" included a lower propensity to accumulate AGEs over time (Klandorf et al., 1999; Rattiste et al., 2015), a lower permeability to glucose and/or a shorter half-life of certain cell types that are sensitive to AGEs accumulation (*i.e.* red blood cells) or specificities of avian protein sequences that may limit potential for

Amadori reactions (Holmes et al., 2001; Zuck et al., 2017). However, only very few studies have investigated protein glycation in birds.

To address the glycation – longevity bird’s paradox, one remaining challenge is our ability to analyze protein glycation. To date, glycated proteins have been measured using chromatography methods (Andersson and Gustafsson, 1995) or using commercial kits implementing a step of glycated protein capture based on boronate affinity (Ardia, 2006; Beuchat and Chong, 1998; Miksik and Hodny, 1992; Rendell et al., 1985) or cation exchange chromatography (Récapet et al., 2016) and a detection using spectrophotometry. However, these methods clearly lack specificity and sensitivity to detect protein-specific modifications, as shown recently by Suo and collaborators (Suo et al., 2019). Moreover, the measure of glycated hemoglobin has sometimes been performed from the whole blood (Ardia, 2006; Beuchat and Chong, 1998), when it should have been done from red blood cells only (Récapet et al., 2016). Therefore, it is highly probable that interferences had come from other blood proteins, glycated or not, thus introducing measurement biases. One of the gold standard methods to specifically analyze glycation events is mass spectrometry (Jeppsson et al., 2002; Priego-Capote et al., 2014; Soboleva et al., 2017), which enables addressing protein site-specificity of glycation (and thereby the intimate mechanisms of AGE formation). To our knowledge, only one bird study used liquid chromatography coupled to mass spectrometry to measure albumin glycation rates *in vivo* (Ingram et al., 2017). In the present paper, our main objective was to develop the use of mass spectrometry-based methods for the non-invasive study, from blood sampling in captive zebra finches, of glycation levels of several proteins. To do so, we used known-age individuals in which glucose and glycated forms of albumin, serotransferrin and carbonic anhydrase 2 from plasma and hemoglobin from hemolysates were accurately measured.

## **2 Materials and methods**

### **2.1 Bird and human samples**

We used blood sample aliquots of two sub-groups of captive zebra finches (*Taeniopygia guttata*) that were part of an experiment on social stress conducted in our laboratory (Quque et al., unpublished). Our individuals all belong to the experimental control group (not socially stressed). This study complied with the ‘Principles of Animal Care’ publication no. 86-23, revised 1985 of the National Institutes of Health, and with current legislation (L87-848) on animal experimentation in France. It was approved by an independent ethical committee and authorized by the French Ministry of Research (authorization reference APAFIS#12019-2018012511525879).

All birds, kept in unisex cages (0.57 x 0.31 x 0.39 m) and housed in standard conditions (24°C, 13:11 h light-dark light cycle), came from an in-house colony. They were fed ad-libitum with a commercial mix of seeds for exotic birds (tropical finches Prestige Premium Versele laga©), completed weekly with vitamins and proteins. The first group was of 11 individuals (6 males, 5 females, age 48-60 months) and was used to assess glycation proteins proportion in whole blood (hemoglobin and plasma proteins). Because we wanted to avoid freezing-melting cycles that may alter proteins structures, a second group of 8 birds was later used to assess hemoglobin isoform proportions (4 males, 4 females, age 19-60 months). In both cases, whole blood was collected (originally for the social stress experiment) in the early morning (50 µL from the brachial vein using heparinized syringe) then immediately centrifuged at 4°C (10

minutes, 2000 x g), and aliquots of red blood cells and plasma were stored at -80°C until utilization.

In parallel, self-collecting blood from a finger prick enabled us to obtain specimens from three healthy and two diabetic human individuals. Briefly, blood was collected using heparinized capillaries and immediately centrifuged (10 minutes, 2000 x g, 4°C) for preparing red blood cells before they were stored at -70°C until utilization.

## **2.2 Plasma glucose measurement**

Plasma glucose levels were measured enzymatically for all zebra finch individuals using the GLUC-PAP kit from Randox (UK). The intra-individual variability was assessed using the intra-class coefficient (ICC value = 0.849; (Cicchetti, 1994)).

## **2.3 Protein glycation measurement in zebra finch red blood cells using a commercial kit**

The fraction of glycated hemoglobin was measured using the Biocon® Diagnostik HbA<sub>1</sub> kit (Biocon Diagnostik, Germany) in red blood cells from a sub-sample (due to blood volume constraints) of our first group of zebra finches (n=9). In a first step, glycohemoglobin (HbA<sub>1</sub>) has been separated from non-glycated hemoglobin using a cation-exchanged resin. It is worth noting here that this cation-exchanged resin is not specific to hemoglobin as any other positively charged molecule can also interact with it. Then, the percent of glycohemoglobin was calculated, as indicated by the manufacturer, from the measurement of the absorbance (415 nm) of the two fractions and the kit calibrator. Again, the specificity can be disputed here, because although the absorption of the heme molecule is maximum at 415 nm, one cannot exclude the possible absorption of other molecules at this wavelength. Because of volume constraint, samples have not been run in duplicate. Intra-assay coefficient of variation of the HbA<sub>1</sub> kit used in birds has already been reported (Récapet et al., 2016).

## **2.4 Protein glycation measurement in zebra finch and human samples using mass spectrometry**

Unless otherwise specified, all chemicals and reagents were purchased from ThermoFisher Scientific (Waltham, MA, USA).

Protein glycation was assessed in red blood cells of one human individual taken as a positive control, and in the plasma and red blood cells of each zebra finch individuals from our first group (n= 11). Twenty-two and 199 µL of water containing 0.1% of formic acid were used to dilute 3 µL of plasma and 1 µL of red blood cells, respectively. Then, 5 µL of each sample were injected on an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, USA) coupled to a quadrupole-time-of-flight (Q-TOF) mass spectrometer equipped with an electrospray source (maXis II, Bruker Daltonik GmbH, Bremen, Germany). This mass spectrometer was selected here because of its [high resolution \(e.g. twice that of the Synapt G2\), which allows accurate determination of m/z values for intact proteins in denaturing conditions](#). Protein separation was achieved using a Vydac 208TP C8 HPLC column (i.d. 2.1 x 250 mm,

300 Å, 5 µm particle size, Grace, Columbia, MD, USA) maintained at 60° C. The solvent system consisted of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B). The elution was performed at a flow rate of 250 µL/minute, using a 40-minute linear gradient from 5% to 40% of solvent B. The mass spectrometer was operated in positive mode, with the source temperature set to 220 °C, the dry gas flow to 4 L/minute, and the electrospray voltage to 4500 V. Mass calibration of the TOF was achieved using ESI-L low concentration Tuning Mix (Agilent Technologies) in the mass range of 322-2722 m/z. Full scan MS spectra (500–3000 m/z) were acquired while the focus option was activated. The system was fully controlled by Hystar v3.2 (Bruker Daltonik GmbH).

MS raw data were processed using DataAnalysis v4.3 (Bruker Daltonik GmbH). Before deconvolution, MS spectra were smoothed using a Gaussian algorithm. All studied proteins eluted at different times (serotransferrin: 31 minutes, albumin: 34 minutes, carbonic anhydrase 2: 39 minutes), each within a narrow period of less than 1 minute. From the analysis of red blood cell samples, charge states ranging from +9 to +18 were consistently detected with a high intensity for hemoglobin in both species. For plasma samples, the charge state of the 10 most intense ions ranged from +47 to +56, +41 to +50, and +21 to +30, for serotransferrin, albumin, and carbonic anhydrase, respectively. For each non-glycated, mono- or multi-glycated protein forms, an extracted ion chromatogram was obtained by summing the signals from the ten most abundant charge state ions. Areas under curves were then used to obtain the relative abundances of each protein form of interest. The proportion of a given protein form was calculated as the ratio, multiplied by 100, of its abundance to the sum of abundances of all its forms.

## **2.5 *In vitro* forced glycation of zebra finch and human hemoglobin**

Unless otherwise specified, all chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO, USA).

Hemoglobin glycation (intact β-chain) was assessed *in vitro* under forced conditions, in both a sub-sample of zebra finches from our first group (again because of volume constraints, n=5) and humans (n=2 diabetic and n=3 non-diabetic). Hemolysates were obtained after one freeze-thaw cycle, and then they were centrifuged at 12000 x g for 10 minutes at room temperature. Hemoglobin-containing supernatants (1 µl) were diluted (1/100, V/V) either in the control buffer (50 mM Tris, pH = 7.5) or glucose buffer (50 mM Tris, 30 mM glucose, pH = 7.5). Forced glycation was then performed by incubation at 37°C. From twenty-five µL of sample, collected either immediately (d0), after 24h (d1) or 72h (d3) of incubation, proteins were reduced by addition of 10 µL of denaturing solution (56mM tris(2-carboxyethyl)phosphine, 50mM Tris, pH = 7.5) and 65 µL of guanidine buffer (3.5 M guanidine, 50mM Tris, pH = 7.5). After incubation at 57°C for 60 minutes, quenching was performed by addition of 1 µl of trifluoroacetic acid. Samples were then stored at -80°C until analysis. Mass spectrometry analysis was performed following the same procedure as for native bird and human samples (see above), except for the chromatographic gradient. Here, elution was performed using the following steps: 5% of solvent B for seven minutes, from 5% to 33% of solvent B in one minute, and finally a 20-minute linear gradient from 33% to 70% of solvent B.

## 2.6 Determination of the oligomeric state of zebra finch and human hemoglobin using nMS and nTD-MS

Hemoglobin quaternary structure was assessed in our second group of zebra finch (n=8) and human (n=2 diabetic and n=3 non-diabetic) red blood cells diluted (1/50, V/V) in a 10 mM ammonium acetate buffer (pH 6.8). After a 5-minute centrifugation (3000 x g), supernatants (5  $\mu$ L for human and 10 $\mu$ L for zebra finch samples) were injected on an Acquity UPLC H-Class system equipped with an UV detector operating at 280 nm and 214 nm (Waters, Manchester, UK), and coupled to a Q-TOF mass spectrometer equipped with an electrospray source (Synapt G2 HDMS mass spectrometer, Waters, Manchester, UK). This mass spectrometer is particularly adapted and tuned to conduct native MS analyses, allowing the transmission of molecular ions of proteins with a high molecular weight and reduced net charge state without inducing the dissociation of the non-covalent interactions of the analytes. Hemoglobin oligomers were separated using an Acquity BEH SEC column (i.d. 4.6 x 150 mm, 200 Å, 1.7  $\mu$ m particle size; Waters) maintained at room temperature. The mobile phase was composed of 10 mM ammonium acetate (pH 6.8). The elution was performed at a flow rate of 250  $\mu$ L/minute, according to an isocratic gradient. Native mass spectrometry (nMS) was performed to identify the different oligomeric populations with the mass spectrometer operating in the sensitivity mode and positive polarity with a capillary voltage of 3.0 kV. Desolvation and source temperatures were set to 200°C and 70°C, respectively. Desolvation and cone gas flow rates were 750 L/hour and 60 L/hour, respectively. The cone voltage was set to 60 V and the backing pressure of the Z-spray source to 6 mbar. Spectra (500-10000 m/z) were acquired without ion selection in the quadrupole. In a second step, native top-down mass spectrometry (nTD-MS) was used to fragment the hemoglobin tetramers and further identify tetramer subunits. For this purpose, the mass spectrometer source was operating under harsh conditions, i.e. with increased source and desolvation temperatures (90°C and 450°C, respectively), giving rise to the dissociation of oligomeric hemoglobin. Thereby, the experimental masses of the obtained monomers along with the experimental masses of the intact scaffolds were used to determine the composition of the different hemoglobin populations. The system was controlled by MassLynx v4.1 software (Waters, Manchester, UK). A multiple Gaussian fitting of the 280 nm chromatographic profiles was used to determine the proportions of the different hemoglobin populations (IGOR Pro, WaveMetrics, Oregon, USA).

## 2.7 Statistics

All statistics were done using R v 4.0.2. We followed five complementary analytic steps to analyze the mass spectrometry glycation measurements in birds. First, using Chi-square proportion comparisons (Pearson's Chi-squared test *from chisq.test* function), we tested for each protein target whether the proportions of non-glycated *versus* glycated forms were differing from an even distribution. Secondly, using pairwise comparisons of proportions (*prop.test* R function), we tested whether the proportion of glycated forms differed among proteins, by running successive two by two comparisons (*i.e.* glycated serotransferrin *vs.* glycated albumin, glycated serotransferrin *vs.* glycated carbonic anhydrase and glycated albumin *vs.* glycated carbonic anhydrase). To control for multiple testing, we applied Bonferroni correction to all tests. Thirdly, using standardized data of gathered glycation levels (thereafter Total Glycation Level [TGL], as the summed weighted proportion of multiple glycated protein forms, *i.e.* glycated serotransferrin, albumin and carbonic anhydrase, see Jeffs et al. (Jeffs et al., 2017)), we tested whether individual levels of glycation were predicted



by plasma glucose concentration, sex or body mass. While doing this, we also checked for existing correlations between plasma glucose levels and the glycated proportion of each protein, using Pearson's correlation coefficients. Using then TGL as the independent variable, we ran a General Linear Model (GLM) using the *glm2* package, with glucose levels, age, sex and body mass as fixed factors. TGL normal distribution was checked using the *shapiro-Wilk normality.test* and *qqnorm* R functions (*fitdistrplus* R package, (Delignette-Muller and Dutang, 2015)). Model selection was based on Akaike Information Criterion (AIC) corrected for small sample sizes (*Mumin* R package, (Barton, 2020)). The same procedure was used to check for any significant correlation between the glycated hemoglobin levels measured using the commercial kit and individual age, sex, body mass or glucose levels. Fourthly, we checked for differences in the dynamic of glycation rates in our experiment of forced glycation of human and bird hemoglobin. Because of the small sample size for the human part of the experiment, we could not test for the effect of diabetic (n=2) compared to non-diabetic status (n=3). We restricted our analyses to distinguish human (n=5) and bird (n=5) dynamics of hemoglobin glycation. Distribution of data satisfied with normality (*qqplot*, Shapiro-Wilk normality test, see results). We then used a General Linear Mixed Model with individual glycation levels as the response variable and time of exposure to glucose (day 0, 1 and 3) and species (Human and Bird) as fixed factors, with their interaction. Repeated measures on the same sample at different times (d0, d1, d3) were controlled using sample identity as random factor. Posthoc comparisons were conducted using *Emmeans* R package (Lenth et al., 2018), with Tukey's correction for multiple testing. As a last step, we used the same PCA and GLM – *Mumin* approach to test for any correlational link between hemoglobin isoforms' proportion and individual sex, body mass, age and plasma glucose levels. After checking for normality of the isoforms distributions (*i.e.* monomer, dimer, tetramer I, II and III) using the *shapiro-Wilk normality.test* and *qqnorm* R functions, we merged those variables using a Principal component analysis (PCA; (Le et al., 2008)) that extracted two principal axes (eigenvalue > 1, explaining 75.8% of the total variance, Supplementary Figure 1a): PC1 positively loaded with hemoglobin dimer proportion (0,857) and negatively loaded with tetramer III proportion (-0,943) and PC2 positively loaded with hemoglobin monomer (0.858). In fact, tetramer III and dimer isoforms' proportions are significantly negatively correlated (Supplementary Figure 1b, Pearson's correlation coefficient,  $r = -0,815$ ,  $t = -3,449$ ,  $P = 0,014$ ). GLM models included either PC1 or PC2 as response variables and individual sex, body mass, age and plasma glucose levels as fixed factors. In all models, collinearity among fixed factors were checked to be < 5 using the *vif* function of the *car* R package (Fox and Weiberg, 2019).

### 3 Results

#### 3.1 Glycaemia and protein glycation in zebra finch and human samples

Detailed raw mass spectra along with LC chromatograms are given in Supplementary Figures 2-4.

Plasma glucose concentrations in zebra finches ranged from 146.5 to 417.5 mg/dl (mean  $\pm$  sem:  $242.5 \pm 21.1$  mg/dl; Figure 1A). Concerning glycation, isolating the glycohemoglobin fraction from red blood cells using a cation-exchanged resin then performing spectrophotometric measurements enabled us to calculate the percent of glycated hemoglobin in zebra finches at values of  $2.99 \pm 0.14$  % (Figure 1B). Those glycated hemoglobin levels were not significantly associated with individual body mass, age, sex or glucose levels (GLM,  $t = 1,459$ ,  $P = 0.149$ ;  $t = 1,648$ ,  $P = 0.099$ ;  $t = 0.565$ ,  $P = 0.572$ ,  $t = 0,355$ ,  $P = 0,723$ , respectively). However, only the

non-glycated form of hemoglobin was detected using mass spectrometry (Figure 1C). Indeed, no peak was detected with a mass shift of +162Da, and the three small peaks that were detected (16210.98 Da, 16236.6 Da, and 16331.49 Da) exhibit mass shifts of, respectively, +30.43 Da, +56.05 Da, and +150.94 Da. Such mass shifts do not correspond to [glyoxal-modified \(carboxymethylamine +58 Da\)](#) or [methylglyoxal-modified \(carboxyethylamine +72 Da\)](#) haemoglobin forms. [Either they may correspond to minor proteins other than hemoglobin or they could just reflect deconvolution artefacts due to their low intensity.](#) Conversely, we were able to easily detect both the non-glycated and glycated (HbA1c) hemoglobin forms from human hemolysates, the latter representing 3.4% of total hemoglobin (Figure 1D).

Mass spectrometry analysis enabled us to visualize all the zebra finch plasma proteins we decided to focus on, both in their non-modified form (i.e. non-glycated) and in their mono- or multi-glycated forms (Figure 2A). Hence, proteoforms bearing up to one, two, and three glucose molecules were observed for plasma carbonic anhydrase 2, albumin, and serotransferrin, respectively. The proportions of each plasma protein form was calculated (Figure 2B). For serotransferrin, the higher proportion was for the mono-glycated form ( $46.0 \pm 1.5 \%$ ), the doubly glycated form ( $29.6 \pm 1.0 \%$ ), and the non-modified form ( $22.9 \pm 1.1 \%$ ). It should be noted that we have detected the serotransferrin triply glycated form only from one individual, where balanced proportions of the non-glycated and triply glycated forms (15% and 17 %, respectively), and of the doubly and triply glycated forms (33% and 34 %, respectively) were observed. For albumin and carbonic anhydrase 2, the proportion of the non-glycated form was of  $57.3 \pm 1.6 \%$  and  $86.2 \pm 0.4 \%$ , respectively. Mono-glycated forms were detected for these two plasma proteins ( $31.5 \pm 0.5 \%$  and  $13.8 \pm 0.4 \%$ , respectively), and a doubly glycated form was finally also detected for albumin ( $11.2 \pm 1.8 \%$ ). For those three targeted proteins, all proportions were found to significantly differ from even distributions (serotransferrin, Chi-square = 39.6; albumin, Chi-square = 31.8; carbonic anhydrase, Chi-square = 51.8; all  $P < 0,001$ ). It should be noted here that the small peak that can be seen for carbonic anhydrase 2 at 27772.18 Da (mass shift +55.45 Da) could correspond to a deconvolution artefact, or to an eventual sequence variant where an Arginine was replaced a Threonine. However, no such sequence variant is known, e.g. in the sequence of the human chicken carbonic anhydrase 2.

When comparing the proportion of glycated *versus* non-glycated forms (i.e. irrespective of the number of glycated sites) for given proteins, we found that there was a higher proportion of glycated serotransferrin than of glycated albumin (77% vs. 43%; Two-sample test for equality of proportions, chi-square = 22.69, 95% confidence interval (0.203 – 0.477);  $p < 0.001$ ). The proportion of glycated serotransferrin also turned out to be higher than the proportion of glycated carbonic anhydrase (77% vs. 14%; chi-square = 77.51, 95% CI (0.513 – 0.765);  $p < 0.001$ ). Finally, the proportion of glycated albumin was higher than of glycated carbonic anhydrase (43% vs. 14%; chi-square = 19.24, 95% CI (-0.419 – -0.162);  $p < 0.001$ ).

Proportion of total glycated levels (weighted for multiple glycatations) were found to follow a normal distribution (Shapiro-Wilk normality test,  $w = 0.998$ ,  $p = 0.231$ ). The output of the GLM analysis testing for relationships between individual TGL and age, sex, glucose or body mass are given in Table 1. Selection model did not find any model significantly better than the null model. None of the fixed factors was significantly linked to TGL, despite the fact that TGL and glucose levels or age tended to be positively related. When we checked for correlations between plasma glucose levels and the proportions of each glycated protein, we only found a significant correlation for glycated serotransferrin ( $r = 0.731$ ,  $t = 3.211$ ,  $df = 9$ ,  $P = 0.011$ ; glycated albumin,  $r = 0.07$ ,  $t = 0.211$ ,  $df = 9$ ,  $P = 0.837$ ; glycated carbonic anhydrase 2,  $r = 0.393$ ,  $t = 1,281$ ,  $df = 9$ ,  $P = 0.232$ ). When checking for age correlations, we only found

a positive linear link with glycated carbonic anhydrase 2 to be significantly affected ( $r = 0.700$ ,  $t = 2.938$ ,  $df = 9$ ,  $P = 0.017$ ; glycated albumin,  $r = 0.329$ ,  $t = 1.045$ ,  $df = 9$ ,  $P = 0.323$ ; glycated serotransferrin,  $r = 0.500$ ,  $t = 1.732$ ,  $df = 9$ ,  $P = 0.118$ ). Very similar results were obtained in the case where multiple glycations were not weighted (data not shown).

### 3.2 Protein glycation in forced glycated samples

To go further in the understanding of bird protein glycation, we next focused on hemoglobin modification dynamics under *in vitro* conditions of experimentally forced glycation. The experiment compared mass spectrometry-based measurements for human and zebra finch beta-globins. Sequences of human beta-globin (or hemoglobin subunit beta; P68871 in UniprotKb database) and zebra finch beta-globin (B5G106 in UniproKb or NP\_001232396 in NCBI database) are both of same length (147 amino acids), and their very similar amino acid composition leads them to share 70.7% sequence identity (83.7% sequence similarity). Finally, all known glycation sites in the human protein are conserved in the bird homologue (Figure 3A).

Mass spectrometry analysis enabled us to show that percent of glycated human beta-globin (HbA1c) remained at stable levels (mean Coefficient of Variation (CV) of 0.36% for humans and 1.97% for diabetic humans) in the *in vitro* control conditions over the three days of the experiment (Figure 3B). In the presence of glucose, the percent of glycated hemoglobin increased from  $4.5 \pm 0.3 \%$  at day 0 to  $7.3 \pm 0.7 \%$  at day 3 for humans, and from  $9.1 \pm 2.1 \%$  at day 0 to  $11.4 \pm 2.2 \%$  at day 3 for diabetic humans. Concerning zebra finch hemoglobin, no glycated form was detected in the control condition from day 0 to day 3, whereas detection was recorded in the presence of glucose (Figure 3C) at day 1 for only 3 birds of 5 ( $1.44 \pm 0.03 \%$ ) and at higher levels for the five birds at day 3 ( $3.7 \pm 0.3 \%$ ). Table 2 shows the output of the GLMM analysis: the *Day x Species* interaction was significant, indicating that the dynamics of glycation reactions differed between human and bird hemoglobin. Posthoc Tukey tests indicated that birds had lower levels of glycated hemoglobin than humans did at day 0 (estimate  $-6.310 \pm 1,308$ ,  $t = -4,825$ ,  $P = 0.017$ ), day 1 ( $-6.566 \pm 1,308$ ,  $t = -5,021$ ,  $P = 0.013$ ) and day 3 (estimate  $-5.278 \pm 1,308$ ,  $t = -4,825$ ,  $P = 0.050$ ). In humans, the level of glycated hemoglobin increased steadily at each time (day 0-day1,  $-1.122 \pm 0,258$ ,  $t = -4,344$ ,  $P = 0.008$ ; day 1 – day 3,  $-1.500 \pm 0,258$ ,  $t = -5,808$ ,  $P < 0.001$ ) while it only increased significant at day 3 in birds (day 0-day1,  $-0.866 \pm 0,258$ ,  $t = -3,353$ ,  $P = 0.061$ ; day 1 – day 3,  $-2.788 \pm 0,258$ ,  $t = -10,795$ ,  $P < 0.001$ ).

### 3.3 Oligomeric states of zebra finch and human hemoglobin

Using nMS in combination with nTD-MS, information about the quaternary structure of zebra finch versus human hemoglobin was obtained. In the case of the human homologue, no difference was seen between plasma samples from diabetic and non-diabetic patients, and we therefore present here combined data for all human samples. Tetramers' composition was determined using the experimental masses of the intact oligomers along with the observed monomers upon dissociation of the tetrameric hemoglobin populations (Figure 4, Supplementary Figures 5-6, and Supplementary Table 1). For zebra finches, the experimental masses of the subunits obtained during tetramer fragmentation were also compared to the relative circulating abundance of their six known different globin chains (Alev et al., 2009).

Hence, a tetramer population, identified as composed of two  $\alpha$ -chains and two  $\beta$ -chains, and a dimer population were found to represent  $80.7 \pm 0.8$  % and  $19.3 \pm 0.8$  % of total human hemoglobin, respectively (Figure 4). Hemoglobin profiles in zebra finches were much more complex, as three different hemoglobin tetramer populations were detected, representing  $43.6 \pm 0.6$  % (two  $\alpha_3$  and two  $\beta_3$  chains, tetramer III),  $34.2 \pm 0.2$  % (one  $\alpha_2$ , one  $\alpha_3$ , and two  $\beta_3$  chains, tetramer II), and  $6.6 \pm 0.2$  % (two  $\alpha_2$  chains and two  $\beta_3$  chains, tetramer I) of total hemoglobin, respectively (Figure 4). Additional signals corresponding to populations of dimers ( $12.5 \pm 0.5$  %) and monomers ( $3.1 \pm 0.1$  %) were also observed in zebra finch samples (Figure 4). Table 3 gives the results of the GLM testing for relationship between hemoglobin isoforms (PC1 and PC2 axes) and individual phenotypes. Model selection indicated that only PC1 was significantly related to individual body mass (Pearson's correlation,  $r = -0.752$ ,  $t = -2.795$ ,  $df = 7$ ,  $P = 0.031$ ): heavier individuals present lower PC1 values, *i.e.* higher hemoglobin tetramer and lower dimer proportions. There was no significant effect of age, plasma glucose levels or sex on PC1. PC2 (*i.e.* hemoglobin monomer proportion) was not significantly related to any of the fixed factors.

## 4 Discussion

To gain insight into the extent protein glycation may affect birds, this study investigated structural features of several proteins in the zebra finch, and how they are related with circulating glucose levels or body mass. The first observation confirmed that, as in other birds (Polakof et al., 2011), glycaemia is high in zebra finches, reaching values that are usually recorded in human diabetic patients (Rhee et al., 2019). In humans, blood glucose and glycated hemoglobin (HbA1c) levels are clearly linked together and a positive relationship is observed (Beltran Del Rio et al., 2016; Lledo-Garcia et al., 2013; Lo et al., 2014; Rohlffing et al., 2002). Conversely in birds, blood glucose and blood hemoglobin concentrations have been shown to negatively correlate in adult great (*Parus major*) and blue tits (*Cyanistes caeruleus*) (Kalinski et al., 2016), and the use of chromatography- or affinity-based methods has revealed low values for glycated hemoglobin despite elevated circulating glucose concentrations in few bird species. For instance, the high glycaemia (17 mM) measured in hummingbirds was associated with levels of glycated hemoglobin (*Calypte spe.*, *Archilocus colubris*, 3.7-4.5%) close to those in non-diabetic humans (Beuchat and Chong, 1998). Low percentages of glycated hemoglobin have also been reported in mute swans and rooks (*Cygnus olor*, *Corvus frugilegus*, 1.4-1.8%) (Miksik and Hodny, 1992), as well as chickens, turkeys and ducks (0.5-0.9%) (Rendell et al., 1985), American kestrels (*Falco sparverius*, 0.6-2.7%) (Ardia, 2006), and collared flycatchers (*Ficedula albicollis*, 0.7-3.7%) (Récapet et al., 2016). Using the same type of method, we found low percentages (2.6-4.0%) of glycated hemoglobin in zebra finches as well. Glycated hemoglobin has been proposed as a measure of condition in flycatchers, its percentage (0.9-2.1%) being not significantly influenced by age, sex or body mass but positively correlated with reproductive performance proxies, suggesting that it reflects pre-breeding condition in migrating birds (Andersson and Gustafsson, 1995). Our data do not support a link between glycated hemoglobin levels in zebra finches and individual body mass, sex or glucose levels.

It is worth reminding at this stage that all of the above-mentioned assays in birds were performed using non-specific methods (see Introduction). Therefore, to get rid of those potentially erroneous measures, we set up a specific method based on the use of mass spectrometry, which did not detect any signal corresponding to any form of glycated hemoglobin in zebra finches. It would be surprising this is due to a lack of sensitivity and a

matter of limit of detection. Indeed, the glyated hemoglobin form we targeted is the major one with the higher abundant glycation adduct, *i.e.*, the  $\beta$ -chain N-terminal fructosyl-valine residue (Wang et al., 2014; Zhang et al., 2001). Although of lower abundance, one cannot exclude that the  $\beta$ -chain of bird hemoglobin bears other modifications such as carboxymethyl-valine or carboxyethyl-valine (Jagadeeshaprasad et al., 2016; Lee et al., 2021). However, we could not detect any of these forms suggesting they may be absent or below our detection threshold.

Our method allowed detection of glyated hemoglobin in one human sample, with a proportion as low as 3.4% of total hemoglobin. Very low levels or the absence of glyated hemoglobin in zebra finches may reflect the existence of *in vivo* mechanisms of protection peculiar to bird species. First, the lower half-life of bird *vs.* human red blood cells (30-42 and 60-120 days, respectively; (Beltran Del Rio et al., 2016; Ottesen, 1948; Reddy et al., 1975; Rodnan et al., 1957) likely do not favor accumulation of glyated hemoglobin in bird's erythrocytes. Secondly, in sharp contrast with most mammal red blood cells including human ones, avian erythrocytes retain a nucleus (Jones, 2015). They might therefore have been hypothesized to express enzymes able to remove fructosamine glycation adducts, like fructosamine-3-kinase (Delpierre et al., 2002; Szwegold and Beisswenger, 2003), at higher levels than their mammalian counterparts do. Contradictory to this hypothesis, fructosamine-3-kinase appears active in human erythrocytes but only at very low levels in chicken erythrocytes (Delplanque et al., 2004). It is not known if this is a peculiarity of chickens or non-flying (captive laboratory strains) birds, or if this is a general feature in all birds. Also, it cannot be ruled out that other deglycation enzymes may exist and be active in bird red blood cells. This hypothesis may be supported by an earlier report that suggested the existence of a still unknown fructosamine deglycation mechanism independent of fructosamine-3-kinase (Szwegold and Beisswenger, 2003). Thirdly, reduced deglycation in bird erythrocytes may be linked to the near absent transport of glucose across the plasma membrane, as shown for chicken red blood cells (Johnstone et al., 1998). Less glucose in red cells would be in line with lower glycation levels. Furthermore, avian erythrocytes are equipped with functional mitochondria, including those of the zebra finch (Stier et al., 2013), and their survival can consequently depend on oxidizable substrates other than glucose. Nucleosides and glutamine have notably been reported as main substrates for chicken erythrocytes (Mathew et al., 1993).

The evolutionary origin of the differences between red blood cells of birds and mammals appears linked to the independent emergence of homeothermy in different geological ages in these two groups (Gavrilov, 2013). Amongst the differences, lower affinity for oxygen has been reported for hemoglobin in birds compared to mammals (Ajiloo et al., 2002), with the exception of diving (Meir and Ponganis, 2009) and high-altitude flying bird species (Natarajan et al., 2018). Therefore, the high-energy demand in birds, which turns into high-oxygen demand, is likely satisfied not only by the high efficiency of the avian respiratory system in uptaking oxygen, but also by facilitating the release of oxygen from hemoglobin into the tissues. Structural characteristics may be responsible for such peculiarities of bird hemoglobin, which have notably a more tense conformation than mammalian ones (Ajiloo et al., 2002; Natarajan et al., 2018). A different conformation may indicate that, although hemoglobin  $\beta$ -chain glycation sites appear conserved, *e.g.*, in the zebra finch compared to the human (Figure 3A), they could remain less accessible in the avian protein. This hypothesis is reinforced by the results we obtained in our *in vitro* experiment. The gradual increase in the levels of human glyated hemoglobin (HbA1c) upon *in vitro* exposure to glucose was observed for both the samples from non-diabetic and diabetic individuals, however, the magnitude of variation was less pronounced for the latter and the levels did not exceed 13.5%. This may suggest a limitation, at least a slowdown, in the glycation rate of hemoglobin beyond about 10-12%. In zebra finch samples, we show here that exposure to glucose allows mass spectrometry-based

detection of glycated hemoglobin *in vitro* at a proportion lower than 4%, which confirms that there was no lack of sensitivity in our *in vivo* assays (see above). However, we did not detect glycated hemoglobin *in vitro* in bird control samples, and the increased levels in bird glycated hemoglobin upon glucose exposure was delayed and not very pronounced. Overall, these data, which support the absence of hemoglobin glycation *in vivo*, may be explained by structural peculiarities of bird hemoglobin that interfere with its glycation.

Structural differences between birds and humans regarding the quaternary structure of hemoglobin was determined in our study. In its mature form, hemoglobin is generally reported to be made up of four subunits (two  $\alpha$ - and two  $\beta$ -chains in adults), including in the few birds that were studied (Abbasi and Lutfullah, 2002; Grispo et al., 2012; Manconi et al., 2007; Mohamed Abubakkar et al., 2014). From these previous works, it is also known that two tetramer isoforms differing in their alpha chains, namely HbA and HbD, may coexist in birds. Whereas we observed that the expected tetramer composed of two  $\alpha$ - and two  $\beta$ -chains was dominating the human hemoglobin signal, we made an unprecedented observation of the presence of three different tetramers along with dimer and monomer populations from the analysis of zebra finch hemoglobin. The expected tetramer with two  $\alpha_3$ - and two  $\beta_3$ -chains was identified as the most abundant (tetramer I, TI), the two  $\alpha_3$ -chains being successively replaced by a  $\alpha_2$ -chain in the two other tetramers (tetramers II and III, TII and TIII). It is possible that  $\beta$ -chain glycation sites in these bird tetramers are less accessible than in the human tetramer. The  $\alpha$ -chain substitution we suspect from one to another tetramer (Figure 4) seems to correspond to transitions from the most abundant  $\alpha$ -globin in adults (alpha3) to a more primitive one (alpha2). Moreover, while using the masses of monomers and that of the heme molecule to reconstitute the composition of each tetramer, we still could not explain an additional mass of 580 Da per tetramer (see Supplementary Figure 5). This could correspond to the presence of a cofactor, which association with globin chains could remain highly stable in zebra finches due to a putatively highly tense conformation of hemoglobin tetramers. It could be a phosphate compound like inositol pentaphosphate, already found in few bird species (Isaacks and Harkness, 1980; Riera et al., 1991). Inositol pentaphosphate modulates hemoglobin affinity for oxygen and possibly protects it from oxidative stress, as do 2,3-diphosphoglycerate in mammals (Tellone et al., 2019). The relative intensities of the monomeric populations obtained during nTD-MS analysis (Supplementary Figure 5) suggest that TIII of bird hemoglobin is more prone to dissociation compared to TII and TI. This experimental evidence could be tentatively correlated with the hydrodynamic volume, and hence to the retention time (Figure 4), of each individual tetrameric population. Thereby, TIII, with greater hydrodynamic volume (more extended structure and lower retention time) is more easily fragmented (higher monomer/tetramer ratio) in comparison with the more compact TII and TI tetramers.

The ‘modern’ hemoglobin tetramer has evolved from a noncooperative homodimer (itself evolved from an ancient monomer) with high oxygen affinity that existed before the gene duplication that generated distinct  $\alpha$ - and  $\beta$ -subunits (Pillai et al., 2020). As suggested elsewhere for few birds species about the coexistence of hemoglobin A and D isoforms (Storz, 2016a, b), and reinforced here by our detailed data about three hemoglobin isoforms in zebra finches, the multiplicity of these isoforms and adjustments in their relative abundance may allow to fine tune oxygen carrying capacity to match changing environmental and physiological conditions (as suggested by our significant link between body mass and hemoglobin tetramer and dimer proportions), and/or energy expenditure levels. Our results also raise the possibility for the hemoglobin cofactor inositol pentaphosphate to add another controlling mechanism. Overall, these mechanisms should be of key importance to ensure that bird physiology remains optimal when facing any challenge to energy expenditure levels imposed by, *e.g.*, changes in food

availability or an immune challenge, or simply due to life stage transitions (from the inactive nestling to the active fledgling) (Cornell et al., 2017). In addition, inter-specific differences in hemoglobin isoforms may also be expected, as birds live in much contrasted environments in terms of, *e.g.*, the partial pressure of oxygen in the atmospheric air, and they do not have all the same expected lifespan or ability to fly or migrate (Minias et al., 2013). A phylogenetic and comparative analysis of hemoglobin isoform structures, relative abundances, and oxygen binding capacity in many bird species should help better understand how such major traits supporting aerobic metabolism have coevolved with flying performances or if they are related to particular environmental constraints only. Further, because glucose levels are negatively related to individual lifespan (Montoya et al., 2018) and glycation, involved in the loss of proteostasis (Lopez-Otin and Kroemer, 2021), is one of the hallmarks of ageing (Chaudhuri et al., 2018), the evaluation of whether glycation eventually occurs in any bird species hence disturbing potentially the functionality of hemoglobin isoforms (or any other plasma protein) will add insight into how glycation impacts on bird's fitness and life expectancy. Despite our small sample size, we notably could see that PC1 was significantly related to individual body mass: heavier individuals seemed to present a larger proportion of hemoglobin tetramer III and a lower proportion of dimer, independently of sex, age or glycaemia. This may suggest that individuals in better body condition may also have a higher capacity of oxygen delivery to the organs. Exploring further how individual differences in hemoglobin isoform proportions is related to the organism's oxygen carrying and delivering capacities may be of key importance in the evolutionary context of individual quality (Wilson and Nussey, 2010) (*i.e.* why some individual perform better than others). This may reveal itself particularly important in determining how the transition from nestling to fledging is done successfully because: (i) hemoglobin concentration in whole blood was highlighted as a proxy of fledging physiological maturity defining survival chances (Cornell et al., 2017), (ii) glycated hemoglobin levels was previously found to be higher in nestlings that grew faster (Ardia, 2006), and (iii) we hypothesized that glycation levels were low in zebra finches, maybe due to specific structural particularities (see above). Whether surviving fledging present a higher proportion of tetramer III hemoglobin, which may be more resistant to glycation, is a tempting hypothesis.

Unlike the analysis of zebra finch hemoglobin, the application of our mass spectrometry-based method to plasma samples has shown that these birds are not free of glycation. Indeed, glycation events were detected, at single or multiple sites, for select highly abundant plasma proteins. Of these, high levels were measured for mono- (32-46%) and doubly-glycated (11-30%) forms of albumin and serotransferrin, and only a mono-glycated form (14%) was detected for carbonic anhydrase 2. In humans, non-enzymatic glycation has been reported as one of the most frequent post-translational modification occurring in the carbonic anhydrase family, with adverse consequences on enzymatic activity that can be considered among the initial steps of altered metabolism in diabetes (Di Fiore et al., 2020). Of the six glycation sites that were described for human carbonic anhydrase 2, only two appear to be conserved in the zebra finch counterpart (Supplementary Figure 7). This is consistent with the fact that, in contrast to mammals, diabetes is rarely diagnosed in birds. However, we did not find any value in the literature for the proportion of plasma glycated carbonic anhydrase 2 to be compared to the 14% we measured in zebra finches. For the two other plasma proteins we examined, the proportion of glycated forms in zebra finches was higher than that in healthy persons. The highest extent of glycation (all forms together) we observed was 77% for zebra finch glycated serotransferrin, whereas the percentage of glycated serotransferrin was of 5% in diabetic patients and only 2% in healthy people if one takes Lys-534, the major site of glycation, as a surrogate estimate of the glycated protein form (Golizeh et al., 2017). Five of the six known glycation sites in human serotransferrin are conserved in the zebra finch (Supplementary Figure

8). It could be that more lysine residues are easily accessible to glycation in the avian counterpart. Not only the zebra finch serotransferrin contains a high number of lysines (64) but its structure may be significantly different from that of the human protein, therefore favouring glycation. This is supported by the low percentage (only 66%) of amino acid identities between the human and avian sequences.

The value of 43% found here for glycated albumin in zebra finches is higher than in humans, except from severe diabetic patients. Indeed, albumin is usually reported as being glycated at about 1-10% and up to 15% in healthy people, 12-25% in diabetic patients, and up to 94% in severe diabetic patients with poor glycemic control (Anguizola et al., 2013; Kisugi et al., 2007; Rondeau and Bourdon, 2011), thus being an alternative biomarker to glycated hemoglobin for monitoring glycaemia (Soboleva et al., 2019). The eleven sites known to be possibly glycated in the human albumin sequence are also present in the avian sequence (Supplementary Figure 9). As argued above for serotransferrin, a higher number of glycation sites may exist in the zebra finch albumin, which contains numerous lysines (66) and shares only 64% of amino acid identities with the human protein. However, a recent study reported contrasting results where resistance of recombinant chicken albumin compared to recombinant bovine albumin was imputed to fewer exposed lysine residues (Anthony-Regnitz et al., 2020). Glycated albumin values were even higher abundant in zebra finches than in other bird species, like the bald eagle (24%), barred owl (24%), great horned owl (19%), and red-tailed hawk (14%), their glycaemia being also high (20-22 mM) putatively due to a diet promoting gluconeogenesis (Ingram et al., 2017). Despite less sophisticated methods were used, an earlier study also found glycated albumin at higher levels in the plasma from chickens, turkeys and ducks compared to humans (Rendell et al., 1985). In addition to possible structural features peculiar to zebra finches, differences between species may be imputed to a different half-life of this protein or more global differences in protein turnover rates.

In humans, albumin levels are positively correlated with glycated albumin levels (Mo et al., 2016) but negatively with glycated hemoglobin (Rodriguez-Segade et al., 2005; Tiwari et al., 2015). This link between hemoglobin glycation and the abundance and glycation status of albumin has further been demonstrated *in vitro* (Jagadeeshaprasad et al., 2018). Therefore, through mechanisms that remain to be determine, high levels of glycated albumin in zebra finches might contribute to the absence of hemoglobin glycation we depicted (see above).

A relationship between glycated albumin levels and circulating glucose has been reported in humans (Inaba et al., 2007). Conversely in our study, neither glycated albumin nor TGL were related to plasma glucose levels, in contrast to glycated serotransferrin levels. Therefore, serotransferrin appears as a possible better target to monitor glycation events and the response to elevated plasma glucose concentrations in birds. However, only glycated carbonic anhydrase 2 seemed to be higher in older individuals. Again, a phylogenetic and comparative future work should help better conclude on how glycaemia and glycation interact and relate to aging in more bird species.

## 5 Conclusion

The formation of a glycated protein is usually considered to depend on the amount of glucose it has been in contact with over the last months in humans. Using a specific mass spectrometry-based method, our study clearly shows that hemoglobin in the zebra finch is not glycated *in vivo*, and only at very low levels after an *in vitro* treatment with glucose. Our results



then support the hypothesis that structural avian hemoglobin characteristics may limit or prevent glycation, a possible prerequisite for the ability to fly, and they may help adjust the aerobic capacity of birds. Thus, they may have played a key role in the evolution of slow pace of life in flying birds. Concerning glycation of bird plasma proteins, differences appear to exist between species, which calls for a deeper analysis of their structure in the future. The identification of all glycation sites as well as the determination of the extent to which they are glycated and the evaluation of the links between inter-specific structural differences and contrasting life history traits in many bird species should help better understand the determinants of avian protein glycation.

## 6 Figure legends

### Figure 1. Glycemia and hemoglobin glycation in zebra finch and human samples

Plasma glucose concentration in zebra finches is expressed as the mean  $\pm$  sem of 11 independent determinations (panel A). The fraction of glycosylated hemoglobin (mean  $\pm$  sem of 9 independent determinations) was measured using the Biocon® Diagnostik HbA<sub>1</sub> kit (Biocon Diagnostik, Germany). (panel B; \* this method lacks specificity; because hemoglobin is by far the most abundant protein in red blood cells, it is assumed that the signal is actually mostly due to glycosylated hemoglobin). Examples of deconvoluted mass spectra are shown for zebra finch (panel C) and human (panel D) hemoglobin  $\beta$ -chains. The number of glucose molecules bound to proteins is indicated for each peak.

### Figure 2. Protein glycation in zebra finch plasma

Examples of deconvoluted mass spectra, obtained through liquid chromatography online with mass spectrometry, are shown for serotransferrin, albumin, and carbonic anhydrase 2 (panel A). The number of glucose molecules bound to proteins is indicated for each peak. For a given protein, the proportion of each of its detected forms is expressed as the mean  $\pm$  sem of 11 independent determinations (panel B).

### Figure 3. Human and zebra finch hemoglobin sequences and their glycation under forced *in vitro* conditions

Amino acid sequences of human and zebra finch  $\beta$ -globins were aligned using Clustal W program (panel A). An asterisk (\*) indicates positions that have a single and fully conserved residue. A colon (:) indicates conservation between groups of strongly similar properties with a score higher than 0.5 in the Gonnet PAM 250 matrix. A period (.) indicates conservation between groups of weakly similar properties with a score lower or equal to 0.5 in the Gonnet PAM 250 matrix. The amino acids in green highlight the known glycation sites of human hemoglobin and whether they appear to be present in the avian counterpart. The percent of glycosylated hemoglobin is expressed as the mean  $\pm$  sem of 3 independent determinations for humans, 2 for diabetic humans (blue circle), and 5 for zebra finches (panel B). An example of deconvoluted mass spectrum is shown for zebra finch hemoglobin (panel C).

### Figure 4. Oligomeric states and proportions of human and zebra finch hemoglobins

LC-UV-MS analysis of whole hemoglobins allowed visualizing different oligomer states (see the representative chromatograms in panel A) and determining their relative proportions (mean  $\pm$  sem of 8 independent determinations, panel B). Mass spectra obtained after dissociation of zebra finch tetramers inside the mass spectrometer are shown in Supplementary Figure 5.

## **7 Data availability statement**

Electronic supplementary materials are provided for the principal component analysis (PCA) of hemoglobin isoforms (Supplementary Figure 1), Chromatogram and representative raw mass spectra for zebra finch (Supplementary Figure 2) and human (Supplementary Figure 3) hemoglobin chains, and for zebra finch plasma proteins (Supplementary Figure 4), representative nTD-MS mass spectra of zebra finch and human hemoglobin (Supplementary Figures 5-6), human and zebra finch carbonic anhydrase 2 (Supplementary Figure 7), serotransferrin (Supplementary Figure 8) and albumin (Supplementary Figure 9) sequences, and for the calculation of the mass of hemoglobin tetrameric forms according to experimental subunit masses (Supplementary Table 1), and bird individual data (Supplementary Table 2).

## **8 Ethics statement**

Our experimental study was conducted according to EU regulation (Directive 2010/63/EU). The protocol was approved by the ethical committee CREMEAS Strasbourg (#AL/02/02/01/13).

## **9 Author Contributions**

FB and FC designed the study. MS-based glycation analysis was performed by CB and AH under the supervision of CS-R, and structural analyses were performed by CB and OH-A. Statistical analyses were generated by FC and FB and FC wrote the manuscript with the input of all authors.

## **10 Funding**

This work was supported by the Mission pour l'Interdisciplinarité of the CNRS (PEPS, Exomod 2014), the French Proteomic Infrastructure (ProFI; ANR-10-INSB-08-03), and the the Agence Nationale de la Recherche (Programme AGEs). CB was supported by a grant from the French Ministry of Higher Education, Research and Innovation.

## **11 Abbreviation list**

## **12 Acknowledgments**

This is a short text to acknowledge the contributions of specific colleagues, institutions, or agencies that aided the efforts of the authors.

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