BIOLOGY LETTERS

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Journal:	*Biology Letters*
Manuscript ID	RSBL-2016-0243.R2
Article Type:	Research
Date Submitted by the Author:	n/a
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Subject:	Evolution < BIOLOGY, Molecular Biology < BIOLOGY, Systems Biology < BIOLOGY
Categories:	Physiology
Keywords:	ageing, diabetes, glycation, survival, collared flycatcher, Ficedula albicollis

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

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

1.Introduction

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

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

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

probability of survival.

2. Material and methods

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

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

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

3. Results

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

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

4. Discussion

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

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

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

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

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

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

Data accessibility. Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.87035

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

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

Competing interests statement

The authors declare that they have no competing interests.

Acknowledgements. Many thanks to Lars Gustafsson and the landowners of Gotland for access to the study sites, to Alice Terrier for her help in collecting samples, to numerous field assistants for their help in monitoring the population, to Ryan Germain for proofreading, and to François Criscuolo and two anonymous reviewers for helpful feedback on an earlier version of this manuscript.

Funding statement. This work was supported by the ANR (ANR-06-JCJC0082 to BD), the CNRS (PEPS INEE and PICS France-Switzerland to BD), the French Ministère de l'Enseignement Supérieur et de la Recherche (PhD fellowship to CR), the Région Rhône-Alpes (Explora'doc mobility grant to CR), the University of Aberdeen (stipend to CR), the L'Oréal Foundation-UNESCO "For Women in Science" program (fellowship to CR), the Rectors' Conference of the Swiss Universities (grant to CR and PB).

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Figure 1. Age-related variation in glycated haemoglobin from 274 adult collared flycatchers

Figure 2. 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).

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

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

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

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

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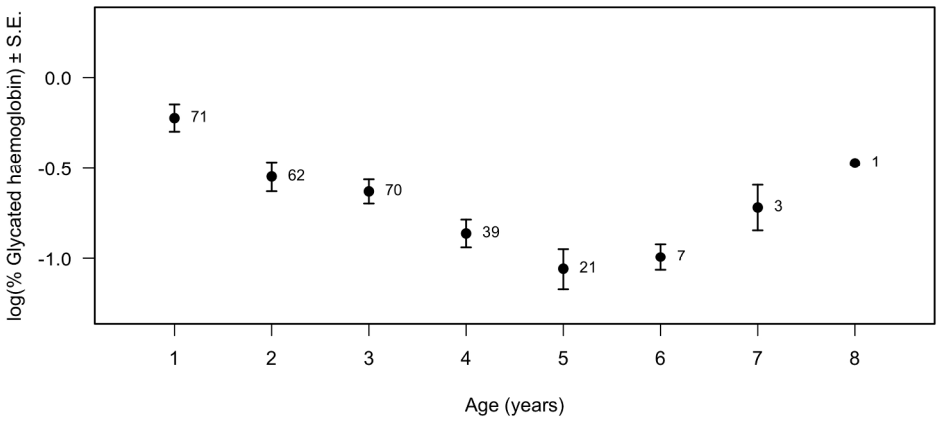

Age-related variation in glycated haemoglobin from 274 adult collared flycatchers
figure 1
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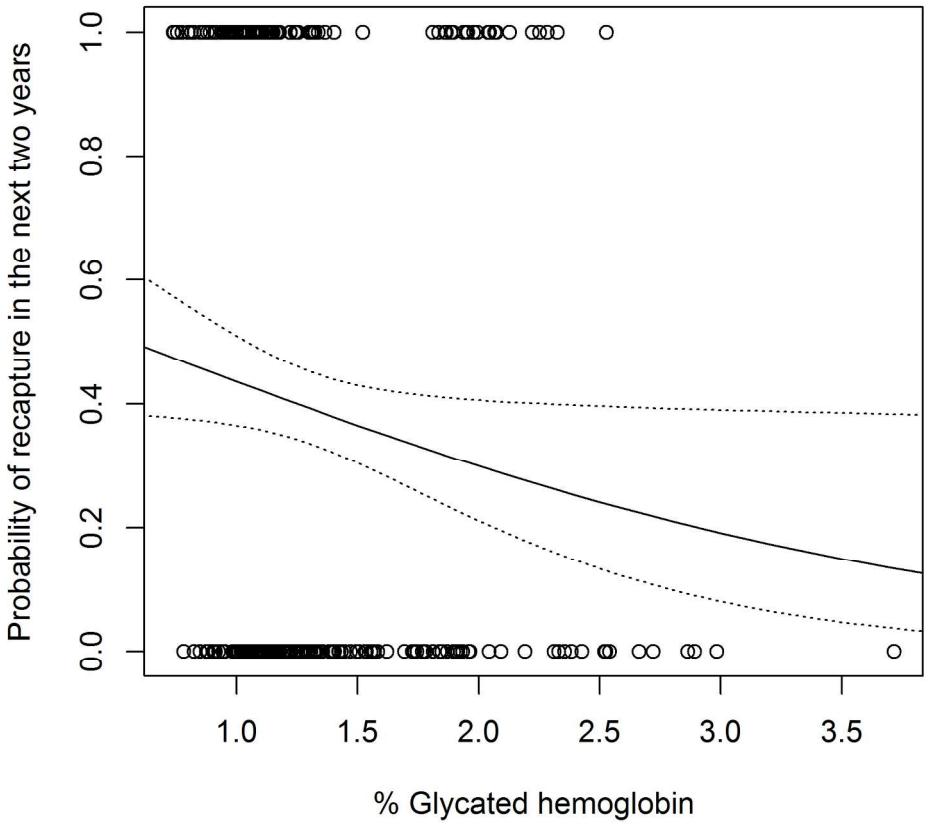

Probability of recapture in the two years following the measurement of glycated haemoglobin. The fitted solid line shows the probability of recapture estimated from a binomial GLM together with 95% confidence interval (dashed lines).
figure 2