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# BIOLOGY LETTERS

## Selective disappearance of individuals with high levels of glycated haemoglobin in a free-living bird

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3

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13

14

15

16 **Abstract.** Although disruption of glucose homeostasis is a hallmark of ageing in humans and  
17 laboratory model organisms, we have little information on the importance of this process in free-  
18 living animals. Poor control of blood glucose levels leads to irreversible protein glycation. Hence,  
19 levels of protein glycation are hypothesised to increase with age and to be associated with a decline  
20 in survival. We tested these predictions by measuring blood glycated haemoglobin in 274 adult  
21 collared flycatchers of known age and estimating individual probability of recapture in the following  
22 two years. Results show a strong decrease in glycated haemoglobin from age one to five years and an  
23 increase thereafter. Individuals with high levels of glycated haemoglobin had a lower probability of  
24 recapture, even after controlling for effects of age and dispersal. Altogether, our findings suggest  
25 that poor control of glucose homeostasis is associated with lower survival in this free-living bird  
26 population, and that the selective disappearance of individuals with the highest glycation levels could  
27 account for the counter-intuitive age-related decline in glycated haemoglobin in the early age  
28 categories.

29

## 30 1.Introduction

31 Glucose is a major source of energy for cellular processes and its transport, storage and metabolism  
32 are tightly regulated in vertebrates [1]. Low glycaemia results in stress and starvation, whereas high  
33 glycaemia leads to cellular damage [2,3]. Glucose can indeed react spontaneously with proteins to  
34 form advanced glycation end-products. This non-enzymatic glycation process, referred to as Maillard  
35 reaction [3], impairs protein function. Advanced glycation end-products are often irreversible and  
36 glycation could thus be responsible for the frequently observed link between disruption in glucose  
37 homeostasis and ageing [3,4].

38 Because haemoglobin is the most abundant protein in red blood cells, haemoglobin glycation  
39 is a standard marker of exposure to damaging levels of glucose in medical research [5]. In agreement  
40 with the hypothesis that disruption of glucose homeostasis and ageing are closely related, glycated  
41 haemoglobin in humans increased during the life course of individuals [6] and high levels of glycated  
42 haemoglobin were associated with increased mortality [7]. Surprisingly few studies have investigated  
43 age-related variation of glycated proteins in non-human vertebrates. They provide conflicting results,  
44 showing an increase with age in some free-living and captive mammal and bird species, but a  
45 decrease between **juvenile** and mid-age individuals in another free-living bird species [8,9]. Hence,  
46 variation of glycated proteins in relation to age in free-living populations may be more complex than  
47 initially thought.

48 Here, we described the age-related variation of glycated haemoglobin using cross-sectional  
49 data from a free-living bird population. Because senescence in survival, reproduction and immunity is  
50 only observed after five years in our study species [10,11], we expected an increase in glycated  
51 haemoglobin in individuals above this age. However, age-related variation in haemoglobin glycation  
52 at the population level can also be shaped by between-individual variation and changes in the  
53 composition of the population with age, rather than to within-individual variation and thus ageing  
54 per se [12]. We explored the individual-level processes shaping age-related variation of glycated  
55 haemoglobin by testing whether glycated haemoglobin was associated with a proxy of individual

56 probability of survival.

57

## 58 **2. Material and methods**

59 The study was conducted in May-June 2009 to 2011 on a breeding population of collared flycatchers  
60 (*Ficedula albicollis*) on the island of Gotland, Sweden (57°10'N, 18°20'E). Nest boxes were checked  
61 regularly to monitor reproduction and parents were trapped in their nest box 6 to 12 days after the  
62 onset of incubation for females and 5 to 13 days after hatching for males (i.e. during nestling  
63 provisioning). Upon capture, adults were identified or ringed with aluminium rings, weighed,  
64 measured (tarsus length) and blood sampled (100 to 130µl from the brachial vein in EDTA-coated  
65 Microvettes; Sarstedt, Germany). In 2009, we sampled 274 adult birds of known age, from one to  
66 eight years (table 1): 193 were ringed as nestlings and 81 were ringed as yearlings. Their subsequent  
67 return rate, dispersal within the study area, and reproductive success were monitored in 2010 and  
68 2011. Dispersal within the study area was defined as a change of breeding plot, either between birth  
69 and the first breeding event or between two consecutive breeding events [13]. Breeding plots were  
70 also separated into two categories: central plots and peripheral plots, because the probability of  
71 dispersing outside of the study area is higher at the edge [14].

72 The fraction of glycated haemoglobin was measured using the Biocon Diagnostik© HbA1 kit  
73 (Biocon Diagnostik, Germany), after minor adaptation of the manufacturer protocol to analyse small  
74 samples. 5µl red blood cells were suspended in 150µl PBS, then 100µl of this suspension was mixed  
75 with 500µl of the lysis reactant and centrifuged. To quantify total haemoglobin, 40µl supernatant  
76 was diluted in 1000µl ultrapure water before reading the absorbance at 440 nm ( $A_{\text{Hbtotal}}$ ). To quantify  
77 glycated haemoglobin, 100µl supernatant was mixed with 1.2mL of cation-exchange resin, and then  
78 separated by filtration. The absorbance was read at 440nm ( $A_{\text{HbA1}}$ ). The absorbance ratio  
79 ( $A_{\text{HbA1}}/A_{\text{Hbtotal}}$ ) was standardized using the kit calibrator. Each sample was analysed in duplicate in a  
80 first assay, then once in a second assay. The inter- and intra-assay CV were respectively 13.7% and  
81 7.9% (N = 364 samples).

82 Levels of glycated haemoglobin were log-transformed before analysis using a linear model  
83 with sex, body mass, tarsus length, and linear and quadratic age as explanatory variables. The return  
84 rate (i.e. the probability to be caught again in 2010, or 2011 for individuals missed in 2010) was  
85 analysed using binomial generalized linear models (GLMs). To check whether return rate was a  
86 reliable proxy of survival, we investigated how glycated haemoglobin related to other sources of  
87 non-detection, such as dispersal outside of the study area and early breeding failure. We tested  
88 whether glycated haemoglobin was related to the probability of dispersal within the study area  
89 between 2009 and 2010 and the probability of successfully fledging at least one offspring in 2010  
90 with binomial GLMs, as well as the number of fledglings for successful nests with a linear model.  
91 Return rate, dispersal within the study area and reproductive output were modelled as a function of  
92 glycated haemoglobin, linear and quadratic age, sex, body mass and tarsus length, as well as the  
93 position of the breeding plot for return rate and dispersal. For analyses of reproductive output, both  
94 pair members were sampled for 8 breeding pairs and their reproductive data were thus not  
95 independent; however, excluding these pairs did not qualitatively change our results. Because  
96 females and males were sampled during two distinct stages (incubation and nestling rearing,  
97 respectively), we tested for sex-specific patterns in each model (tables S1-S3). Dispersal and timing of  
98 egg laying varied with age (tables S4-S5) and could relate to differences in resource use, and thus  
99 glycated haemoglobin, during migration and settlement, but including laying date or dispersal status  
100 between 2008 and 2009 as covariates did not alter our results (tables S4-S5). Analyses were based on  
101 type-II F-tests using the function *Anova* of the R package *car* [15].

102

### 103 3. Results

104 The fraction of glycated haemoglobin varied between 0.73% and 3.72% (median = 1.15%, mean  $\pm$  SE  
105 =  $1.33 \pm 0.47$  %). The log-transformed fraction of glycated haemoglobin followed a quadratic  
106 relationship with age, showing a strong significant decline between 1 and 5 years of age and a slight  
107 but significant increase between 5 and 8 years of age (table 2a, figure 1). The return rate was 39.1%

108 on average (95% confidence interval: 33.2% – 45.1%), i.e. lower than annual survival in this  
109 population estimated via capture-mark-recapture as 56.8% (95% confidence interval: 52.9% – 60.7%)  
110 [16]. Only eight returning individuals out of 107 (7.5%) were caught again in 2011 but not in 2010.  
111 Return rate decreased with increasing fraction of glycated haemoglobin (table 2b, figure 2). Return  
112 rate was not explained by the position of the breeding plot within the study area (table 2b). The  
113 probability of dispersal within the study area between 2009 and 2010, of successfully fledging at  
114 least one offspring in 2010, as well as the number of offspring fledged for successful nests, were  
115 independent of the fraction of glycated haemoglobin (table 2c, d and e). The variation in glycated  
116 haemoglobin with age, as well as the effects of glycated haemoglobin on return rate, dispersal and  
117 future reproduction, did not differ significantly between sexes (tables S1-S3).

118

#### 119 **4. Discussion**

120 In agreement with the positive association between disruption of glucose homeostasis and mortality  
121 in humans [7], breeding collared flycatchers with higher level of glycated haemoglobin were less  
122 likely to be caught again in the two years following their sampling, independent of age. In natural  
123 populations, a lower observed return rate might be due to higher early breeding failure and/or  
124 higher dispersal out of the study area. We found no support for such alternative mechanisms  
125 underlying the association between glycated haemoglobin and return rate. Indeed, levels of glycated  
126 haemoglobin did not predict breeding failure (after the first capture of the parent) or dispersal within  
127 the study area the following year. Return rate was also not biased by the position (i.e. periphery vs.  
128 centre) of the breeding plot within the study area. Altogether, these findings provide strong evidence  
129 that high levels of glycated haemoglobin are associated with increased mortality risk.

130 At the population level, we observed a counter-intuitive significant decrease in glycated  
131 haemoglobin from one to five years of age and an expected increase thereafter (but with small  
132 sample sizes). This decline at early age is possibly driven by the selective disappearance of individuals  
133 with the highest glycation levels [12]. Demonstrating changes in the fraction of glycated haemoglobin



134 with age requires a longitudinal analysis using repeated sampling of the same individuals over their  
135 lifetime. Nevertheless, senescence patterns in physiological markers were detected in small cross-  
136 sectional datasets in this species ([11] and this study), likely because they measure individual  
137 performance more accurately than binary (survival, breeding failure) or ordinal (fledglings number)  
138 traits.

139 Interestingly, previous research in natural bird populations has reported positive  
140 relationships between glycated haemoglobin and fitness-related traits such as nestling growth [17]  
141 and adult reproductive success [18], suggesting that high levels of glycated haemoglobin could reflect  
142 higher nutritional state. Our results however show that glycated haemoglobin is negatively  
143 associated with a proxy of survival. Taken together, these results suggest that increased metabolic  
144 demands through growth and reproduction translate into increased costs in terms of glycation and  
145 subsequent mortality. Glycated haemoglobin could thus mediate life-history trade-offs.

146 Although senescence in free-living animals is now well demonstrated [19], we often lack  
147 information on the factors associated with age-related mortality in nature [20]. This is however  
148 essential to identify conserved mechanisms of senescence in the animal kingdom [20]. Our study  
149 supports the idea that disruption of glucose homeostasis decreases survival and could contribute to  
150 ageing in natural populations.

151

152 **Ethics statement.** Birds were caught, handled and ringed under a license from the Stockholm  
153 Museum Ringing Center (license number 471) and blood samples were collected under a general  
154 license from the Swedish Committee for Experiments on Animals for all experiments on the site  
155 (license number C 108/7).

156 **Data accessibility.** Data are available from the Dryad Digital Repository:  
157 <http://dx.doi.org/10.5061/dryad.87035>

158 **Authors' contributions.** CR, BD and PB designed the study; LC and BD performed the fieldwork; CR  
159 and AS carried out the laboratory and statistical analyses; CR, BD and PB drafted the manuscript and

160 AS and LC revised it for significant intellectual content. All authors approved of the manuscript and  
161 agree to be held accountable for its content.

162 **Competing interests statement**

163 The authors declare that they have no competing interests.

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173

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For Review Only

237 Figure 1. Age-related variation in glycated haemoglobin from 274 adult collared flycatchers  
238 Figure 2. Probability of recapture in the two years following the measurement of glycated  
239 haemoglobin. The fitted solid line shows the probability of recapture estimated from a binomial GLM  
240 together with 95% confidence interval (dashed lines).

241

242

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For Review Only

244 Table 1. Distribution of age and sex categories in the study

245

| Sex        | Age (years) |    |    |    |    |   |   |   |
|------------|-------------|----|----|----|----|---|---|---|
|            | 1           | 2  | 3  | 4  | 5  | 6 | 7 | 8 |
| Females    | 43          | 39 | 45 | 20 | 11 | 3 | 0 | 0 |
| Males      | 28          | 23 | 25 | 19 | 10 | 4 | 3 | 1 |
| Both sexes | 71          | 62 | 70 | 39 | 21 | 7 | 3 | 1 |

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247

For Review Only

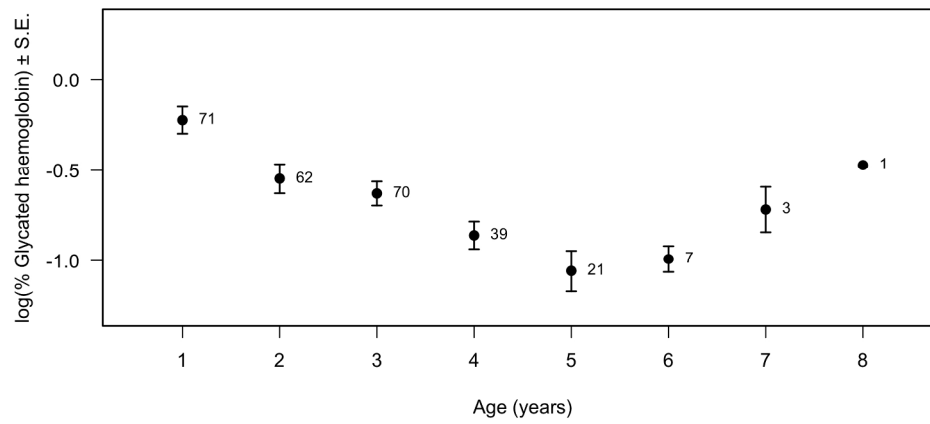
248 Table 2. Models describing age-related variation in glycated haemoglobin (a) and its relationship to  
 249 return rate (b), dispersal (c) and future breeding success (d-e). The effect of sex is expressed as males  
 250 compared to females and that of breeding plot position as peripheral compared to central ones.  
 251 Significant effects are shown in bold.

| Response  | Effect                      | Estimate $\pm$ S.E.                  | F            | P                 |
|---|-----------------------------|--------------------------------------|--------------|-------------------|
| <b>(a) Log-transformed fraction of glycated haemoglobin in 2009</b>                                       |                             |                                      |              |                   |
| N = 274, Adjusted R <sup>2</sup> = 0.160, test statistic F <sub>1,268</sub>                               |                             |                                      |              |                   |
|   | <b>Age</b>                  | <b>-0.391 <math>\pm</math> 0.086</b> | <b>20.51</b> | <b>&lt; 0.001</b> |
|   | <b>Age<sup>2</sup></b>      | <b>0.034 <math>\pm</math> 0.013</b>  | <b>7.00</b>  | <b>0.009</b>      |
|   | Sex                         | 0.282 $\pm$ 0.145                    | 3.79         | 0.053             |
|   | Body mass                   | 0.083 $\pm$ 0.047                    | 3.20         | 0.075             |
|   | Tarsus length               | 0.003 $\pm$ 0.071                    | < 0.01       | 0.961             |
| <b>(b) Return rate in 2010 or 2011</b>  |                             |                                      |              |                   |
| N = 274, Deviance explained = 3.5%, test statistic F <sub>1,266</sub>                                     |                             |                                      |              |                   |
|   | <b>Glycated haemoglobin</b> | <b>-0.749 <math>\pm</math> 0.322</b> | <b>5.80</b>  | <b>0.017</b>      |
|   | Age                         | 0.155 $\pm$ 0.352                    | 0.19         | 0.661             |
|   | Age <sup>2</sup>            | -0.047 $\pm$ 0.053                   | 0.82         | 0.365             |
|   | Sex                         | 1.069 $\pm$ 0.551                    | 3.82         | 0.052             |
|   | <b>Body mass</b>            | <b>0.366 <math>\pm</math> 0.177</b>  | <b>4.37</b>  | <b>0.038</b>      |
|   | Tarsus length               | -0.365 $\pm$ 0.259                   | 1.97         | 0.161             |
|   | Position of breeding plot   | 0.071 $\pm$ 0.256                    | 0.08         | 0.784             |
| <b>(c) Dispersal between 2009 and 2010</b>  |                             |                                      |              |                   |
| N = 99, Deviance explained = 24.0%, test statistic F <sub>1,91</sub>                                      |                             |                                      |              |                   |
|   | Glycated haemoglobin        | -0.667 $\pm$ 0.801                   | 0.51         | 0.479             |
|   | Age                         | -1.291 $\pm$ 0.866                   | 1.53         | 0.219             |
|   | Age <sup>2</sup>            | 0.175 $\pm$ 0.132                    | 1.13         | 0.290             |
|   | <b>Sex</b>                  | <b>-4.433 <math>\pm</math> 1.473</b> | <b>8.59</b>  | <b>0.004</b>      |
|   | Body mass                   | -0.333 $\pm$ 0.339                   | 0.70         | 0.404             |
|   | Tarsus length               | -0.630 $\pm$ 0.625                   | 0.73         | 0.397             |
|   | Position of breeding plot   | 0.045 $\pm$ 0.561                    | < 0.01       | 0.947             |
| <b>(d) Probability of fledging at least one offspring in 2010</b>   |                             |                                      |              |                   |
| N = 96, Deviance explained = 14.6%, test statistic F <sub>1,89</sub>                                      |                             |                                      |              |                   |
|   | Glycated haemoglobin        | -1.679 $\pm$ 1.255                   | 1.87         | 0.174             |
|   | Age                         | -2.402 $\pm$ 1.636                   | 3.05         | 0.084             |
|   | Age <sup>2</sup>            | 0.286 $\pm$ 0.246                    | 1.95         | 0.166             |
|   | Sex                         | 1.494 $\pm$ 1.828                    | 0.73         | 0.394             |
|   | Body mass                   | -0.082 $\pm$ 0.483                   | 0.03         | 0.862             |
|   | Tarsus length               | -0.401 $\pm$ 0.907                   | 0.21         | 0.644             |
| <b>(e) Number of fledglings (for successful nests, i.e. where at least one offspring fledged) in 2010</b> |                             |                                      |              |                   |
| N = 88, Adjusted R <sup>2</sup> = 0.113, test statistic F <sub>1,81</sub>                                 |                             |                                      |              |                   |
|   | Glycated haemoglobin        | 0.299 $\pm$ 0.355                    | 0.71         | 0.403             |
|   | Age                         | 0.203 $\pm$ 0.351                    | 0.33         | 0.565             |
|   | Age <sup>2</sup>            | -0.004 $\pm$ 0.053                   | 0.01         | 0.935             |
|   | Sex                         | -0.757 $\pm$ 0.536                   | 2.00         | 0.162             |
|   | <b>Body mass</b>            | <b>-0.470 <math>\pm</math> 0.163</b> | <b>8.28</b>  | <b>0.005</b>      |
|   | Tarsus length               | -0.033 $\pm$ 0.260                   | 0.02         | 0.898             |

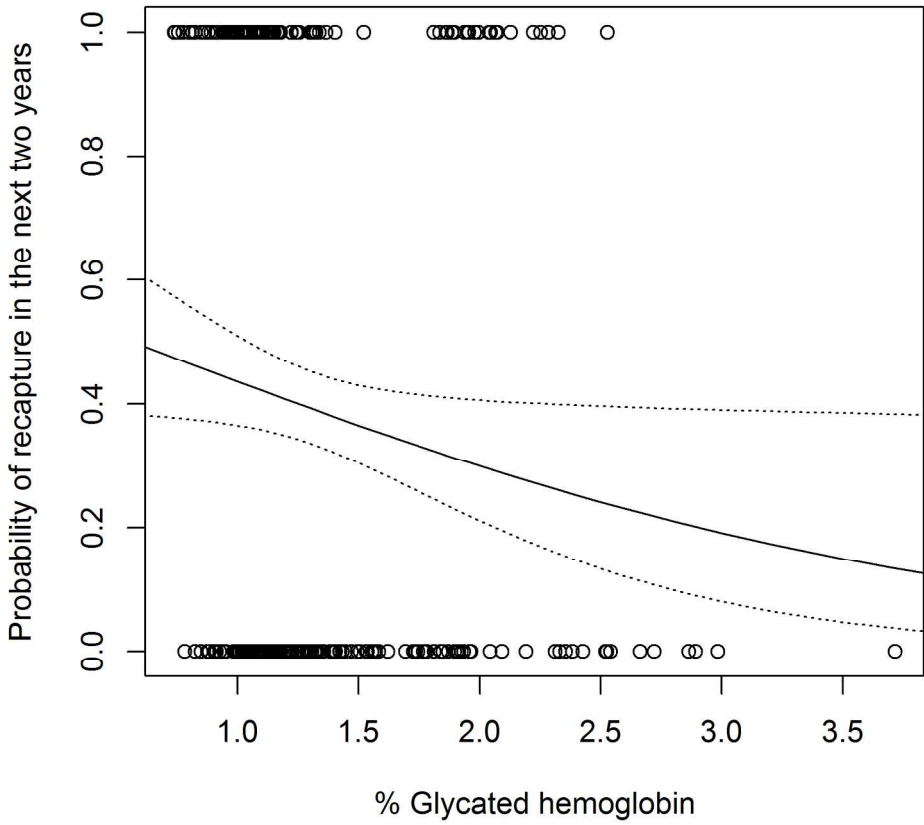


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For Review Only



Age-related variation in glycosylated haemoglobin from 274 adult collared flycatchers  
figure 1  
916x416mm (72 x 72 DPI)



Probability of recapture in the two years following the measurement of glycated haemoglobin. The fitted solid line shows the probability of recapture estimated from a binomial GLM together with 95% confidence interval (dashed lines).  
figure 2