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Glycation: an Additional Biomarker of Aging in mammals? Insights from Non-Conventional Species

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Je vais m'arrêter là, sinon cette section de remerciements risque d'être plus longue que la thèse elle-même ! Je terminerai avec un mot dans la langue du pays du matin calme, qui résume bien l'état d'esprit de ces trois années de thèse : 화이팅 !

Metal bats illustrations by Timothée Gérard

Résumé en français

Les réactions de glycation, une chaîne de réactions non-enzymatiques impliquant la liaison initiale d'un hexose à une molécule organique (protéine dans notre cas), fût pour la première fois décrite en chimie alimentaire par Louis C. Maillard en 1912. Il fallut néanmoins attendre les années 1980 pour que cette réaction soit observée in vivo et que soit soulevée sa potentielle contribution aux mécanismes du vieillissement chez les organismes vivants. Depuis, le nombre d'études à ce sujet n'a cessé de croître de manière exponentielle. On sait aujourd'hui que les produits de glycation jouent un rôle crucial dans le processus de sénescence, que ce soit par le biais de composés produits tôt dans la chaîne de réaction (composés d'Amadori tel que l'hémoglobine glyquée ou l'albumine glyquée, principaux marqueurs diagnostics du diabète chez l'homme), ou par le biais des produits finaux de la réaction, communément appelés « produits de glycation avancée » ou AGEs. Altération de la conformation des protéines, accumulation et formation d'agrégats protéiques dans les cellules et tissus, augmentation du stress oxydatif… tout cela ne représente qu'une petite partie des mécanismes connus par lesquels les glycations contribuent au vieillissement et aux pathologies chroniques qui y sont liées (complications du diabète, pathologies neurodégénératives ou cardiovasculaires etc.).

Jusqu'à ce jour, la majorité des études sur les glycations ont été conduites en médecine humaine et sur quelques modèles animaux de laboratoire (rats et souris). Or, il est intéressant de noter que certaines espèces animales sauvages ou non-modèles de laboratoire (que nous regrouperons ci-après sous le terme d'espèces « non-conventionnelles ») semblent échapper aux effets délétères des glycations sur la santé. L'exemple le plus connu et le plus étudié est celui des oiseaux, animaux longévifs qui présentent des niveaux de glycémie semblables ou supérieurs à ceux observés chez des humains diabétiques, mais sans les effets glucotoxiques et pathologies associées. Il a été suggéré que cela pouvait passer entre autres par une résistance de certaines protéines aviaires à la glycation. Un paradoxe similaire existe chez les chauves-souris frugivores et nectarivores, qui font partie des mammifères les plus longévifs proportionnellement à leur taille, mais ne semblent pourtant pas présenter de signes évidents

de sénescence ou de pathologies associées, et ce malgré un régime alimentaire riche en sucres. A ce jour, les études comparatives sur le vieillissement ont exploré le plus souvent les mécanismes de résistance au stress oxydatif ou de réparation de l'ADN particulièrement efficaces pour expliquer ces longévités paradoxales. Cependant, la question des glycations n'a jamais été abordée chez ces mammifères volants.

S'il a déjà été montré par le passé que la glycémie corrèle avec des marqueurs de la fitness individuelle ou du rythme de vie des animaux, nous pouvons émettre l'hypothèse que l'existence de telles relations soient également le fruit d'un processus évolutif ayant favorisé la sélection de mécanismes de résistance ou de régulation des glycations spécifiques aux chauves-souris. Résister à la glucotoxicité via une résistance aux glycations (alors que le glucose reste la principale source d'énergie dans l'environnement) pourrait s'inscrire dans une nouvelle vision de l'évolution de la sénescence, qui ne se baserait pas uniquement sur des compromis, mais également sur des conjonctions de caractéristiques structurelles, cellulaires ou physiologiques particulières.

Malgré cela, force est de constater que les glycations et leurs liens avec la sénescence n'ont fait l'objet de quasiment aucune étude chez des espèces animales non-conventionnelles. Or l'étude de ces modèles pourrait apporter de nouvelles perspectives, aussi bien pour la recherche de traitements contre les effets néfastes des glycations chez l'homme que pour la compréhension des mécanismes qui auraient co-évolué avec une résistance aux glycations chez certaines espèces. Dans ce contexte et au travers de cette thèse, nous nous sommes proposés de contribuer à combler ce vide en nous intéressant aux glycations et à leurs liens avec la sénescence et l'histoire de vie chez différentes espèces animales non-conventionnelles. Nous avons fait le choix de nous focaliser sur les mammifères, qui ont fait l'objet de moins d'études sur ce sujet que les oiseaux, avec une attention plus particulière aux chauves-souris, pour les raisons évoquées précédemment. Après avoir réalisé un état de l'art sur la question des glycations chez les espèces animales non-conventionnelles (qui a donné lieu à la publication d'une review au sein de Biology Letters), nos objectifs principaux ont été de déterminer si 1) les glycations peuvent être considérées comme un biomarqueur supplémentaire du vieillissement chez les mammifères, 2) si les chauves-souris présentent une résistance et/ou tolérance aux glycations par rapport aux autres mammifères non-volants

et 3) si les glycations varient entre individus faisant face à des contraintes environnementales différentes.

Pour mener à bien ce travail, il nous a d'abord fallu affiner la méthodologie de mesure des glycations par spectrométrie de masse utilisée au sein dans notre laboratoire. En effet, il faut noter qu'à ce jour, la plupart des études ayant mesuré des taux de glycations chez les animaux ont utilisé des techniques peu spécifiques ou des kits commerciaux développés pour l'Homme ou des modèles de laboratoire comme le rat ou la souris. La spectrométrie de masse couplée à la chromatographie liquide permet de cibler plus spécifiquement certains produits de glycation, et elle présente l'avantage de pouvoir être appliquée à toutes les espèces. Elle est néanmoins très chronophage, et il a donc fallu trouver un compromis entre bon rendement temporel et bonne précision des analyses. Nous nous sommes plus précisément intéressés aux formes glyquées de deux protéines présentes dans le sang : l'albumine et l'hémoglobine.
Nous avons échantillonné plus d'une quinzaine d'espèces de chauves-souris à la fois issues de populations sauvages européennes et sud-américaines, ou de population captives (zoos). Nous n'avons en revanche pas pu détecter de glycations chez la totalité d'entre elles, ce qui nous a conduit par la suite à ne sélectionner que certaines espèces pour les différentes études de cette thèse.

Dans un premier temps, nous avons mené des analyses comparatives interspécifiques en nous basant sur l'albumine glyquée. La première analyse portait sur neuf espèces de mammifères incluant cinq espèces de chiroptères (Pteropus rodricensis, Rousettus aegyptiacus, Carollia perspicillata, Myotis daubentonii et Nyctalus noctula), deux espèces de rongeurs (Arvicanthis ansorgei et Rhabdomis pumilio), un artiodactyle (Capreolus capreolus) et un carnivore marin (Mirounga leonina). Nous avons émis l'hypothèse que les chauves-souris présentent une tolérance aux glycations qui se traduirait par des taux inférieurs d'albumine glyquées comparés à ceux d'autres espèces. Nous avons également regardé si les différences de valeurs moyennes d'albumine glyquée entre ces espèces pouvaient être influencées par un rythme de vie plus ou moins lent, la masse corporelle ou la glycémie. Nous avons pu mettre en évidence que les chauves-souris présentent effectivement des niveaux d'albumine glyquée inférieurs à ceux des autres mammifères. En revanche, et contrairement à nos attentes, les taux d'albumine glyquée étaient légèrement plus élevés chez les espèces présentant un rythme de vie plus lent et une

longévité plus importante, suggérant l'existence d'une tolérance aux glycations chez ces dernières. Suite à cela, nous avons momentanément quitté le seul prisme des mammifères pour étendre notre analyse comparative aux vertébrés volants. Nous avons regardé si sur le plan des glycations et de leurs liens avec certains traits, les chauves-souris étaient comparables aux oiseaux, pour lesquels une résistance aux glycations a déjà été démontrée chez certaines espèces. Ces deux groupes présentent en effet de nombreuses similitudes, comme l'évolution du vol actif, une grande longévité malgré une petite masse corporelle, ou encore une apparente tolérance aux effets délétères d'un régime alimentaire riche en sucre. Nous avons pour cela sélectionné cinq espèces d'oiseaux (dont les données de d'albumine glyquée étaient disponibles dans une autre étude), de masses et régimes alimentaires similaires à nos cinq espèces de chiroptères (à savoir frugivores et insectivores). Nous avons pu mettre en évidence que d'une façon générale, les oiseaux présentaient une glycémie et des taux d'albumine glyquée supérieurs à ceux des chauves-souris. De plus, il est apparu qu'entre espèces de même longévité maximale, les oiseaux avaient plus d'albumine glyquée que les chiroptères. Il est également ressorti qu'au sein de ces deux groupes, les espèces insectivores présentent des taux d'albumine glyquée supérieurs à ceux des espèces frugivores. Si ce résultat peut paraître surprenant à première vue, il va dans le sens de certaines études ayant mis en évidence l'existence de glycémies supérieures chez les oiseaux carnivores, attribuant cela à la néoglucogenèse et/ou à une moindre tolérance au glucose des carnivores comparativement aux frugivores. Si certaines recherches avaient également montré une tendance à une glycémie supérieure chez les chauves-souris insectivores par rapport aux frugivores, notre étude est à notre connaissance la première à montrer cela sur le plan des glycations.

Nous nous sommes ensuite intéressés à la façon dont les glycations se comportent à l'échelle intraspécifique, et en regard de certains paramètres connus pour influer sur la sénescence. Ainsi, dans un deuxième chapitre, nous nous sommes focalisés sur deux espèces de chauves-souris frugivores, la Roussette d'Égypte (Rousettus aegyptiacus) et la Roussette de Rodrigues (Pteropus rodricensis), pour lesquelles nous avons pu échantillonner de nombreux individus (n=24 et n=15, respectivement). Ces individus étant issus de populations captives, nous avons pu suivre un plan d'échantillonnage précis incluant des proportions équivalentes entre mâles et femelles ainsi que juvéniles et adultes pour chaque espèce. Nous avons également pu récolter des données de masse individuelle et de stade reproducteur, ainsi que l'âge

chronologique précis des individus de P. rodricensis. En plus des mesures de glycémie et de glycations (hémoglobine glyquée et albumine glyquée), nous avons évalué l'intensité du stress oxydatif via des mesures de 8-hydroxydésoxyguanosine (8-OHdG), ainsi que l'état des défenses antioxydantes via des tests OXY-adsorbent. Le stress oxydatif est en effet un mécanisme connu pour contribuer au processus de sénescence chez de nombreux mammifères, ainsi que pour être intensifié par les glycations dans le cas de l'homme. On sait également que les chauves-souris présentent de nombreux mécanismes de protection vis-à-vis du stress oxydatif. Nous avons donc étudié comment les niveaux de glycations évoluent avec le stress oxydatif chez ces chauves-souris, en plus de vérifier s'ils diffèrent en fonction de la masse, du sexe ou de l'âge des individus. Chez la Roussette de Rodrigues, les taux de glycation ne différaient pas significativement en fonction de la masse, du sexe ou de l'âge, malgré une glycémie significativement plus élevée chez les mâles et un stress oxydatif plus important chez les femelles. En revanche, nous avons pu mettre en évidence une corrélation positive entre intensité de la barrière antioxydante et taux d'hémoglobine glyquée. Concernant la Roussette d'Égypte, nous avons montré que les mâles présentaient des taux d'hémoglobine glyquée supérieurs à ceux des femelles. Si aucun des deux marqueurs du stress oxydatif ne corrélaient avec les niveaux de glycation chez cette espèce, il est en revanche apparu que les mâles et les juvéniles présentaient un stress oxydatif plus élevé que les femelles et les adultes, respectivement. Notre échantillonnage étant un échantillonnage transversal à l'échelle de la population, on ne peut exclure que seuls les individus les plus résistants au stress oxydatif aient survécu à un âge plus avancé, ce qui expliquerait qu'ils présentent un stress oxydatif inférieur à celui des juvéniles. L'intensité du stress oxydatif corrélait également positivement avec les défenses antioxydantes chez cette espèce.

Enfin, dans un dernier chapitre, nous nous sommes demandé si (de la même manière que cela a déjà été suggéré pour la glycémie) les taux de glycation pourraient varier entre des populations d'une même espèce faisant face à des contraintes environnementales différentes. Pour cela, nous nous sommes concentrés sur un mammifère terrestre, le chevreuil d'Europe (Capreolus capreolus), pour lequel nous avions accès à deux populations vivant dans des environnements radicalement différents (forêt de Trois-Fontaines, milieu de vie riche en ressources et au climat tempéré vs forêt de Chizé, milieu de vie pauvre en ressources et au climat océanique, en France). Ces populations présentent d'autant plus d'intérêt pour notre

sujet d'étude qu'il a été montré que de nombreux paramètres hématologiques et immunologiques présentent une sénescence chez le chevreuil, et que les trajectoires de sénescence diffèrent entre les deux populations étudiées. Dans un premier temps, nous nous sommes donc intéressés aux liens existants entre glycation, glycémie, sexe et âge, avant de vérifier si les glycations corrélaient avec des paramètres hématologiques connus pour être sénescents chez cette espèce. Nous avons également vérifié si ces liens différaient entre les deux populations. Nous avons montré que les taux d'albumine glyquée augmentaient avec la masse des individus, et que leurs liens avec des marqueurs inflammatoires différaient entre les deux populations. Ainsi, dans la population vivant dans l'environnement le plus contraignant, les taux de glycation diminuaient avec un état inflammatoire et infectieux plus important, alors que ce lien était légèrement positif chez les individus vivant dans l'environnement le plus riche en ressources, suggérant que les glycations relèveraient donc plutôt d'un marqueur de qualité individuelle ou d'état de santé chez cette espèce.

Ainsi, les différents résultats de ce travail de thèse semblent indiquer que le lien entre glycation et senescence qui existe chez l'Homme ne serait pas présent chez tous les mammifères. Les taux de glycation légèrement plus élevés chez les espèces au rythme de vie plus lent et à la longévité plus grande, l'absence de lien systématique entre âge et glycations chez toutes les espèces étudiées ici, ou encore l'hétérogénéité des résultats liant le stress oxydatif ou l'état inflammatoire aux taux de glycation entre espèces et individus… tout semble indiquer que les glycations ne sont pas un marqueur de sénescence universel chez les mammifères. Cette thèse a également permis de mettre en évidence que les chauves-souris présentent effectivement un comportement particulier vis-à-vis des glycations comparativement aux autres mammifères, suggérant l'existence d'une résistance ou d'une tolérance de ces dernières aux effets néfastes du glucose. Enfin, il apparaît que les glycations diffèrent entre individus d'une même espèce faisant face à des contraintes environnementales différentes. L'ensemble des points soulevés par ce travail mériteraient d'être approfondis dans le futur, notamment au travers de suivis longitudinaux chez certaines espèces de mammifères, ou encore d'études mécanistiques à l'échelle cellulaire chez les chauves-souris.

LIST OF COMMUNICATIONS

Articles Published in Peer-Reviewed Journals

Cyrielle Duval, François Criscuolo & Fabrice Bertile (2024). Glycation resistance and life-history traits: lessons from non-conventional animal models. Biology Letters 20, 20230601.
DOI : 10.1098/rsbl.2023.0601

Articles in Preparation

Cyrielle Duval, Jean-François Lemaître, Benjamin Rey, Sarahi Jaramillo-Ortiz, Christine Schaeffer-Reiss, Fabrice Bertile & François Criscuolo. In prep. Glycated albumin levels in roe deer: a marker of body condition which is influenced by environment quality.

Cyrielle Duval, Sarahi Jaramillo-Ortiz, Philippe Christe, Etienne Chalet, Carsten Schradin, Benjamin Rey, Jean-François Lemaître, Laura Charlanne, Thierry Petit, Christine Schaeffer-Reiss, Fabrice Bertile & François Criscuolo. In prep. Glycation and life-history: comparative analysis among non-conventional animal species.

Cyrielle Duval, Sarahi Jaramillo-Ortiz, Thierry Petit, Christine Schaeffer-Reiss, François Criscuolo & Fabrice Bertile. In prep. Links between glycation levels and oxidative stress in two species of fruit bats.

Conferences

6ème Colloque d'Ecophysiologie et Physiologie Animale (CEPA), Tours
2-4 Novembre 2023
Cyrielle Duval, Sarahi Jaramillo-Ortiz, Christine Schaeffer, Fabrice Bertile & François Criscuolo. Increased glycation levels: a biomarker of aging in mammals? Présentation orale (en anglais).

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Egyptian Fruit Bats *Rousettus aegyptiacus* (Photo 2 p.34) - Photo by S. Meys

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ABBREVIATIONS

8-OHdG: 8-hydroxy-2-deoxyguanosine

AGEs: Advanced Glycation End-products

AICc: Corrected Akaike Information Criterion

BMR: Basal Metabolic Rate

C: Chizé

CEL: carboxyethyl-lysine

CML: N-carboxymethyllysine

GHb: Glycated Hemoglobin

GA: Glycated Albumin

HbA1c: Hemoglobin A1c

HClO: hypochlorous acid

LC: Liquid Chromatography

MS: Mass Spectrometry

MLSP: Maximum Lifespan

NS: Non-significant

OXY: Antioxydants Capacities

PBS: Phosphate-Buffered Saline

PCA: Principle Component Analysis

PCs: Principle Components

PGLS: Phylogenetic Least Square Model

POL: Pace of Life

RAGE: Receptor for Advanced Glycation End-products

ROS: Reactive Oxygen Species

SOD: Superoxyde Dismutase

TF: Trois-Fontaines

TPGP: Total Plasma Glycated Protein

GENERAL INTRODUCTION

Nectarivorous hummingbird (aquarelle by Cyrielle Duval)

Most of this general introduction was the subject of a review entitled "Glycation resistance and life-history traits: lessons from non-conventional animal models" published in Biology Letters in April 2024. The version presented in this general introduction is slightly expanded from the published version, to provide additional context. The original publication is available in the appendixes of the manuscript (Appendix n°4).

A Foreword on Senescence and its Importance in Evolutionary Biology

In many “languages”, the terms “aging” and “senescence” are commonly confused and used synonymously. While in evolutionary biology these two notions are quite distinct, it must be said that there is no strict consensus on the definition of “aging” among scientists, in contrast to the term “senescence”, which is therefore preferred in this branch of science. Another notion that is intrinsically linked to the previous two is “longevity”. First and foremost, we therefore need to define these terms and how they are used in this thesis.

Starting with “longevity”: it is a finite concept, representing how long an organism has lived, from birth (zero age) to death (death age). However, assessing the longevity of species, especially in the wild, presents several challenges. There is no absolutely reliable technique for determining the age of an individual first encountered in the wild (but see the promising avenue opened by DNA methylation [1], and long-term longitudinal studies of wild populations are difficult to conduct [2]). In addition, the longevity of a population in the wild is affected by several external and environmental factors, such as infant mortality, predation or anthropogenic effects, and may not always reflect the true potential lifespan of a species (i.e., the age at which an individual would die from senescence alone - see definition below) [3]. For this reason, certain longevity metrics are more commonly used in evolutionary biology, each with their advantages and limitations. One of these is “maximum lifespan” (MLSP), which is the longest recorded lifespan of an individual within a species and reflects the potential longevity of the species under optimal conditions. However, it is often based on exceptional cases (whether in captivity or in the wild) and may not represent the average lifespan of individuals in a species. Another commonly used metric to address this issue is the median lifespan of a population, which is the age at which half of the individuals in a population have died, thereby filtering out extreme outliers. Unfortunately, this metric needs long-term effort to record individual life trajectories over years and is not often available.

“Aging”, in its purest definition, represents the fact of “advancing in age”, and is therefore inherently linked to the mobile notion of the passing of time. In evolutionary biology, according to Prof. R. Salguero-Romez’s definition, the notion of “aging” encompasses all the “mechanisms that either shorten or lengthen the longevity” of an organism [4]. On the

contrary, senescence is defined as the progressive deterioration of structural integrity and function of cells and tissues while growing old, ultimately leading to a decline in fertility and survival (known as reproductive and actuarial senescence respectively) [5]. Just as with longevity, senescence at the individual or population level can be assessed in a variety of ways. When discussing senescence it is essential to distinguish the age at onset of senescence, which is the age at which an organism begins to experience a decline in specific physiological functions (e.g., reproduction), from the rate of senescence, which refers to the rate at which mortality and reproductive decline increase with age [6].

In most species, the notions of “aging” and “senescence" are both (conf)used, such as for humans, because performances decrease with age [4]. However, there are species such as the hydra, the anemone or the jellyfish *Turritopsis nutricula* [7,8] that show no apparent signs of senescence as they age. Some species maintain constant performance with age [9], while some even show improvements in physiological functions while growing old (negative senescence) [10]. We therefore understand that there is only a partial correlation between aging and senescence (and by extension between longevity and senescence) within living organisms [11–13]. Indeed, studies have shown that senescence seems to be a feature shared by endotherms (mammals and birds), but is not obligatory in ectotherms [14,15]. Among species experiencing senescence, it appears that the rate and the age at onset of senescence vary considerably from one species to another, but also in between individuals of a same species [9,16]. It has been reported that rates of senescence can also differ between sexes [17], environmental conditions [18,19] or social organisation (solitary *versus* group living [20]). Thus, the age at onset of senescence allows us to determine when individual fitness starts to be affected and how it may differ among species, populations or individuals, while the rate of senescence quantitatively informs how quickly traits of interest are differently modified, thus linking senescence to evolutionary or individual strategies of fitness optimisation.

There are thus a large number of theories that propose to explain senescence persistence over evolution (i.e., despite the effect of natural selection) and its variability across species and individuals, all grouped together under the heading of “theories of aging” [21]. They can be divided into evolutionary theories on the one hand, and mechanistic theories on the other. Senescence is a key concept in evolutionary biology because it directly affects an organism's

longevity and individual fitness [5,6,13]. It is often viewed as a trade-off between investing energy in reproduction and maintaining or repairing the body for long-term survival [22]. One of the most prominent theories is based on the central hypothesis that the force of natural selection weakens with age because most reproduction occurs early in life [23]. As a result, deleterious mutations affecting older individuals are less efficiently eliminated, allowing senescence to accumulate (also known as the "Mutation accumulation hypothesis") [24]. Therefore, species with slower rates of senescence tend to have longer lifespans, while those with rapid senescence have shorter lifespans.

It is also important to consider the role of environmental and ecological factors that can shape senescence [5,6]. Species living in environments with high predation or external mortality pressure may experience selection for early reproduction and high current investment in breeding (e.g., greater number of offsprings), thereby increasing senescence rate and shortening lifespan [26]. On the contrary, low predation risks will select for long-lived individuals as reproduction effort can be distributed over a longer lifespan with lower current investment in breeding.

On the other hand, there are mechanistic theories like the "Disposable soma theory", which proposes that organisms preferentially allocate resources to reproduction rather than somatic maintenance [25,26]. The study of aging mechanisms in the context of evolutionary biology is essential to better understand how organisms make trade-offs between short-term reproduction and long-term survival [5]. This sheds light on the evolutionary strategies employed by different species. It also provides insights into the variation in lifespan across species and helps to explore how different species optimize fitness under specific ecological and environmental conditions [27]. This, in turn, can help explain how senescence evolves and what factors promote or delay senescence.

Mechanistic theories are the most numerous and cover different aspects, making them complementary and underlining the multifactorial nature of senescence [28]. In a bid to bring all of this knowledge together and establish a common framework for researchers working on the issues of senescence and aging, Lopez-Otin et al. proposed nine major hallmarks of aging in 2013 [29], which they extended to twelve in 2023 [30]. Those are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, disabled macroautophagy, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell

exhaustion, altered intercellular communication, chronic inflammation, and dysbiosis. Among these different mechanisms proposed to contribute to the senescence process, glycation is attracting significant attention.

I. Interest of Studying Protein Glycation in the Context of Senescence in Different Animal Species

1. What Are Glycations?

The glycation reaction (also known as Maillard reaction) was first discovered in food by Maillard in 1912 as the browning reaction which occurs between protein amino acids and reducing sugars [31]. However, it took a few decades before the exact chemical pathways of the reaction could be described [32], and even more time for the glycation reaction to be observed occurring *in vivo* in living systems [33–35]. Since then, huge progress has been made and we now know that this reaction also occurs between reducing sugars and certain nucleic acids or amino phospholipids [36].

The glycation reaction (see Figure 1) begins with the binding of a carbonyl function of a reducing sugar (such as glucose) to a free amino group of a macromolecule, usually a protein (mainly lysine or arginine residues, or N-terminal group). This first spontaneous reaction occurs quickly (within a few hours) and leads to the formation of a reversible Schiff base. The Schiff base then undergoes a series of intra-molecular rearrangements resulting in the formation of a much more stable product: the so-called Amadori compound (in the case of glucose), which belongs to the early-stage products of glycation. Some of those Amadori compounds are essential biomarkers in human medicine and serve as health indicators, such as glycated hemoglobin HbA1c [37] or glycated albumin (GA) [38] used in the monitoring of chronic hyperglycaemia, notably for diabetics (HbA1c and GA, with their different half-lives, reflect glycemia over the past 2 to 3 months and 2 to 3 weeks, respectively [39]).

Importantly, Amadori compounds can then undergo further spontaneous but irreversible reactions (such as rearrangements, oxidative cleavages, dehydration, condensation or

fragmentations) to finally form stable advanced glycation end-products called AGEs, within a timelapse ranging from a few weeks to a few months. During those series of reactions leading to AGEs, some reactive dicarbonyl intermediates are formed, such as glyoxal or methylglyoxal. These compounds are highly reactive, much more than reducing sugars, and can also react with the amino groups of proteins, hereby increasing the diversity and the rate of AGEs production.

It is also interesting to note that, there are two other pathways leading to the formation of AGEs, apart from the Maillard reaction. The first one, known as “polyol pathway”, consists in the conversion of glucose into fructose, which may be further transformed into alpha-oxoaldehydes (such as 3-deoxyglucose and fructose-3-phosphate) [40]. These in turn can react with proteins, leading to the formation of AGEs. AGEs can also be synthesized through protein incubation with lipid peroxidation products [41].

AGEs are therefore a heterogeneous group of compounds which nevertheless share the common feature of being stable and accumulate in cells and tissues. Some have the particularity of being fluorescent, and some of forming inter- or intra-molecular cross-links. Well-studied AGEs include N-carboxymethyllysine (CML) [42], carboxyethyl-lysine (CEL) [43], pentosidine (a cross-linking AGE) [44] or pyrraline [45]. The biochemistry and the nomenclature of AGEs are vast and complex. Comprehensive reviews exist on the subject [40,43,46], and given that AGEs have not been studied in the context of this thesis, I will not go into further detail.

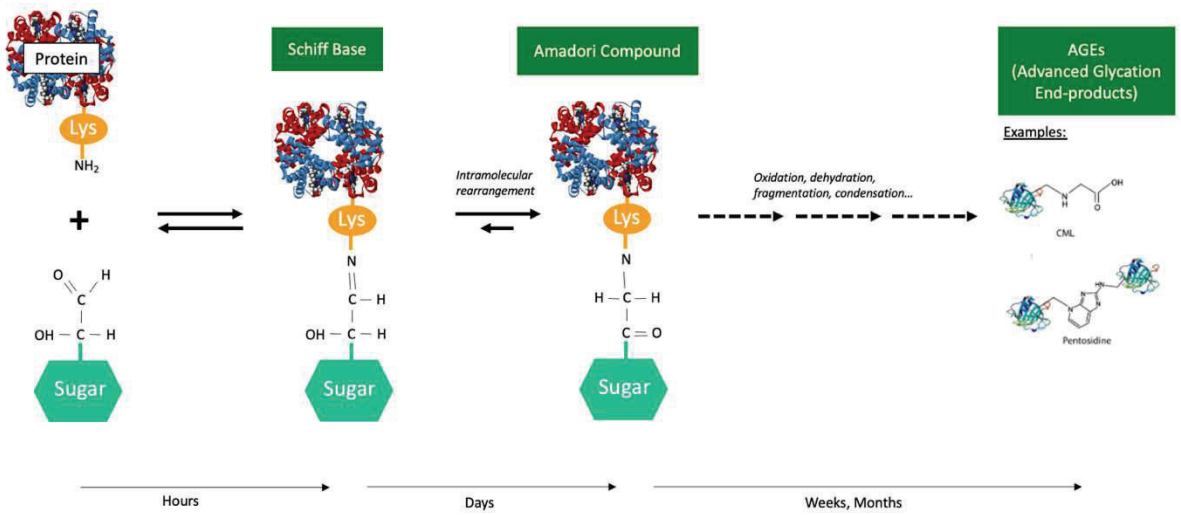


Figure 1. The glycation reaction and its products. CML: N-carboxymethyllysine.

Figure 2. Hallmarks of aging and examples of glycation's contribution (adapted from Lòpez-Otin et al., 2023)

Although protein glycation is a non-enzymatic reaction (which is what primarily distinguishes it from glycosylation), certain factors have been shown to influence its rate of occurrence [46–48]. It has for example been shown in studies on processed food that higher temperatures or lower water concentration (dehydration) favor the first steps of the reaction. It is also known that low pH increases the reactivity of basic free amino groups in proteins, thus promoting glycation. However, these findings are not really transposable *in vivo*, since temperature or pH remain mostly constant in living organisms. Another effective parameter is a protein's half-life, (and its related turnover rate). Proteins with half-lives of several weeks are more likely to be glycated (and to give rise to AGEs for long-lived ones) than proteins with half-lives of a few days [49,50]. Similarly, proteins that are more exposed to circulating glucose (such as plasma proteins or extra-cellular proteins) are more likely to undergo this reaction. Since glycation is a non-enzymatic glycation based on reducing sugars, and since glucose is the major extracellular reducing sugar in the body, one can assume that mean blood sugar concentrations are likely to influence the extent of the reaction. Indeed, it is known that in hyperglycemic patients Amadori products levels are higher [51,52]. It is also interesting to note that not all sugars have the same susceptibility to undergo the Maillard reaction [48]. Open chain forms of sugars are the ones which are favored in the glycation process, rather than cyclic forms, which happen to be more stable [53].

Moreover, several cellular and physiological mechanisms exist to prevent and/or limit the rate of glycation. These mechanisms are described in greater detail in Part III of this introduction.

2. The Involvement of Glycation in the Senescence Process

The number of studies showing the involvement of glycation products in different physiological and cell mechanisms well known to take part in the senescence process is continually increasing [54–56]. Glycation of proteins induce a change in proteins' conformation, thereby altering their functionality [57,58] and degradation [59]. AGEs also tend to accumulate in cells and tissues over time, and by cross-linking with other proteins, some of them contribute to the formation of harmful protein aggregates [60]. AGEs also bind specific receptors (see paragraph III.3.b), thereby contributing to an increased oxidative stress [61] and chronic inflammation [62]. More information on glycation's contribution to the different hallmarks of

aging as described by Lòpez-Otin et al. is given in Figure 2, and there are many comprehensive reviews on the subject (e.g., [63]). While these hallmarks now constitute a reference in the aging research field, it is important to note that they are based on human studies. Since there is a lack of descriptive data for senescence mechanisms over the tree of life, it must be kept in mind that these hallmarks might thus not be applicable to all species (as suggested by Gorbunova et al. for bats [64]).

Through the various mechanisms mentioned above, glycations contribute to several chronic and/or aging-related diseases, such as diabetes mellitus and its complications (*e.g.,* retinopathy, neuropathy, nephropathy) [65], neuro-degenerative diseases (*e.g.,* Alzheimer or Parkinson) [66], cardiovascular diseases (*e.g.,* atherosclerosis) [67,68], renal failure [69], but also inflammatory diseases [70] like rheumatoid arthritis [71] or even cancer [72]. In most of these pathologies, increased levels of AGEs and Amadori compounds are observed in tissues and serum.

3. Why Study Glycation in Non-Conventional Animal Species?

To date, most research on protein glycation has been conducted in human medicine, with the main aim of combating its deleterious health consequences. Interestingly, some wild and/or non-conventional animal models do not seem to suffer from any of the known consequences of glycation on human health. Despite their long lifespans, birds indeed usually express blood glucose levels similar to those observed in hyperglycaemic humans, but without the expected deleterious effects [73]. A similar paradox can be found in nectarivorous and frugivorous bats, which are among the longest-lived mammals and do not seem to show any apparent signs of age-related diseases, despite relying on a high sugar diet [74]. If glycaemia per se has been previously suggested to be correlated with proxies of species' pace of life [75,76] or individual fitness [77] mainly based on energetic explanations, it can be hypothesized that such a relationship also implies that evolution might have acted through the selection of glycation regulatory and/or resistance mechanisms. Resisting glucotoxicity *via* resistance to glycation, while glucose remains the main energy source in the environment, may well fall into a novel view of senescence evolution. This is not only based on trade-offs, but also on the conjunction

of structural, cellular, physiological or behavioural traits pushing further the limits imposed by physiological constraints, and so extending physiological homeostasis in life-threatening conditions [78].

As mentioned earlier, senescence stands as a key variable, and whilst most of its hallmarks have been found in most mammals studied, it seems that some are absent from the Chiroptera order, as highlighted in a study from Gorbunova *et al.* [64]. In this context, any anti-senescence-related mechanism may have been the target of positive selection. How glycation stress covary among and within species with contrasted paces of life or fitness, and under which genetic and environmental influences, remains an open question.

II. Exploring the Links Between Protein Glycation and Life-History Traits in Non-Conventional Animal Models

Evolutionary biologists consider that a trait is susceptible to natural selection if it meets the following conditions: (i) variability in its expression within a population, (ii) heritability and transmissibility between generations, and (iii) impact on fitness [79]. It has been demonstrated in humans that some glycation products (HbA1c, GA and CML) meet the first two conditions [80]. Moreover, given that glycation levels are higher in patients suffering from ageing-related diseases (the latter increasing the risk of mortality), this suggests that glycation most likely has an impact on at least fitness-related traits. If such data concerning glycation are not yet available in animals other than humans, we however know that glycaemia is a trait that varies with life-history stage and environmental conditions [81]. Montoya *et al.* also showed that glucose regulation is a repeatable trait that is affected by handling in zebra finches (*Taeniopygia guttata*) [82]. This regulation capacity can be impaired by stress events, and it has been suggested that individuals' ability to cope with glucose-related physiological challenges could impact their fitness [83]. It therefore appears that regulation of glucose levels and reactivity may be a key physiological trait under selection [83–85]. As glycation rates are usually expected to positively correlate with glycaemia [80,86], we might expect glycation levels and glycation regulation to also be of prime importance in defining individual fitness,

and moreover in shaping species' life-history. However, it is important to mention that the positive relationship between glycation and glycaemia is not systematic [87]. High glycaemia levels may be beneficial for fitness (by relaxing energy-based trade-offs), while low glycation levels may be concomitantly selected as an anti-senescence adaptation. This may account for the lack of (e.g., interspecific) correlation between the two traits, while both are related to individual fitness. Hence, glycaemia and glycation seem to be proxies of individual fitness and not just labile variables that follow the nutritional status of organisms. This may be attributable to their central energetic role and, at the same time, to their chemical characteristics that, hypothetically, make them harmful molecules for fitness. What evidence do we have to suggest such an evolutionary role?

1. Protein Glycation in Relation to Reproduction and Survival

Fitness is optimized differently among species, and local selection pressure has shaped specific trade-offs on life-history traits such as those related to reproduction and survival, thereby defining the pace-of-life of species [88]. Several studies have therefore tested the relationship between glycated haemoglobin (GHb[1]) levels and individuals' reproduction in birds. In collared flycatchers (*Ficedula albicollis*), higher GHb levels corelated with increased adult reproductive success, namely a bigger clutch size for females and a higher number of fledged young for both males and females, after correction for the laying date [89]. A positive correlation between glycated hemoglobin levels and growth rates of nestlings has also been reported in American kestrels *(Falco sparverius)* [90]. Although the links reported are not proven to be causal, those two studies still suggest that high GHb levels reflect a higher investment in reproduction effort and in growth. However, it should be noted that Récapet *et al.* did not find any correlations between GHb and other markers of reproductive success (i.e., probability to successfully fledge at least one offspring and number of total chicks) in collared flycatchers [91]. Still, they found a negative correlation between GHb levels and a proxy of survival of adults (number of recaptures after a capture-marking-recapture protocol). According to the authors, these

[1] As HbA1c is a term used in human medicine, we reserve it for human, preferring the term GHb to refer to glycated hemoglobin in other animal species.

different results suggest that reproduction and growth, which both induce a high metabolic demand, are life-history traits likely to trigger elevated glycation levels, ultimately leading to increased mortality rates. Thus, GHb, more than a simple variable that changes according to nutritional status, should be considered as a physiological variable that modulates individuals' life-history trade-off. However, those previous measurements of GHb levels in birds suffer from various methodological drawbacks (see General Material & Method II.) as discussed in Suo *et al.* [92] and in Brun *et al.*, who showed no detectable GHb in zebra finches using a highly specific method [87]. It remains that little is known about the relationship between glycation levels and individual fitness, and that the few available studies solely focused on birds. Finally, intra-population variability and heritability of glycation levels remain undefined in most animal species. Such data would enable us to assess whether glycation and the proteins (receptors, enzymes) involved in related regulatory pathways can be targeted through natural selection.

2. Correlations Between Glycation and Body Mass

Among the different traits that determine the life history of a species, body mass is one of the most important [93] because it allows to assess interspecific allometric relationships among traits as well as intraspecific variability in relation to individual quality. However, to our knowledge, only one correlational study in zebra finches addressed the links between glycation and body mass at an intraspecific level, and no correlation was found between plasma protein glycation levels and body mass [87]. On the other hand, some interspecific studies have been conducted on this subject. A recent work focusing on plasma AGEs concentrations in 7 bird and 16 captive mammal species of different body sizes showed that birds displayed higher AGEs levels than mammals, but no significant correlation with body mass was detected in either class, even after phylogenetic correction [94]. Similarly, another study in Canidae failed to correlate plasma AGEs levels with average body mass in each species [95]. An identical result was obtained when restricting the analysis to domestic dog strains, although dog breeds vary widely in body size [95]. This suggests that glycation is a variable likely reflecting a biological feature that stands above a simple body condition index, as it has been recently suggested for glycaemia [96,97], a hypothesis that needs a broader exploration at the inter- and intraspecific levels.

3. Glycation in Relation to Age and Longevity

In humans, it is widely admitted that the levels of glycated molecules such as HbA1c and GA or the levels of certain AGEs increase with age [98], thus taking part in the biological modifications that accompany the senescence process (e.g., loss of skin elasticity, crystalline opacification etc.) [56]. Similarly, because glycation is associated with ageing-related diseases, it contributes to an increased probability of death. This is in accordance with a pro-senescent role of glycation and AGEs in humans, presumably due to the lack of selection pressure to set up any anti-glycation protection systems.

In non-conventional animal models, the picture is more mixed. The dynamic of glycation with age has been studied at an intraspecific level in a few species from different taxa. In mammals, while no correlation between age and GHb levels was found in the domestic dog (*Canis familiaris*) [99], a positive correlation between skin pentosidine levels (a specific cross-linking AGEs) and age was highlighted in seven mammal species (respectively least shrew, rat, domestic dog, cow, pig, squirrel monkey and rhesus monkey), with tighter relationships in shorter-lived species [100]. This is in line with the human observations. In insects, it was shown that older flies *Drosophila melanogaster* displayed 44% less AGEs than younger ones [101]. This may reflect a selective disappearance over time of individuals with higher AGEs levels, reinforcing the idea that the rate at which glycated products are accumulated may impact individual fitness and longevity.

When looking at the interspecific level, a negative correlation was underlined between the rate of formation of pentosidine in skin collagen and maximum lifespan in mammals [100]. This highlights that shorter-lived species accumulate pentosidine more quickly than longer-lived ones, which might be coherent with the fact that glycation accelerates actuarial senescence (e.g., the increase in mortality with age), and therefore might contribute partly to a shorter lifespan, both between and within species. Interestingly, in their extensive study of plasma AGEs levels in mammals and birds, Baker *et al.* found no correlation between age and AGEs levels in birds [94]. When focusing on 16 mammal species, after correction for phylogeny, they also did not highlight any correlation between age and plasma AGEs [94]. The absence of correlation between the plasma levels of AGEs or GHb and age was also observed at a smaller scale in wild canid species and domestic dogs of different breeds [95,99].

Taken together, these conflicting results suggest that there is not necessarily a direct link between AGEs accumulation and age. The dynamic of glycation with age is likely to be a species-specific trait, which might be largely dependent on (i) the metabolic adaptation to high sugar diet (i.e., which absolutely needs to be buffered when the effect on fitness is likely to be the more important, i.e., age of reproduction.), (ii) the flying ability (see chapter 4.a. below) or (iii) the methodological issues related to the type of tissue considered and on the nature of the glycated compound used as a marker of senescence. Indeed, we might expect to observe a more marked accumulation of glycation products in tissues with slow-protein turnover rates [102]. Furthermore, there is a higher probability to observe an age-dependent accumulation of AGEs rather than Amadori compounds, as AGEs are stable over the long-term due to their (supposed so far) non-degradable status and difficult clearance. Conversely, Amadori compounds have short lifespans, as they are among the first products formed during the glycation reaction chain, and they are prone to undergo further reactions due to their relative instability. They can also be degraded by specific mechanisms (see chapter 4 below). It therefore seems more likely to observe Amadori compounds correlating with glycaemia in the short term (as observed in diabetic patients) rather than with age. However, a slight increase in their levels with age in the context of a longitudinal follow-up cannot be ruled out, given that glucose tolerance (i.e., the ability of an individual to efficiently regulate its blood glucose level and to avoid potential harmful effects of high glycaemia through specific mechanisms) is known to decline with age [103].

Other important differences, such as the sex-dependent differences in glycation (see [99,104–106]) and their implication in sex-ageing patterns also need further examination. Longevity differences between males and females can be observed in many species, and one could expect the longest-lived sex to exhibit lower levels due to their ability to prevent their early formation. We indeed already know that AGEs levels can differ between genders in patients with ageing-related diseases [104] or in pathological laboratory rats [106].

In conclusion, it appears that the current knowledge on how glycation might be associated to life-history traits and age in non-model species remains highly heterogeneous, and even contradictory in some specific cases, whatever the scale (inter- or intraspecific) or the parameter considered (see Table 1). One possible explanation lies in the methodology of the

studies carried out to date. They are all population-based and therefore cross-sectional, and it cannot be ruled out that the selective disappearance of the weakest individuals or of those with the highest glycation levels does play a role in the heterogenous results obtained. Long-term studies with follow-up of individuals appear necessary to assess the possible links between glycation and age. It is also interesting to note that most studies looked at each life-history trait separately, although all these traits are intrinsically linked and influence each other. A global approach integrating the whole panel of traits defining the species' pace-of-life and several markers of glycation at once is still lacking. Only such an approach might assess how glycation and life-history traits may have been shaped in relation to environmental challenges by evolution. Finally, we need a more precise description of the metabolic and cellular pathways through which glycation resistance may be acquired, and how such mechanisms may be related to a higher individual fitness. Because (hyper)glycaemia levels are different among clades (i.e., mammals and birds), it is likely that the resistance mechanisms that emerged and their importance as modulators of fitness will differ among them.

Table 1. Known correlations between glycation products and age, glycaemia and/or life-history traits in non-conventional animal models at (a) intraspecific and (b) interspecific levels.

(a) Intraspecific studies

Glycation product	Factor	Correl - ation	Species	Comments	Ref.
GHb	Age	NS	*Ficedula albicollis*	Wild, adults	[89]
		NS	*Canis familiaris*	Domestic, all ages	[99]
	Sex	NS	*Ficedula albicollis*	Wild, adults	[89]
		NS	*Falco sparverius*	Wild, nestlings	[90]
		NS	*Canis familiaris*	Domestic, all ages	[99]
	Body mass	NS	*Ficedula albicollis*	Wild, adults	[89]
	Clutch size	+	*Ficedula albicollis*	Wild, adults	[89]
	Number of offspring fledged	+	*Ficedula albicollis*	Wild, adults	[89]
		NS	*Ficedula albicollis*	Wild, adults	[91]
	Arrival date on reproduction site	-	*Ficedula albicollis*	Wild, adults	[89]
	Return rate	-	*Ficedula albicollis*	Wild, adults	[91]
	Nestling growth rate	+	*Falco sparverius*	Wild, nestlings	[90]
	Number of brood mates	NS	*Falco sparverius*	Wild, nestlings	[90]
GA	Age	NS	*Taeniopygia guttata*	Captive, adults	[87]
	Glycaemia	NS	*Taeniopygia guttata*	Captive, adults	[87]
TPGP	Age	NS	*Taeniopygia guttata*	Captive, adults	[87]
	Sex	NS	*Taeniopygia guttata*	Captive, adults	[87]
	Body mass	NS	*Taeniopygia guttata*	Captive, adults	[87]
	Glycaemia	NS	*Taeniopygia guttata*	Captive, adults	[87]
Glycated Serrotransferrin	Age	NS	*Taeniopygia guttata*	Captive, adults	[87]
	Glycaemia	+	*Taeniopygia guttata*	Captive, adults	[87]
Glycated Carbonic Anhydrase	Age	+	*Taeniopygia guttata*	Captive, adults	[87]
	Glycaemia	NS	*Taeniopygia guttata*	Captive, adults	[87]
Pentosidine (skin)	Age	+	*Cryptotis parva*	Captive, 0-3 y.o.	[100]
		+	*Rattus norvegicus*	Captive	[100]
		+	*Canis familiaris*	Domestic	[100]
		+	*Bos taurus*	Captive	[100]
		+	*Sus scrofa*	Captive	[100]
		+	*Saimiri sciureus*	Captive	[100]
		+	*Macaca mulatta*	Captive	[100]

(b) Interspecific studies

Glycation product	Factor	Correl - ation	Species	Comments	Ref.
Plasma AGEs	Age	NS	Mammals	Captive (zoos)	[94]
		NS	Birds	Captive (zoos)	[94]
		NS	Wild canids & domestic dog breeds	Captive (zoos) & domestic	[95]
	Age/MLSP	NS	Mammals	Captive (zoos)	[94]
		NS	Birds	Captive (zoos)	[94]
Plasma AGEs	Age/MLSP	NS	Wild canids & domestic dog breeds	Captive (zoos)	[95]
	Body Mass	NS	Mammals	Captive (zoos)	[94]
		NS	Birds	Captive (zoos)	[94]
		NS	Wild canids & domestic dog breeds	Captive (zoos) & domestic	[95]
Pentosidine (skin)	MLSP	-	Mammals	Captive	[94]

+ and - indicate positive and negative correlations respectively, NS indicates no significant correlation. GA: Glycated albumin; TPGP: Total Plasma Glycated Proteins (includes GA, Glycated Serotransferrin and Glycated Carbonic Anhydrase); MLSP: Maximum Lifespan.

III. Mechanisms of Glycation Resistance and Tolerance that Need to Be Explored in a Broad Range of Animals

Before continuing, we would like to briefly review the use of the terms glycation "resistance" and "tolerance" in the remainder of this manuscript. We define "resistance" as the mechanisms that prevent the glycation reaction from taking place, i.e., the initial attachment of one or more hexose molecules to a protein. Conversely, we will speak of "tolerance" to glycation when glycation products have been formed (Amadori compounds and AGEs), but organisms are able to escape their harmful effects (see Figure 3 and the section below for more details on these different mechanisms).

The apparent resistance and/or tolerance of bats and birds to high blood glucose levels may be explained by specific physiological and molecular mechanisms aimed at mitigating glycation stress. Interestingly, GHb levels measured in some bird and bat species showed lower levels than those of diabetic humans (e.g., 3.9 % in the *Glossophaga soricina* bat [107], between 0.73 and 3.72 % in collared flycatchers [91], no detection in zebra finches [87]), which supports the beforementioned hypothesis. Still, Baker *et al.* [94] found that AGEs levels are higher in birds than in mammals. This completes the tolerance hypothesis by suggesting that tolerating high glycaemia can be reached at different levels (i.e., through early or late-glycation buffering). Thus, a more detailed exploration of the potential regulatory and/or resistance mechanisms (see Figure 3) in apparently high glycaemia and glycation tolerant animal species could lead to a better understanding of the glycation – life-history traits nexus.

1. Fast and Effective Glucose Utilization: The Case of Flying Vertebrates

Active glucose metabolism enables rapid glucose utilization and/or effective storage in cells immediately after food digestion, which can reduce the amount of blood glucose available for glycation reactions. Birds and bats both practice active flight, which is one of the most energy-demanding activities that has evolved in multiple clades [108–111]. It has been shown in

nectar- and fruit-feeding bats that flight is one of the key factors in postprandial blood glucose regulation [107,112]. Studies on bats have shown that post-prandial blood glucose levels reach peaks that would be harmful in other mammals, but they drop quickly and reach fasting levels (similar to those observed in humans) within 60-70 min after the bats spent 70-75% of their time flying [107,112], a behavioural-based mechanism that might almost replace insulin-mediated pathways in the *Glossophaga soricina* bat [107].

In addition to the above-mentioned behavioural adaptations (flight), physiological mechanisms are also primarily involved in glucose metabolism. Floral nectar is roughly composed of a 35:35:30% ratio of glucose, fructose and sucrose [113]. *Phyllostomoid* bats seem to rely on a well-developed arsenal of intestinal sucrases, which are responsible for the efficient hydrolysis of sucrose [114], and therefore favour the rapid absorption of sugar molecules. Birds also seem to be adapted to high-sugar regimes, as the maximal enzymatic activity of sucrases was found to be up to 2 times higher in hummingbirds than in nectarivorous bats [114].

In bats, ingested sugars are usually directly consumed instead of being converted into fat and stored in adipose tissue or as glycogen in liver and muscles. This is notably the case for nectarivorous bats, both at rest and while flying and foraging [115–117]. To explain such a fast utilization of absorbed sugars, muscles would be the ideal compartment to study. To date, we only have data from some studies that highlighted that adult bats' erythrocytes have a higher permeability to glucose than those of most mammals and birds, thanks to a rapid GLUT-1 mediated glucose transport [118]. This is even more remarkable because high permeability to glucose in erythrocytes is a feature usually only observed during foetal and neonatal life in mammals [119,120], except for humans, higher primates, small ondoncete whales and bats, where it continues into adulthood [118]. Still, this metabolic particularity cannot be generalized to other sugars, as bats' red blood cells permeability to fructose seems really low [118], which conversely might reduce exposure of erythrocyte proteins to fructose toxicity. The case of avian erythrocytes is very different from that in bats: studies on domestic geese showed a very low permeability to glucose [121] and facilitated hexose transport seems to be almost absent in pigeons [122]. It should be noted that, unlike mammals, including bats, avian red blood cells are nucleated, and possess functional mitochondria [123,124], thus making these more prone to be fuelled by fatty acids instead of glucose, a possible indirect mechanism of protection against glycation.

While the insulin-dependent cascade is conserved across most vertebrates [125], it differs substantially in birds [126]. Indeed, mammals heavily rely on GLUT-4 for insulin-induced glucose uptake into cells, but some studies mention the absence of the GLUT-4 transporter in birds [127,128], while others claim to have detected it [129,130]. One hypothesis to explain these conflicting results might be because avian genomes remain incompletely annotated due to complexity. The absence of GLUT-4 could also be compensated by other transporters, such as GLUT-12 that appears to play a predominant role in few avian species [126,131,132]. Recent studies have also shown that the Slc2a4 gene encoding GLUT-4 had undergone changes under selective pressure in old world fruit bat species (in comparison to their insectivorous counterparts), favouring an enhanced binding ability of GLUT-4 and consequently a more efficient glucose uptake of the skeletal muscles [133]. Moreover, it is estimated that bats heavily depend on intestine passive paracellular absorption, which constitutes over 70% of their total glucose absorption [134–136]. While this far exceeds what is seen in non-flying mammals [135,137–139], it is worth noticing that similar observations have been made in small birds [138–141]. Some authors suggested that this heightened reliance on passive paracellular transport might stem from the necessity to compensate for a proportionally smaller intestinal tissue mass compared to their overall body mass [138]. In the same vein but among non-flying vertebrates, marmosets, primarily gum-feeders with a smaller intestine relative to mammals of comparable size, also demonstrate a significant 30% paracellular glucose transport [142].

2. Protection of Biomolecules

a) Protein Turnover

Protein turnover contributes to the maintenance of protein homeostasis, which can be defined as the preservation of the stability and functionality of the proteome. Several intracellular mechanisms, notably mediated by the proteasome and the lysosome, are involved in degrading structurally altered proteins to avoid their possible toxicity. Various studies in different animal models have demonstrated that the rate of accumulation of AGEs in cells is positively correlated with their half-life, and therefore negatively with their turnover rate [100,143–145]. One study suggested that enzymes and intracellular proteins with a rapid

turnover may therefore be protected from high rates of glycation [146]. However, it is now widely admitted that senescence is accompanied by a loss of proteostasis, namely a decline in the synthesis of chaperones and a decline in the activity of the proteolytic systems [147], which could then partly explain the accumulation of AGEs in cells and tissues over life [56,148]. In addition, proteostasis itself does not escape glycation stress [149].

b) Specific Amino Acid Composition and 3D Conformation of Proteins

Protein glycation mainly targets lysine and arginine residues [150–153]. However, several studies demonstrated that not all lysines or arginines in a same protein are subject to the same glycation rates. For example, of the 44 amino acids in the alpha and beta chains of human haemoglobin, only five appear to be potential glycation sites, namely 3 lysines and 2 valines [154]. This site specificity obviously depends on the amino acid composition of a given protein, but also and above all on its multidimensional conformation, with theoretically glycatable amino acids being either exposed at the surface or, on the contrary, inaccessible because hidden. Thus, the lower number of lysines in chicken albumin and the fact that most of these residues are located in the inner part of the protein (only one lysine on the surface) seem to explain why it is glycated at a lower rate than bovine or human albumin (three lysines on the surface) when exposed to the same increasing concentrations of glucose [155,156]. Moreover, the presence of certain amino acids (such as aspartic acid or specific binding sites for phosphate or phosphorylated molecules) in the neighbourhood of the preferential glycation sites also seems to be of importance [157]. It is also interesting to notice that birds from the *Psittacidae* family, which includes parrots and parakeets, have more lysine residues than other birds, yet cases of diabetes have been reported in parrots [158].

3. Buffering Glycated Proteins and AGEs

a) Deglycating Enzymes

Several defence mechanisms occur *in vivo* to regulate the accumulation of glycation products at different stages of the Maillard reaction. Although research in this area is still in its infancy, researchers have already identified several enzymes capable of detaching the sugar from the amine function of the fructosamine, thus reversing the formation of Amadori products and stopping the glycation reaction at it early steps [159]. To date, three major classes of deglycating enzymes have been described, which differ in their physiological role and their catalytic mechanisms: fructosamine oxidases (in fungi and bacteria) [160], fructosamine-3-kinase (in bacteria, mammals [109] and birds [161]) and fructoselysine-6-phosphate deglycase (in bacteria) [159].

b) RAGE

Originally known for binding AGEs [162], the Receptor for Advanced Glycation End-products (RAGE) is now recognized as a multi-ligand receptor of the immunoglobulin superfamily [163]. It is expressed in various tissues [164] and is involved in inflammatory and immunological pathways [165]. RAGE is found under two main forms: transmembrane, whose binding activates different signalling cascades, and as a free circulating form that can bind ligands without activating any pathway [166].

Among the AGEs capable of binding RAGE, preferential candidates include carboxymethyl-lysine (CML) and hydroimidazolone [165]. The consequences of AGE-RAGE interactions at the membrane include a significant increase in ROS production, the activation of the NFκB transcription factor, an up-regulation of inflammatory pathways, an increased cell adhesion to the extracellular matrix, as well as facilitated AGEs accumulation [165,167–169]. Studies in diabetic patients have shown higher RAGE levels in the kidneys, particularly in the context of nephropathies, as well as in atheroma plaques of patients suffering from atherosclerosis [170,171]. A positive correlation between RAGE and HbA1c levels has also been highlighted in diabetic atherosclerotic plaques [171]. In addition, genetically modified mice that do not express RAGE appear to resist to ageing-related diseases associated with hyperglycaemia

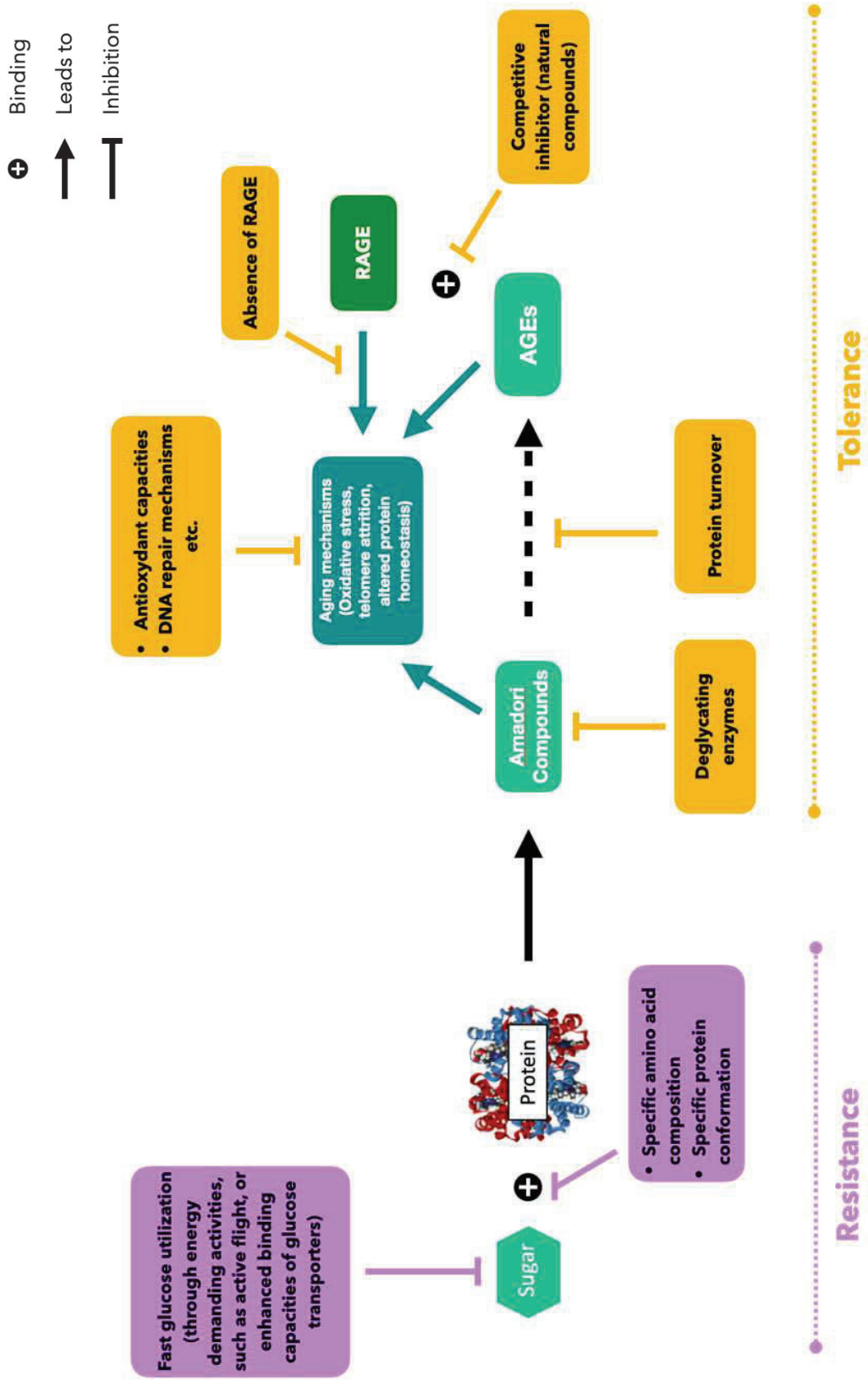


Figure 3. Mechanisms of resistance and tolerance to protein glycation that need to be explored in a broad range of animals

[172–175]. Furthermore, it is worth noting that soluble RAGE is suspected to mitigate the negative impact of AGEs by sequestrating them [176].

For a long time, RAGEs were believed to be unique to mammals, with birds lacking them, which appeared to be a potential explanation for birds' resistance to glycation [177]. An evolutionary study points out that RAGEs emerged in mammals while being lost in birds [178]. However, recent research indicates that some bird species may possess genomic sequences related to RAGE, thus leading to an emerging debate [179,180].

c) Competitive Inhibition and Regulation of Glycation's Deleterious Effects by Natural Compounds

In pharmaceutical and medical research, several natural compounds have been identified as having "anti-glycating" properties [181]. Their mechanisms of action can range from the reduction of glycemia to the competitive inhibition by binding to amino acids of proteins that are typically glycated or to the selective trapping of reactive carbonyl compounds [182]. Other mechanisms involve combating the detrimental effects of glycation, such as oxidative stress [183]. This latter category is of particular interest in the context of adaptations observed in certain animal species to sugar-rich diets (e.g., frugivores and nectarivores). Indeed, fruits are rich in antioxidants, and one of the known harmful mechanisms of glycation is the increase in oxidative damage. It is well known that some species of frugivorous bats exhibit particularly high levels of antioxidants, which are attributed to their diet [184]. Several studies have demonstrated the regulatory effect of antioxidant compounds on glycation products [182]. This could potentially constitute a protective system against the deleterious effects of glycation in frugivorous species.

IV. Aims and Scope of the Thesis

This introduction clearly outlines the critical lack of research on glycation in species other than humans or laboratory mice and rats. However, as we saw before, some taxa such as bats seem to have evolved tolerance and/or resistance to high glycaemia and/or glycation, while having an extended lifespan relatively to non-flying animals.

In this thesis, I therefore set out to explore this vast subject of glycation in non-conventional and/or wild animals, focusing mainly on the order of mammals. I wondered whether glycation levels could serve as an additional biomarker of aging in mammals. To explore this, I aimed to address several specific questions through multiple studies, which form the chapters of this thesis.

Firstly, I questioned whether, among mammals, glycation correlates with the life-history traits of different species. I hypothesized that species with greater longevity and a slower pace-of-life would exhibit lower levels of glycation compared to shorter-lived species with a faster pace-of-life. Based on the current state of knowledge, I also considered whether bats might differ from non-flying mammals in terms of glycation, potentially exhibiting either resistance or tolerance to it. Since another clade of vertebrates has also developed powered flight, birds, I asked whether they differ from bats in their glycation dynamics. Finally, given that some studies have highlighted the evolution of mechanisms conferring resistance to high-sugar diets in frugivorous and nectarivorous birds and bats, I wondered whether glycation levels vary with dietary habits among these flying vertebrates. To answer these questions, I conducted two interspecific comparative studies. The first focused on several mammalian species with differing body masses, longevities, and life history traits, while the second compared bats and birds with similar body masses and longevities, including two dietary groups: frugivores and insectivores. I focused on glycated albumin and examined how it correlates with blood glucose levels, body mass, and pace of life in mammals. In flying vertebrates, I examined the evolution of glycated albumin levels in relation to body mass, maximum longevity, and dietary habits. In both comparative studies, I accounted for phylogenetic relationships among species. These findings are presented in Chapter 1 of this manuscript.

Secondly, I aimed to explore glycation at the intraspecific level and investigate how glycated protein levels might reflect individual quality within several mammalian species. In Chapter 2 of this manuscript, I focused on two frugivorous bat species, the Rodrigues flying fox and the Egyptian Rousette. I then considered whether bats' apparent resistance or tolerance to glycation could be explained by specific protein sequences (as observed in chicken albumin). Thus, I compared the albumin and hemoglobin sequences of the Egyptian fruit bat with those of humans and chickens. To further investigate whether glycation can be a marker of aging in these animals, I examined intraspecific relationships between glycation markers (glycated hemoglobin and glycated albumin) and age, as well as parameters such as body mass, often considered an indicator of individual quality, or sex. I also explored whether individual differences in glycation tolerance could be mediated by oxidative stress regulatory mechanisms.

Finally, in the last chapter, I examined the relationship between the environment and glycation. I hypothesized that individuals living in more favorable environments (in terms of resource abundance or climate) and exhibiting slower senescence would show lower levels of glycated proteins compared to individuals in more challenging environments. To test this, I studied two populations of European roe deer living in distinct environments. I examined how the relationships between glycation and individual parameters, such as age, body mass, sex, or inflammatory status, differed between these two populations.

I conclude this thesis by discussing the results and limitations of the various studies and the exciting future research opportunities opened up by this thesis.

GENERAL MATERIALS & METHODS

Torpid Daubenton's bat Myotis daubentonii (photo by Marc Bastardot)

Twenty mammal species were sampled for this work, with studies at different scales (interspecific *versus* intraspecific). To avoid redundant descriptions of these species between chapters, all mammal species (and the associated sampling protocols) involved in the different studies are presented in the following "Materials and Methods" section.

The following chapter is divided into three parts. The first one focuses on the animal study models. The second part describes the methodology used in this thesis to measure protein glycation levels. The last part covers additional physiological measurements conducted on blood samples.

I. Study Models: Non-Conventional Mammal Species

Twenty mammal species (see Table 2) from different orders, diets and geographical origins were sampled throughout the three years of this PhD work.

Bats, with their different diets and extended lifespans, were particularly relevant for our research subject. However, many challenge arise when one wants to work with bats. First, these animals are very fragile and sensitive to stress, which makes them hard to breed in captivity, and even more so to subject to controlled study protocols. Secondly, these animals can be carriers of many pathogens (more specifically viruses), and the geographical origin of certain species can therefore quickly become a safety constraint, requiring work in laboratories with a higher level of safety (P2 laboratory for captive bat species, and P3 laboratory for wild bat species). Thirdly, as many species have a very small mass, it is difficult to draw a lot of blood from a single individual (limit of 10% of total blood volume, which corresponds to approximately 10% of the individual's body mass), and the number of measurements that can be made on a plasma sample is quickly limited by the quantity. Based on our hypothesis that bats are resistant to glycations, we first put an emphasis at sampling many species from this order (15 in total, see Table 2) and to use both limited plasma volume but also red blood cells (as 5 µL of these two latter tissues is enough for our MS-based glycation measurement), in order to carry out a "scan" on many species of different masses and diets.

Table 2. Mammal species sampled during this thesis.

	Species	Wild/ Captive	Origin	Comment
Bats	*Pteropus rodricensis*	Captive	Zoo de la Palmyre (France)	Plasma & Red blood cells
	Rousettus aegyptiacus	Captive	Zoo de la Palmyre (France)	Plasma & Red blood cells
	Myotis daubentonii	Wild	Switzerland	Plasma & Red blood cells
	Nyctalus noctula	Wild	Switzerland	Plasma & Red blood cells
	Carollia perspicillata	Captive	Papiliorama (Switzerland)	Plasma & Red blood cells
	Sturnira parvidens *	Wild	Belize	Plasma
	Artibeus lituratus *	Wild	Belize	Plasma
	Artibeus jamaicensis *	Wild	Belize	Plasma
	Glossophaga mutica *	Wild	Belize	Plasma
	Carollia sowelli *	Wild	Belize	Plasma
	Molossus nigricans *	Wild	Belize	Plasma
	Uroderma convexum *	Wild	Belize	Plasma
	Lonchophylla concava *	Wild	Belize	Plasma
	*Anoura geoffroyi**	Wild	Belize	Plasma
	*Phyllostomus discolor**	Wild	Costa Rica	Plasma
Non-flying mammals	*Arvicanthis ansorgei* *	Captive	Chronobiotron (France)	Plasma & Red blood cells
	Rhabdomys pumilio *	Captive	IPHC (France)	Plasma & Red blood cells
	Capreolus capreolus *	Wild	France	Plasma (Frozen samples recovered from previous experiments)
	*Mirounga leonina**	Wild	French Southern and Antarctic Territories.	Plasma
	Microcebus murinus *	Captive	MNHN (France)	Plasma (Frozen samples recovered from previous experiments)

* Indicates species that could not be used in the studies and statistical analyses of this thesis, as we have not detected glycated proteins. IPHC: Institut Pluridisciplinaire Hubert Curien. MNHN: Museum National d'Histoire naturelle

Unfortunately, due to the constraints outlined above, as well as methodological issues to be discussed below, we were unable to use all these species as planned. We therefore decided to include several species of non-flying mammals, in order to carry out a comparative analysis with our bats, as well as to paint a broader picture of protein glycation in mammals. We first turned to two rodent species (*Arvicanthis ansorgei* and *Rhabdomys pumilio*). These are in the same body mass range as bats but have a very short lifespan. We also wanted to include a frugivorous mammal whose average mass would fall within the mass range of our bats. For this, we turned to a small, long-lived frugivorous primate (Grey-mouse lemur *Microcebus murinus*), based on existing samples (see below). We then broadened our sampling to include species of much greater mass, and, also based on already available collected samples, added one artiodactyl (European roe deer *Capreolus capreolus*) and one carnivore (Southern elephant seal *Mirounga leonina*) to our species list.

To make it all happen, we have set up several collaborations with other researchers and laboratories (see below). Our final species sampling was distributed as follows: 15 bat species, 2 rodent species, 1 primate, 1 artiodactyl and 1 marine carnivore species.

For most of the species, blood was centrifuged right after sampling to separate plasma from red blood cells (see below for collection details, such as site, tubes etc.; Figure 12 p.57). Our protocol included (with the exception of roe deer, southern elephant seal and grey-mouse lemur, for which we had access to frozen samples collected in previous experiments): two centrifugation cycles (4°C, 10 min, 2500g), with collection of plasma and rinsing of red blood cells with PBS (phosphate-buffered saline) in between (see Appendix n°1). When blood was sampled in sufficient quantities, several aliquots of plasma and red blood cells were made, before being stored in dry ice. Samples were then returned to our laboratory (IPHC, Strasbourg, France) within a maximum of 24 hours, before being stored in freezers at -80°C until analysis.

Photo 1. Adult Rodrigues Flying Fox *Pteropus rodricensis* (photo by S. Meys – Zoo de la Palmyre)

Photo 2. Egyptian Fruit Bats *Rousettus aegyptiacus* (photo by S. Meys – Zoo de la Palmyre)

1. Bats: Species & Sampling

a) Frugivorous Species from Captive Populations

Three of the 15 sampled bat species were issued from captive colonies, and all three of them were frugivorous: the Rodrigues Flying Fox (*Pteropus rodricensis*), the Egyptian Rousette (*Rousettus aegyptiacus*) and the Seba's Short-tailed Bat (*Carollia perspicillata*).

The Rodrigues Flying Fox is a big bat species (adult body length: 35 cm, wingspan: 75-90 cm, adult body mass: 254-300 g) endemic to the Rodrigues Island, to the east of Madagascar [185]. They are classified as "endangered" by the International Union for Conservation of Nature (IUCN), with only ca. 20 000 remaining individuals in the wild [186].
Our individuals came from a captive colony bred at the Zoo de la Palmyre (Royan, France). This colony had the advantage of being strictly monitored, as the individuals were all born in the zoo, fitted with microchips and thus of known age. Their food ration consisted of fruits and vegetables, distributed 4 times a day (see Appendix n°2 for details).
Fifteen individuals were sampled in December 2021, ranging from approximately 4 months to 14 years old, with a ratio of 7 females and 8 males. Animals were first anesthetized using isoflurane, then weighed and blood was collected from either the brachial vein or the uropatagial vein, using a syringe and EDTA as anticoagulant.

The Egyptian Rousette (or Egyptian Fruit Bat) is one of the most studied bat models, for which we have large protein and genomic databases. It is also one of the bat species most frequently encountered in zoos. The range of this species is rather wide and uneven, extending across sub-Saharan Africa, the Middle East, the Mediterranean and southwest Asia. A medium-sized species (body length: 12.1-19.2 cm, wingspan of ca. 60 cm, adult body mass: 108-140 g) [187,188], it has enjoyed an unfortunate reputation ever since it was identified as the reservoir of the Marburg virus [189].
The 25 individuals we sampled were also issued from captive population from the Zoo de la Palmyre. Contrary to *P. rodricensis*, this population is not monitored by the zoo, their population being much larger, and consequently we don't know the exact age of the individuals. Thirteen females and 12 males were sampled in December 2021, to which we

Photos 3. Seba's short-tailed bat *Carollia perspicillata* (Top photo by Emmanuelle Rey, left bottom photo by Cyrielle Duval, right bottom photo by Yves Bilat)

attributed an age class based on their reproductive status: juvenile (n=13) or adult (n=12). The reproductive status was also recorded (in reproduction or not). The animals were euthanized as part of the zoo's population control program, before being weighed and intra-cardiac blood was collected with a syringe and EDTA as anticoagulant.

The last captive species, Seba's short-tailed bat, was sampled at the Papiliorama zoo (Kerzers, Switzerland) in August 2023. Native to Central America and the northern half of the South American continent, this little species (adult body mass ≃ 15 g, wingspan ≃ 21cm, body length ≃ 4.5-6.5 cm) mostly feeds on fruits in the wild, but they also allow themselves pollen, nectar or insects when fruit is less abundant [190].
At the Papiliorama, the bats were fed solely with fruit, twice a day, and water was provided *ad libitum*. The animals in this large population, which also reproduce at a high rate, are not monitored individually. For the same reasons as for *R. aegyptiacus*, the 24 individuals we sampled were first sacrificed before weighing and blood collection was performed using the same method as before. The age class (juvenile *versus* adult) was determined by observing their reproductive status and dentition. 13 females and 11 males were sampled, distributed between 11 juveniles and 13 adults.

b) Insectivorous Species from Wild European Populations

In October 2021, we sampled two insectivorous bat species, the Daubenton's bat (*Myotis baubentonii*) and the Common noctule (*Nyctalus noctula*), both from wild populations living in a forest adjoining the campus of Lausanne University (UNIL) in Switzerland, and which are monitored by Philippe Christe, researcher at this same university.
The Common Noctule's range encompasses a very large part of temperate Eurasia: it is found throughout Europe as far as western Siberia and China. It belongs to the largest bat species found in Europe, with a mean adult body mass between 21 and 30 g, a body length of *ca.* 6-9 cm and a wingspan of *ca.* 32-45 cm [187,191]. After hibernation (which lasts from November to March), females from western European countries migrate towards eastern Europe to give birth, before coming back with the juveniles in autumn for the mating season [187]. Our sampling took place in October, during the mating season. During this time, common noctules like to

Photos 5. Right picture: Common noctule *Nyctalus noctula* (photo by Marc Bastardot); **left picture: Nest box used by common noctules** (photo by Fabrice Bertile)

Photo 6. Daubenton's bats *Myotis baubentonii* (photo by Marc Bastardot)

roost in harems, one male being usually surrounded by several females. Bat nesting boxes have therefore been installed in the forest by UNIL research teams, and we were thus able to benefit from these installations to sample Common noctules. The sampling session took place during the day, when the bats were in torpor in the boxes. Individuals were put in separate cloth bags right after capture, before being transported from the field capture site to the laboratory in the shortest possible time.

Each animal was weighed, its reproductive status and general state of health was recorded, and its age class evaluated. A total of 7 adult common noctules (5 females and 2 males) were captured.

The Daubenton's bat is a tiny bat (adult body mass: 6-10 g, wingspan: 24-27.5 cm, body length: 4 -6 cm) which can be found in almost all of Europe, as far west as Asia [187,192]. This species has the particularity of hunting over bodies of water and rivers. Unlike the common noctule, this species is sedentary [187]. It is one of the bat species that has the highest maximum lifespan [193,194].

Since those bats like to roost in large colonies in caves during the day, we waited for their hunting phase at night to capture them. A harp trap was placed on their usual hunting route, above a river in Lausanne. Once captured, bats were put in individual cloth bags, and the same procedure as for *N. noctula* was followed. Ten individuals were sampled, with the following proportions: 3 females *versus* 7 males, and an equivalent number of juveniles and adults (n=5 for both).

Photos 7. Right picture: blood sampling on the uropathagium of a Daubenton's bat (photo by Clara Castex)**; left picture: harp trap used to capture Daubenton's bats** (photo by F. Bertile)

c) Wild Species from South America

Thanks to a collaboration with Ken Welch, researcher at the University of Toronto, and his PhD Jerrica Jamison, who is regularly sampling wild bats in South America, we have gained access to 9 species of wild bats sampled in Belize (*Sturnira parvidens*, *Artibeus lituratus*, *Artibeus jamaicensis*, *Glossophaga mutica*, *Carollia sowelli*, *Molossus nigricans*, *Uroderma convexum*, *Lonchophylla concava*, *Anoura geoffreyi*), and 1 species sampled in Costa Rica (*Phyllostomus discolor*), in the summer of 2022. As these samples were acquired as part of this collaboration, the plasma was kept for their use by our collaborators, and we recovered only the red blood cell samples, with the aim of measuring glycated hemoglobin levels. However, as it will be detailed the general discussion of this manuscript, we did not detect glycated hemoglobin in these species, which led us not to use them in the studies to be presented in this manuscript. We therefore will not expand on these species any further in this section.

2. Other Mammals: Species & Sampling

a) Rodents

The scope of this thesis being to focus on non-conventional mammals, we reached out for two African exotic diurnal rodent species: the Soudanian unstriped grass rat (*Arvicanthis ansorgei*) and the Four-striped grass mouse (*Rhabdomys pumilio*).

A. Ansorgei is a medium sized omnivorous rodent (adult body mass: 68-180 g, length without tail: 12.9-17.7 cm) found in central Africa [195]. With sleep/wake cycles similar to those of humans, it has been of interest to researchers for some time as a model for studying circadian rhythms and the regulatory mechanisms of sleep [196,197], but also aging [198]. Despite this, little information exists on this rodent and its wild lifestyle.

Our Soudanian unstriped grass rats originated from the Chronobiotron animal facility (UMS 3415, Strasbourg), through cooperation with the researcher Etienne Chalet. Each animal was housed separately in plastic cages with free access to food (standard rat chow) and water. As this breeding also contained diabetic individuals, we were careful to select only non-diabetic animals. All individuals (n=24) were between 5 months and 2 years old. They were

anesthetized by isoflurane inhalation before sacrifice (for other research purposes than ours), and blood was sampled on heparinized tubes. We sampled as many females as males (n= 12 for both), as well as juveniles and adults (n= 12 for both).

Photo 8. Soudanian unstriped grass rat *Arvicanthis ansorgei* (photo by Emma Grosjean)

The Four-striped grass mouse is a small rodent (body length: 18-21 cm, mean adult body mass: 30-55 g) endemic from the southern part of Africa. Its diet is omnivorous, although the green plant material and seeds form the largest portion of its food consumption [199]. Twenty-seven individuals were sampled from a captive colony reared in our animal facility at IPHC-DEPE (UMR 7178, Strasbourg). Animals were housed in small groups in cages, with free access to food and water. They were sacrificed before weighing, and blood was sampled on EDTA tubes. Individuals ranged in age from 21 to 390 days, with an almost balanced proportion of females and males (16 and 11 respectively), and a ratio of 9 adults *versus* 19 juveniles.

Photo 9. Four-striped grass mouse *Rhabdomys pumilio* (photo by Carsten Schradin)

b) Artiodactyl: The European Roe Deer *Capreolus capreolus*

The European roe deer (*Capreolus capreolus*) is a rather small ungulate (adult body mass: 20-25 kg, height at withers: 65-75 cm), widespread in Europe, whose diet is herbivorous [200]. We took advantage of two wild distinct populations (forests from Chizé and Trois-Fontaines, France) which have been extensively monitored over more than 40 years [201] by the Laboratoire de Biométrie et Biologie Evolutive in Lyon (LBBE, France), offering the advantage, among other, of knowing the precise age of the animals.

In order to create homogeneous groups between males and females and between the two study sites, covering a wide age range (from 1-year old juveniles to 15-years old adults), we used data from two capture sessions (March 2021 and February 2022). After being captured using drive netting, each roe deer's identity, sex, body mass, age and overall health condition was recorded (see [201] for further details). For each study site, we tried to select an equal number of males and females (12 individuals for both). We ended up with a total of 48 samples, actually corresponding to 46 individuals (two of them were sampled both in 2021 and 2022). The two populations' characteristics are covered more specifically in the chapter dedicated to the intra-specific study of glycations in roe deer (Chapter 4, p. 130).

Photo 10. Male European roe deer Capreolus capreolus (photo by Lucille Billon)

c) Carnivore: The Southern Elephant Seal *Mirounga leonina*

The species from the order Carnivora that we were able to include in our studies (thanks to a collaboration with Laura Charlanne, PhD in our laboratory) is the Southern Elephant Seal (*Mirounga leonina*), one of the largest pinnipeds. It is found in the southern seas of the globe, from the Antarctic to the south of other continents. This marine species breeds on land during the austral summer, on the beaches of the sub-Antarctic islands, and is widely studied for its extreme diving abilities. The southern elephant seal is characterized by extreme sexual dimorphism [202,203], with males weighing up to 3 or 4 times as much as females.

Sampling took place in wild colonies located at Port-Aux-Français (49°34'S, 70°21'E) in the Kerguelen Archipelago during the austral summer of 2022. For practical and safety reasons, only female elephant seals were sampled. Elephant Seals of two age categories (5 juveniles of ca. 5-6 weeks old and 5 adults at the end of the lactation period over 3-4 years old) were captured and anesthetized using tiletamine and zolazepam (Zoletil®). Blood sampling was performed in the extradural vein with a needle (18G 50mm), using EDTA as anticoagulant. Samples were centrifuged on site within an hour (5 min, 4000 rpm), plasma was extracted into an airtight vial and then stored at -20°C (until being transferred to -80°C).

Photo 11. Female Southern elephant seal *Mirounga leonina* and her pup (photo by Laura Charlanne)

d) Primate: the Gray Mouse Lemur *Microcebus murinus*

Through a collaboration with the researcher Jérémy Terrien from the Muséum National d'Histoire Naturelle, we had access to frozen plasma samples from Gray Mouse Lemurs (*Microcebus murinus*) from a captive colony breaded at CNRS/MNHN in Brunoy, France (MECADEV, UMR 7179). Plasma samples had been taken from these same animals several years previously, as part of a study into the effects of caloric restriction (CR) on longevity. The study showed that caloric restriction in these primates extended their lifespan [204]. We therefore believed that it would be interesting to have access to samples from both the control group (to gain insight into glycations in general in this species) and the group under caloric restriction. We assumed that, if glycations are a marker of aging in this species, the longer-lived individuals in the CR group would have less glycated proteins compared to the control group. These individuals had been followed longitudinally, so for the same individual we had 4 samples: one at two different ages, and for each age two samples taken at different seasons (winter-summer equivalents, this species being a hibernating one). Unfortunately, as will be discussed in detail in the general discussion of this manuscript, we were unable to detect glycated proteins in these samples, which led to us being unable to pursue a study on this species.

Photos 12. Gray mouse lemurs *Microcebus murinus* (photos by Aude Noiret)

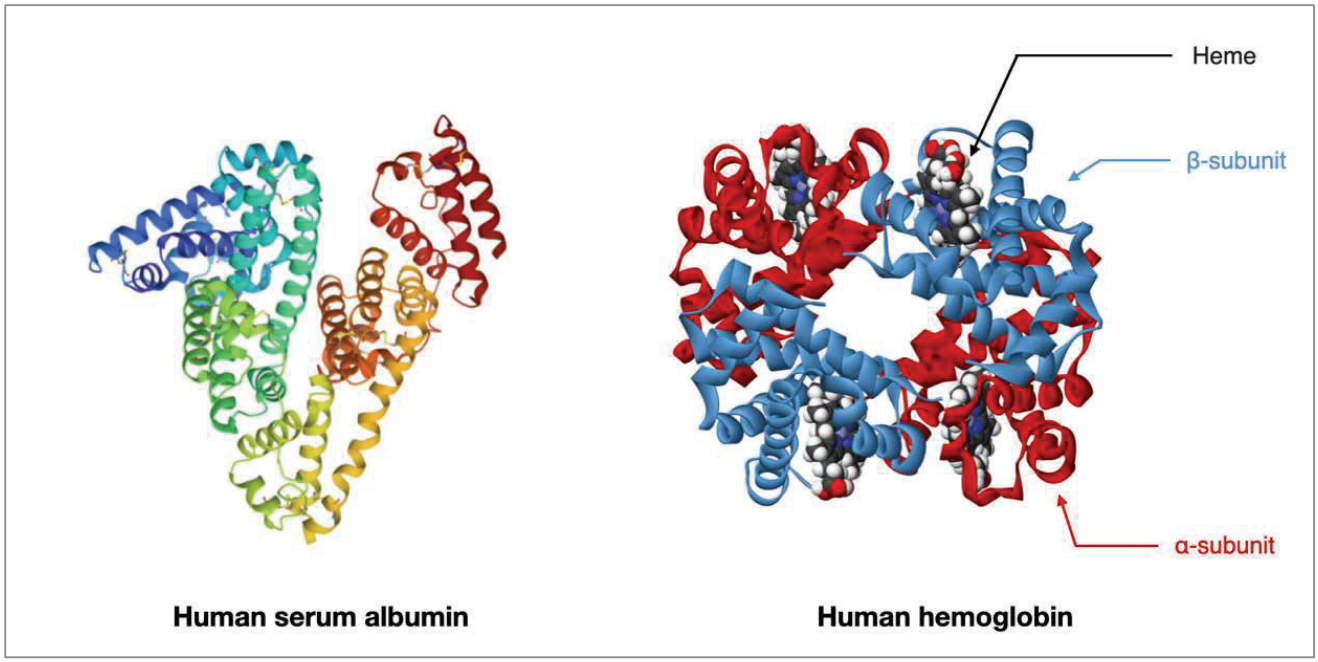


Figure 4. Different levels of protein organization: albumin's tertiary structure and hemoglobin's quaternary structure

Image sources: Albumin - RCSB Protein Data Bank (available at https://www.rcsb.org/structure/1ao6) / Hemoglobin - Ben Mills (available at http://commons.wikimedia.org/wiki/ File:Haemoglobin-3D-ribbons.png)

II. Measuring Glycated Protein Levels

In this thesis, we focused on two glycated proteins: glycated albumin and glycated hemoglobin (see Figure 12 p.57 for a schematic synthesis of the sampling and the associated measurements). These two molecules have the advantage of being the most abundant in the blood, and they are also among the most studied glycated proteins, since they are the reference biomarkers for diagnosing diabetes in humans. Glycated protein levels were measured by mass-spectrometry (MS) coupled to liquid chromatography (LC) using an intact protein approach.

1. An Introduction to the Principles of the LC-MS Method Used in this Thesis

a) A Reminder of Protein Structure

Proteins are organic macromolecules made up of a succession of amino acids linked to one another by peptide bonds. They are the result of the transcription of genes in ribonucleic acid (RNA), which is then translated into proteins.

A protein has different levels of structure. The amino acid sequence of a protein represents the protein's primary structure, which is comparable to a linear chain. These chains are then folded on themselves to form a three-dimensional structure, called tertiary structure or conformation. The latter *in vivo* is also known as "native conformation", and it very often determines the protein's biological function. In some cases, a final level of organization can be observed: when several proteins combine by covalent bonds to form a macromolecular complex, this is referred to as a quaternary structure. This is for example the case of hemoglobin, which consists of four sub-units (two alpha- and two beta-globins chains), each with a heme in its center (Figure 4).

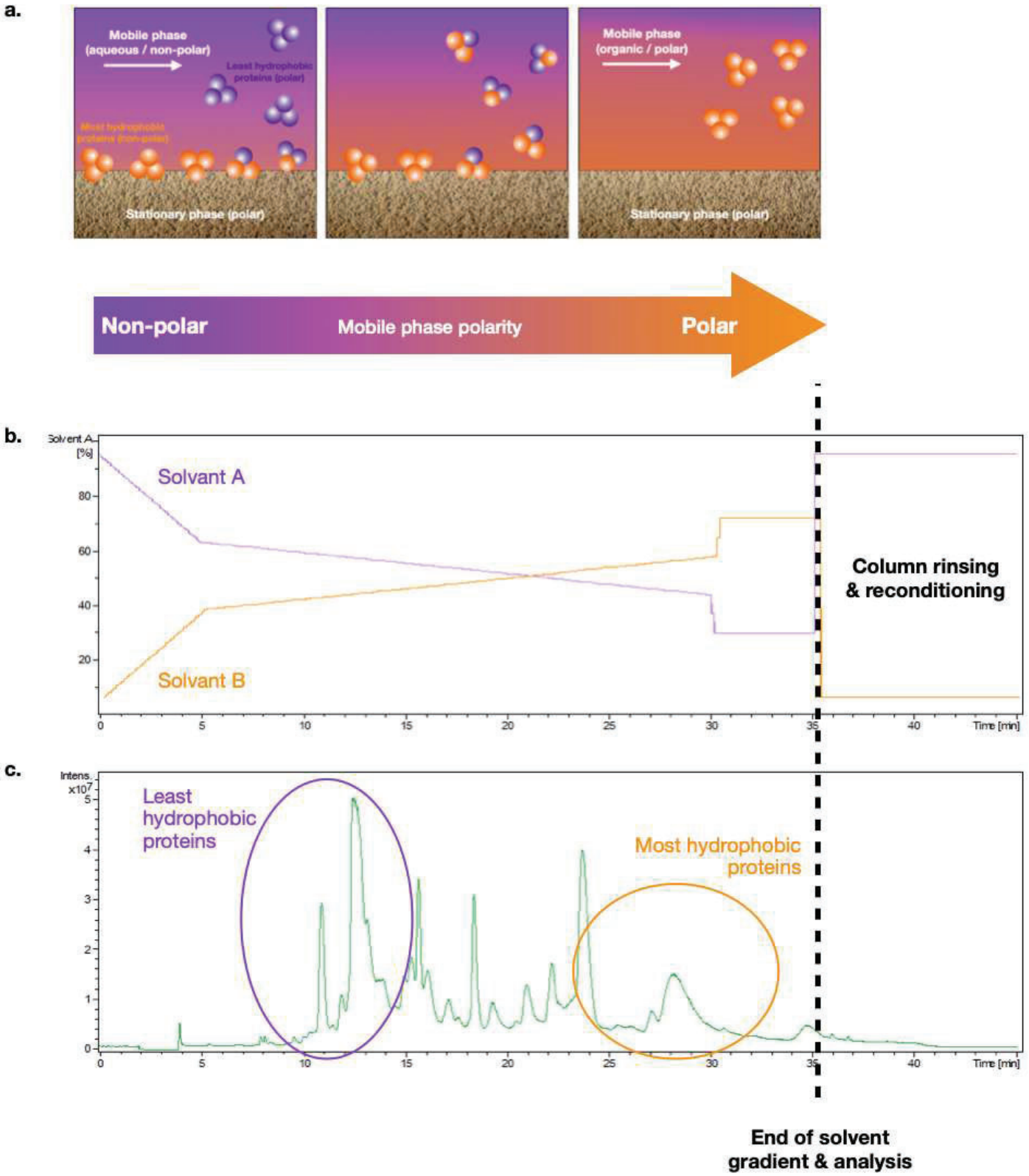


Figure 5. Reversed-phase chromatography: (a) Principle (b) Solvent gradient (c) Chromatogram

b) Liquid Chromatography

In mass-spectrometry, the proteins analyzed are often derived from complex samples (i.e., containing many different proteins). This is the case with plasma, one of the most complex biological matrices to analyze. It has an extremely wide dynamic range, and 60% of its protein mass is represented by albumin alone [205]. It is therefore necessary to separate the proteins before introducing them into the spectrometer, to facilitate their analysis by the latter. In this context, spectrometers are often coupled to chromatography systems [206]. One of the most frequently used in LC-MS is the reversed-phased chromatography (also known as hydrophobic interaction chromatography) [207]. As its name suggests, this technique is based on the existence of hydrophobic zones on the surface of proteins, which associate with polar regions contained in the matrix of a stationary phase, thus retaining the proteins on its surface (Figure 5). Its principle is as follows. The mixture containing the proteins is deposited at the beginning of the stationary phase contained in a column. A mobile phase consisting of acidified water (solvent A) mixed with an organic solvent (solvent B) then passes through the column, the proportion of the two solvents varying over time. The aqueous phase dominates at the start of the process. In this way, proteins with low hydrophobicity are carried along by the mobile phase first, the more hydrophobic ones being retained on the stationary phase. Gradually, the proportion of organic solvent in the mixture is increased along a gradient, thereby reducing hydrophobic interactions between the solid phase and the proteins (Figure 5.b.). They are thus carried along an increasing hydrophobic gradient towards the column outlet, with the more hydrophobic ones last. In this way, the proteins will successively enter the mass spectrometer, increasing the selectivity, sensitivity and coverage of the analyzed proteome (Figure 5.a). We then obtain a chromatogram, on which we can observe a succession of chromatographic peaks corresponding to proteins as a function of time (x axis), and whose height corresponds to the signal intensity (y axis) (Figure 5.c.).

c) Mass-Spectrometer: Composition and Principle

Mass spectrometry is a chemical analysis technique which allows to detect, identify and quantify a chemical or biological compound of interest, and in some cases to characterize its chemical structure. Its principle is based on the separation under gas phase of charged molecules (ions) depending on their mass/charge ratio (m/z) [208]. A mass spectrometer can thus be compared to a very precise balance capable of measuring extremely small masses [209]. Molecules (and here more specifically proteins) are therefore identified on the basis of their molecular weight.

A mass-spectrometer comprises three major components (Figure 6):

- An ion source
- An analyzer
- An ion detector

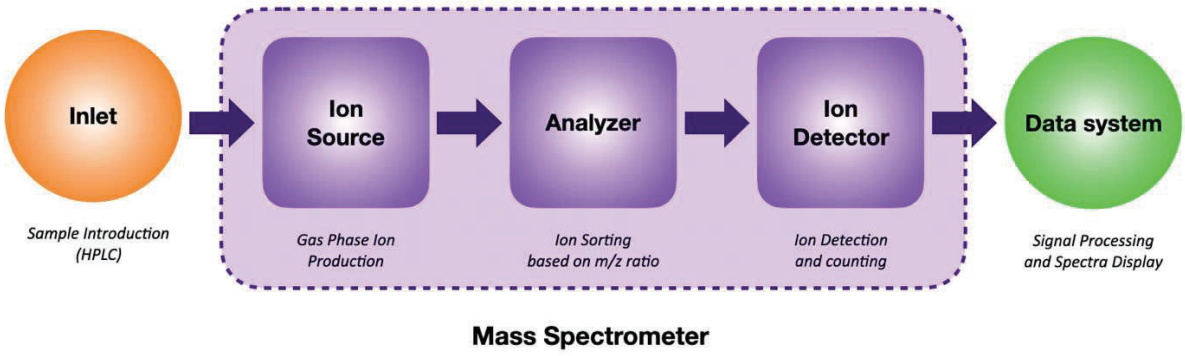


Figure 6. Main components of a mass spectrometer

The role of the ion source is to generate gaseous ions from the sample to be analyzed. There are several ionization methods, adapted to a particular type of sample and/or analysis. Here, we will only present the ionization method we have used, that is, electrospray ionization (ESI), operated in positive mode. This gentle ionization technique is based on the formation of a nebula containing solvated ions (which can be considered as microdroplets) under the effect of an electric field. The solvent in the droplets is then evaporated by collision with an inert gas, resulting in desolvated ions. We obtain ions charged with one or more protons H+ (Figure 7). The ion source extracts the ions thus formed and delivers them to the interface between the ion source and the second part of the spectrometer: the mass analyzer.

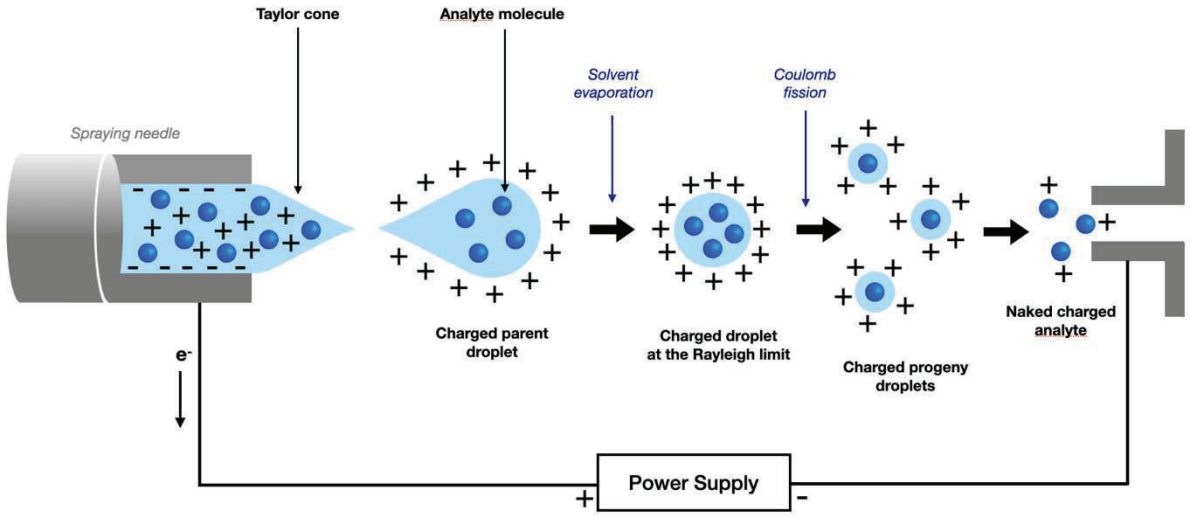


Figure 7. Electrospray ionization (ESI) process *(adapted from Banerjee & Mazumdar, 2012)*

The mass analyzer then sorts out molecules based on their m/z ratio, using electric or magnetic fields. Here too, there are several different types of analyzers, depending on their mechanism, resolution (their ability to separate two ions of similar mass), mass measurement range and measurement accuracy. For this thesis, we used a time-of-flight analyzer (TOF). Its principle is quite simple: on emerging from the ion source, the molecules are propelled with a certain kinetic energy into a field-free chamber (the flight tube), before being collected by the detector. The time they spend in the flight tube is proportional to the square root of their m/z

ratio, which means they are collected by the detector at different times, the lighter (and therefore faster) ones first (Figure 8).

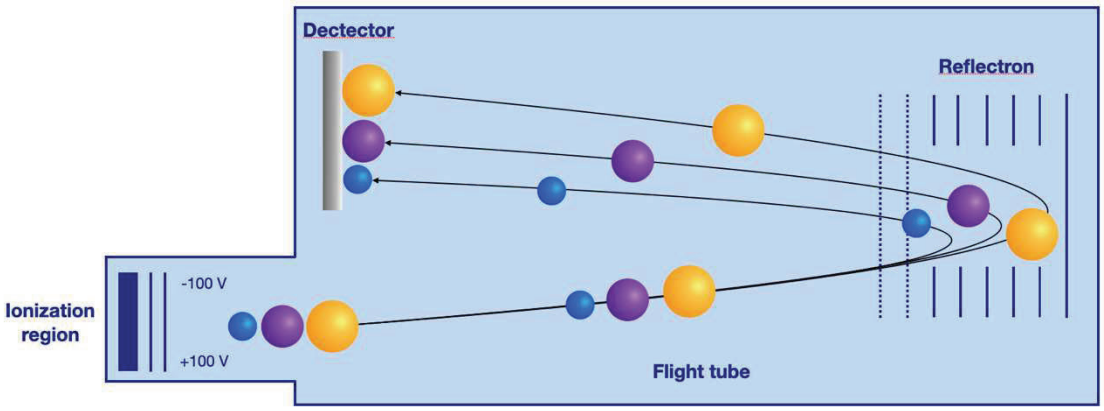


Figure 8. Time-of-flight (TOF) analyzer *(adapted from François Y, ULP)*

The role of the detector is then to collect the ionized molecules and generate an electric current proportional to the number of ions. The detector itself consists of an ion collector and an electronic unit for measuring and amplifying the electrical signal, from which mass information can be extracted through various processes.

Mass spectrometry analysis produces mass spectra, which is a graph showing the ions' intensity as a function of their m/z ratio (Figure 9). For small molecules, we often obtain monocharged ions, so determining molecular mass is straightforward (m = m/z - mass(H+) = m/1 - m(H+)).

For larger molecules, such as proteins, several sites can be ionized. For a given molecule, we thus obtain several ions with multiple different charges (more or less attached protons). On the mass spectra of the molecule of interest (example in Figure 9.a.), each "bar" therefore

corresponds to the ionized molecule with a different charge state (1H+, 2H+, 3H+ etc.), and its intensity is proportional to its relative abundance in the mixture. A special mathematical algorithm is required to determine the actual molecular mass of the compound from the m/z data. This process is known as deconvolution (Figure 9.b).

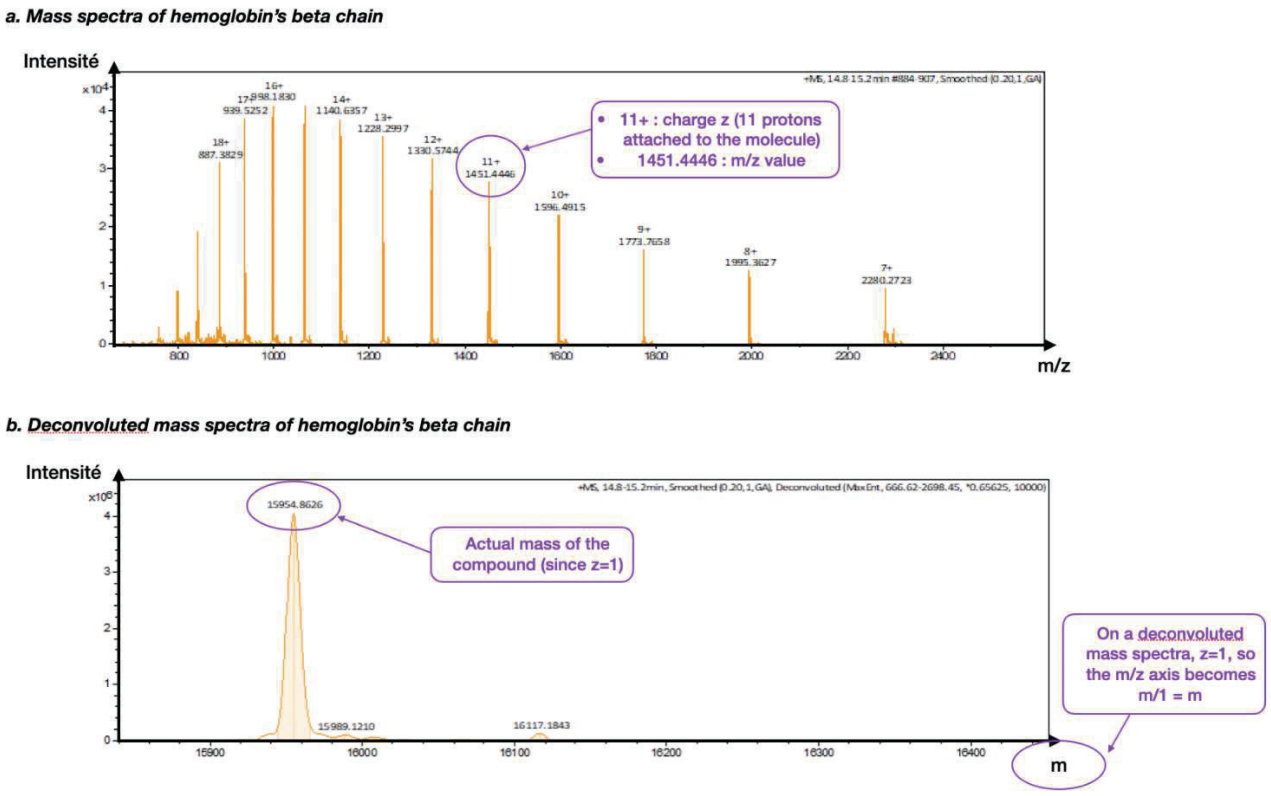


Figure 9. Mass spectra: example for hemoglobin. (a) Raw data showing the multicharged ions for the beta-globin chain. (b) Deconvoluted mass spectrum on the true mass scale after software transformation.

Obtaining the molecular mass of the protein of interest enables us to identify it, thanks to the existence of protein databases listing proteins and their characteristics (such as mass, amino acid sequence, etc.).

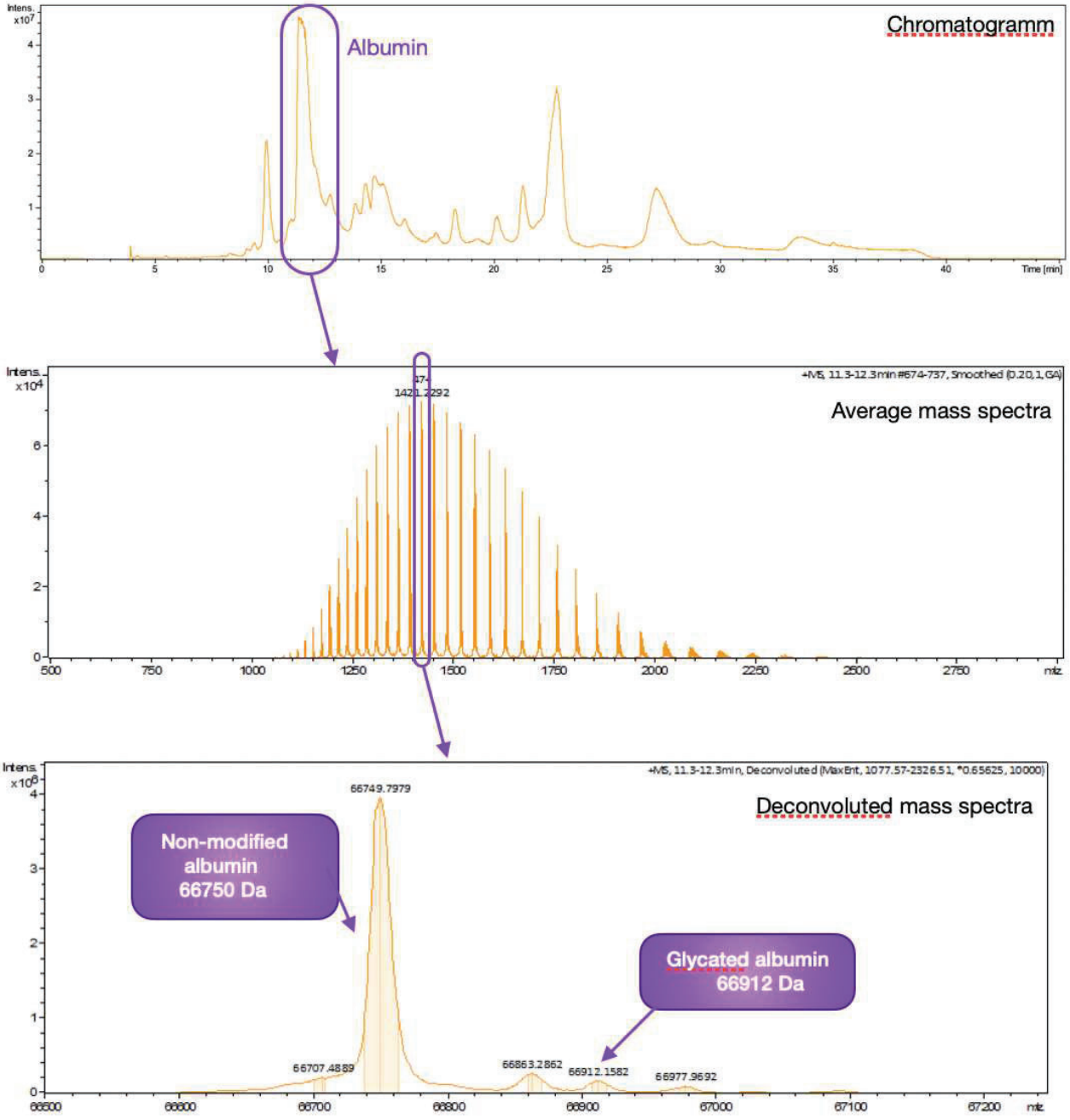


Figure 10. Glycated protein identification – Example with albumin. 1) select a peak on the chromatogram 2) extract the average mass spectrum 3) generate the deconvoluted mass spectrum 4) identify the major peak corresponding to the normal protein (without PTMs) and a secondary peak with a mass shift of +162 delta corresponding to the protein's glycated form.

2. Identification and Quantification of Glycated Proteins

As described previously, proteins analyzed using mass-spectrometry are identified on the basis of their molecular weight. When a glucose molecule binds to a protein during the first steps of the glycation reaction, the tertiary or quaternary conformation of the protein is altered, as is its mass. The mass of an hexose being 162 Da [210], adding a glucose molecule to a given protein will thus change its mass by +162 Da. A mass spectrometer is able to detect this mass difference, allowing us to determine if a protein is glycated or not.

To make the link with the LC-MS principle described above, the determination is carried out as follows (Figure 10):
First, a peak is selected on the chromatogram, from which the average mass spectrum is extracted. The signal is then smoothed, and a deconvolution algorithm applied. Based on the main peak corresponding to the unmodified protein, we then search for a secondary peak with a mass shift of +162 Da. If we detect one, it means that we have identified an Amadori compound (simple addition of one sugar to a protein). If we do not detect one, we consider this a “non-detection” of glycation for the considered protein.

By defining the “areas under the curves” for each protein form, we were able to quantify glycated proteins, i.e., evaluate the proportion of each form of a given protein. We tried to develop automated scripts to speed up the process, but these attempts failed due to the different (and unknown) molecular weights of the targeted proteins depending on the species studied. Considering the intensity of the signal obtained as a proxi of the abundance of protein species, we first extract the ion currents of the 10 most intense ions from the raw mass spectrum of the protein of interest without PTM. In doing so, we generate a chromatogram of extracted ions. We repeat the same operation for the glycated form of the protein. We thus obtain two extracted ion chromatograms (EIC) and integrate the area under the curve. We then calculate the ratio between these two areas, giving us a relative quantification of glycations (expressed as a percentage of glycated form for a given protein) (Figure 11).

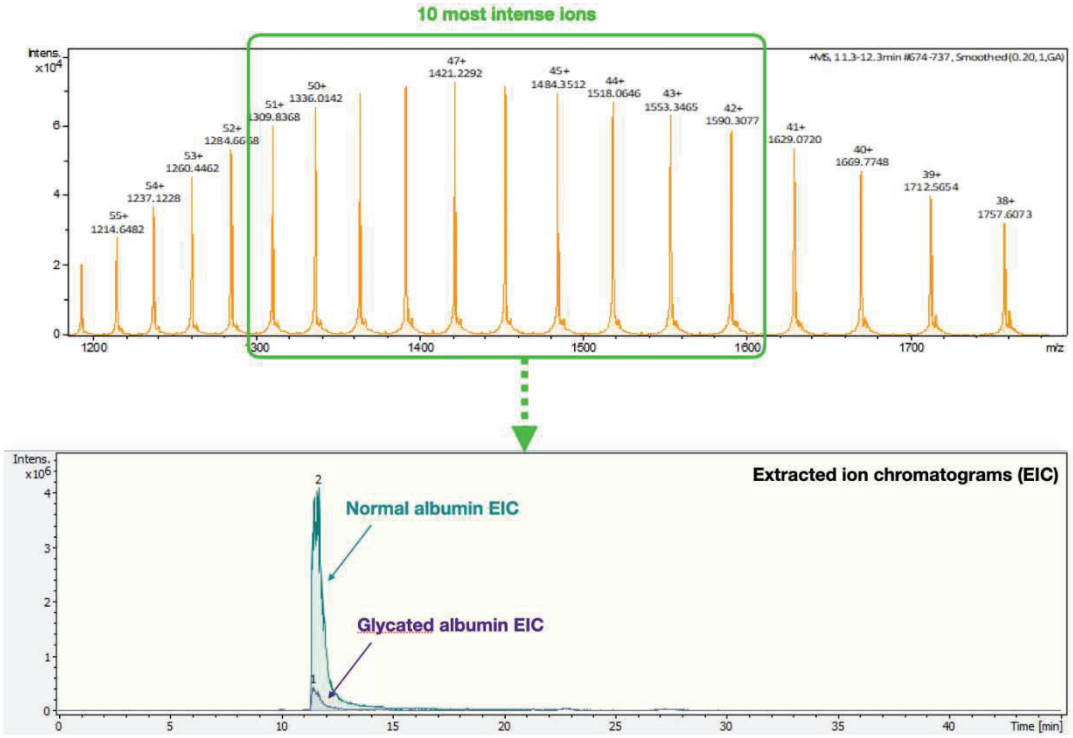


Figure 11. Glycated protein quantification. 1) select the 10 most intense ions on the average mass spectrum 2) generate the two extracted ion chromatograms and select the area under the curve 3) calculate the ratio between the two areas.

3. Instruments and Parameters Used in this Thesis

Glycated albumin and hemoglobin were assessed through LC-MS using an intact protein approach under denaturing conditions. To do so, 3 µL of plasma and 1 µL of red blood cells (whose intramolecular disulfide bonds have previously been reduced with TCEP and Guanidine chloride, see Appendix n°3 for the reduction protocol) were diluted in 22µL and 199µL of water containing 0,1% of formic acid, respectively. 5 µL of each were then injected on a high-performance liquid chromatography system (Agilent 1200 Series HPLC system, Agilent Technologies, Paolo Alto, USA) equipped with a C8 column maintained at 60°C (Vydac 208TP C8 HPLC column i.d. 2.1 × 250 mm, 300 Å, 5 µm particle size, Grace, Columbia, MD, USA). The HPLC system was coupled to a quadrupole-time-of-flight (Q-TOF) mass spectrometer equipped with an electrospray source (maXis II, Bruker Daltonik GmbH, Bremen, Germany) and operated in positive mode (for further technical details, see [87]). The acquired raw MS spectra were then processed through DataAnalysis v4.3 (Bruker Daltonik GmbH).

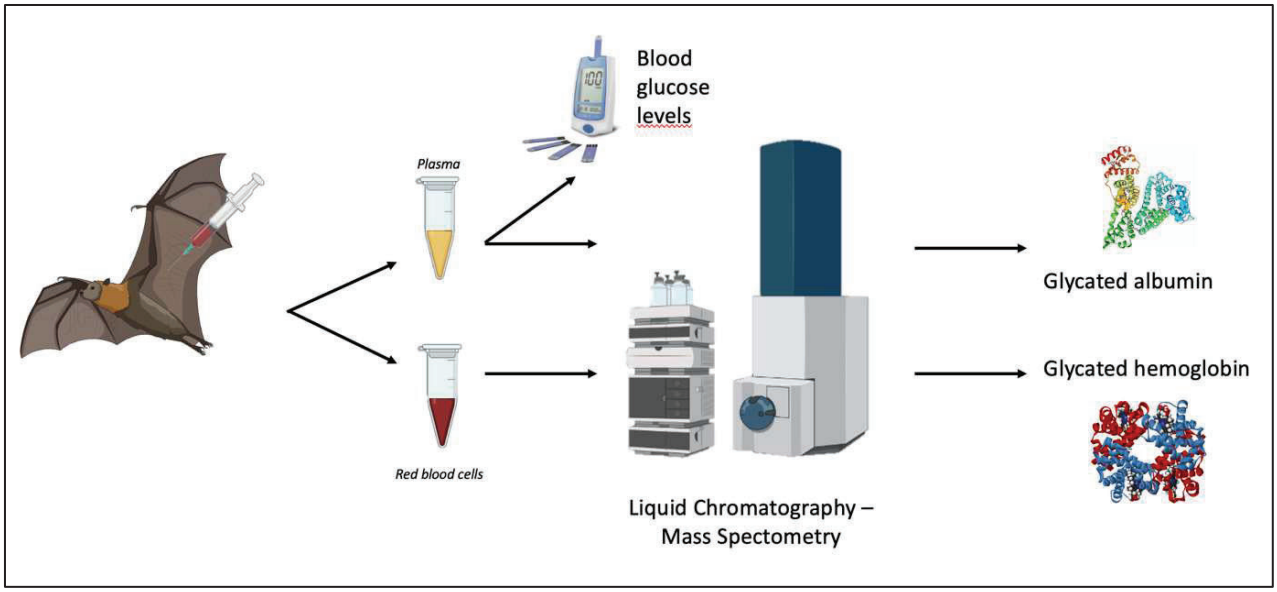


Figure 12. Schematic illustration of the sampling and the associated measurements made in each species

III. Other Biological Measurements

1. Glycemia

For each individual in each species (except for the two European bat species *M. daubentonii* and *N. noctula*, for which all plasma was used for LC-MS measurements), glycemia was measured on plasma samples using a Contour Plus® 115 portable glucometer (Ascensia diabetes solutions, Basel, Switzerland). The repeatability of the glucometer was evaluated on 43 individuals from different bird species, and for each individual the blood glucose measurement was repeated 3 times. The mean coefficient of variation for all these individuals was of 9.86 ± 12.25. To assess measurement accuracy, we made a comparison with measurements from a colorimetric glucose assay kit (absorbance measurement), on the same samples as for repeatability assessment. The linear regression equation between glycemia measurements made with the two techniques was 0,8138x + 34,888 and $R^2 = 0.77$.

2. Oxidative Stress

Oxidative stress markers were measured in two bat species, *Pteropus rodricensis* and *Rousettus aegyptiacus*. These are described in the Materials & Methods section of Chapter 2 of this manuscript (p. 98).

3. Hematological and Immune Parameters

Several hematological and immune parameters were measured as part of the roe deer study. A detailed description is available in the Materials & Methods section of Chapter 3 of this manuscript (p. 130).

CHAPTER 1 - Exploring Glycation and Life-History Traits Associations: Comparative Insights from Two Interspecific Analyses

Juvenile Bolivian Squirrel Monkey Saimiri b. boliviensis (Aquarelle by Cyrielle Duval)

I. Introduction

According to the pace-of-life (POL) hypothesis, an organism's life history co-evolved with specific physiological and behavioral traits, collectively termed pace-of-life syndromes (POLS), as an adaptive response to particular ecological constraints [211,212]. POLS are defined along several axes [213], with the “fast-slow continuum”, initially described by Stearns in 1989 [214], emerging as the principal axis of life-history variation among vertebrates [215]. Organisms exhibiting a slow pace-of-life are characterized by larger body mass, slower growth rates, delayed reproduction, infrequent reproductive attempts, fewer and larger offspring per litter, and greater longevity [214,216]. Conversely, species at the “fast” end of this continuum exhibit the opposite traits.

It has been suggested that the longer lifespans observed at the slower end of the continuum may be due to slower rates of senescence. In both mammals and birds, faster senescence rates correlate positively with earlier reproduction, higher fecundity, and shorter generation lengths, while senescence onset is delayed in species with slower POLS [217]. Additionally, the relatively lower senescence rates in birds compared to mammals may be attributable to slower life histories given similar metabolic rates [217]. Some physiological traits associated with this continuum include metabolic rate, which tends to be higher in species with faster life histories and lower in those with slower life histories [218–220]. Basal glycemia has been identified as a significant physiological component of POLS in birds, demonstrating close negative or positive correlations with some variables (like body mass or reproductive investment, respectively) characterizing the fast-slow continuum, potentially even more so than basal metabolic rate (BMR) [75]. However, such studies in mammals remain scarce.

Given the significant role of glycation in the senescence process in some mammalian species, such as humans, it is pertinent to investigate whether different levels of sensitivity to glycation might also be a key physiological variable that may covariate along the POLS, analogous to the role of glycemia in birds [75,84]. However, as discussed in the general introduction of this manuscript, there is a notable lack of research on the relationship between glycation and life-history traits in non-model animal species [221]. To date, only three studies in birds have

explored the association between GHb levels and few (but main) traits defining POL, i.e., reproductive and survival proxies [89–91]. Furthermore, research on the relationship between glycation and maximum longevity is limited. To the best of our knowledge, only one study investigated the link between maximum longevity and the rate at which pentosidine, a biomarker for AGEs, is formed in mammalian species [100], and a second one in birds which looked at glycated albumin [222]. Both studies highlighted divergent results, with a negative correlation for the first one, and an absence of correlation for the second one. Similar gaps exist regarding the relationship between body mass—one of the major traits defining the fast-slow continuum[93]—and glycation [87,89,94,95] (see General Introduction p.13).

A notable observation is the predominance of studies focusing on birds. This emphasis is understandable given that many bird species have basal glycemia levels that would be considered hyperglycemic in humans [223], yet they exhibit long lifespans and lower senescence rates compared to mammals of similar mass and/or BMR
[224–228]. Nevertheless, this focus may overlook a mammalian group with similar characteristics: bats. Bats challenge the general life-history patterns observed in mammals. Despite their small body mass and high metabolic rates, bats display exceptionally long lifespans [74,229], up to three times greater than those of comparable-sized mammals [219], which contradicts the rate-of-living theory. For instance, the Brandt's bat (*Myotis brandtii*) has been documented to live up to 41 years in the wild [230]. Despite their small size, bats exhibit life-history traits typically associated with large, long-lived mammals [231], such as producing few, large offspring [232,233] that require extended gestation periods [234], and showing slow growth and sexual maturation rates [231,235,236]. Interestingly, bats exhibit trade-offs between longevity and reproduction, with species that have higher reproductive rates and earlier sexual maturation often displaying reduced lifespans [231,237], aligning with the disposable soma theory of aging. While flight is widely recognized as a major factor contributing to their extended longevity by reducing predation and non-condition-dependent mortality risks [238,239], bats also possess unique adaptations that mitigate senescence mechanisms prevalent in other mammals [240]. These adaptations include reduced oxidative stress [241–245], specialized DNA repair mechanisms [246,247], and enhanced protein homeostasis [184,248,249]. Recent research has also highlighted that nectarivorous and frugivorous bats do not seem to suffer from the adverse effects of a sugar-rich diet usually

observed in humans or domestic animals (such as chronic hyperglycemia or reduced lifespan) [107,116,117]. In this context, studying glycation in bats, particularly in comparison with other mammals, presents a compelling avenue for further research both in the evolution or medicine fields.

The questions and analyses in this chapter were carried out in two stages.
Our first objective was to determine if, among different mammal species, glycation levels are influenced by pace-of-life, body mass and glycemia, as well as if they differ between bats and other mammals (i.e., flying vs. non-flying mammals). We also checked whether glycemia could be influenced by the pace-of-life or body mass among our mammals. Bats being exceptionally long-lived for their body mass, our hypothesis was that glycation levels would be lower in bats than other mammals. We also expected mammals with slower pace-of-life and longer longevity to have lower glycation levels compared to mammals with faster pace-of-life and shorter longevity.

In a second step, we wanted to broaden our questioning to flying vertebrates in general, by comparing glycation between bats and birds of similar mass and diet. We first checked if glycemia differed between the two classes, and if it could be explained by diet or maximum lifespan. Our hypothesis was that bats and birds sharing many similarities in terms of mode of life or resistance to senescence, glycation levels would not significantly differ between them. Based on previous studies on glycemia in birds and bats [250,251], we however expected insectivorous species to have more glycations than frugivorous ones. Lastly, we were anticipating longer-lived species to exhibit lower glycation levels than shorter lived ones, independently from the class.

II. Materials & Methods

1. Species & Sample Collection

a) Mammals

All the mammal species described in the General Materials and Methods section of this manuscript were used in this study. To summarize briefly, we used data from nine mammal species: five Chiroptera species and four non-flying mammal species. Three of the bat species are frugivorous (Egyptian rousette *Rousettus aegyptiacus*, Rodrigues flying fox *Pteropus rodricensis* and Seba's short-tailed bat *Carollia perspicillata*) and the other two are insectivorous (Daubenton's bat *Myotis baubentonii* and Common noctule *Nyctalus noctula*).

As for non-flying mammals, we had access to three terrestrial species and one marine species. Among the terrestrial species, we worked with two African diurnal rodent species, the Soudanian unstriped grass rat (*Arvicanthis ansorgei*) and the Four-striped grass mouse (*Rhabdomys pumilio*), and one European artiodactyl, the roe deer (*Capreolus capreolus*). The marine mammal species involved in this study is the Southern elephant seal (*Mirounga leonina*), which belongs to the order Carnivora. More details on the provenance of the species and the sampling method can be found in the General Materials and Methods section.

b) Birds

To compare differences in terms of glycated albumin in bats and birds, we selected five bird species of similar diets and mean adult mass (range from 12 to 360g) as our bat species (mass range from 8.5g to 350g). We selected three predominantly insectivorous species (*Sylvia communis, Tachymarptis melba* and *Acrocephalus scirpaceus*) and two predominantly frugivorous species (*Leucopsar rothschildi* and *Musophaga rossae*). All data on birds were taken from a recent study from Moreno-Borrallo et al. [222]. Apart from *Tachymarptis melba* (which were issued from a wild population in Switzerland [252,253]), all data for the other species were obtained from captive populations reared at the Mulhouse Zoo (France). Blood samples were collected from the brachial or tarsal vein, or from the feet in the case of swifts, using heparin-lithium as anticoagulant. More information on the provenance of bird samples is available in the original study [222].

Table 3. Mean glycated albumin levels, glycemia and life-history traits values for the nine sampled mammal species used in the first part of this chapter (dataset mammals).

Species	GA (%)	Glycemia (mg/dL)	Adult body mass (g)	Maximum Lifespan (years)	Gestation Time (days)	Female sexual maturity (days)	Nb litters/ year	Nb Pups/ litter
Pteropus rodricensis	13.98	140.07	350	28	198	755	1	1
Rousettus aegyptiacus	10.09	149.65	125	22.9	116	365	2	1
Myotis daubentonii	17.84	*160 [97]*	8,5	28	54	730	1	1
Nyctalus noctula	15.39	*115.2[254]*	27.75	12	60	90	1	1,5
Carollia perspicillata	8.20	64.27	15	17	95	258	2	1
Arvicanthis ansorgei	17.46	174.33	95.8	6,7	22	51.89	3.5	5
Rhabdomys pumilio	NA	92.59	51	4,5	25	42	4	6
Capreolus capreolus	26	183	21670	17.5	153	413	1	1.6
Mirounga leonina	23.2	128.6	*500000[255]*	25	220	1059	1	1

Unless otherwise specified in the table (in italics and corresponding source in brackets), GA levels and glycemia per species were calculated as the mean from the individual data, and life-history data were obtained from the The Animal Ageing and Longevity Database (AnAge) [193,194].

Table 4. Mean glycated albumin levels, glycemia and life-history traits values for the ten bat and bird species used in the second part of this chapter (dataset flying vertebrates).

Species	Class	GA (%)	Glycemia (mg/dL)	Adult body mass (g)	Maximum Lifespan (years)	Diet
Pteropus rodricensis	Bat	13.98	140.07	350	28	Frugivorous
Rousettus aegyptiacus	Bat	10.09	149.65	125	22.9	Frugivorous
Myotis daubentonii	Bat	17.84	*160.00 [97]*	8,5	28	Insectivorous
Nyctalus noctula	Bat	15.39	*115.2[254]*	27.75	12	Insectivorous
Carollia perspicillata	Bat	8.20	64.27	15	17	Frugivorous
Sylvia communis	Bird	16.32	328.60	15,1	12.6	Insectivorous
Tachymarptis melba	Bird	24.87	350.45	100,38	26	Insectivorous
Acrocephalus scirpaceus	Bird	20.00	332.50	12.3	19.08	Insectivorous
Leucopsar rothschildi	Bird	18.89	488.00	91.3	30	Frugivorous
Musophaga rossae	Bird	19.66	262.85	361.42	28	Frugivorous

Unless otherwise specified in the table (in italics and corresponding source in brackets): GA levels and glycemia per species were calculated as the mean from the individual data, life-history data for mammals were obtained from the Animal Ageing and Longevity Database (AnAge) [193,194], and life-history data for birds were obtained from Moreno-Borallo et al. [222].

2. Data Collection & Formatting

a) Data Collection

Glycemia and glycated protein levels were measured following the protocol described in the general Materials & Methods section of this manuscript. As it will be shown in the Results section, we focused solely on glycated albumin (GA) as, surprisingly, glycated hemoglobin remained undetectable in most mammal species other than bats.
Unless otherwise specified (see Tables 3 and 4), GA levels and glycemia per species were then calculated as the mean from each species individuals' data.

The following life-history traits were considered: adult body mass, number of litters per year, number of pups per litter, gestation time, female age at sexual maturity and maximum lifespan. When available, life-history data were obtained from the The Animal Ageing and Longevity Database (AnAge) [193,194]. Where data was not available on the latter, we retrieved data from various previous studies (see Table 3 and 4).

b) Datasets Creation

Two different subsets of data were created, one for each of the two parts of this study. For the first part focusing solely on mammals, we included the nine mammal species described earlier, and used mean values per species for life-history traits, glycemia and GA levels (Table 3).
The second dataset for the flying vertebrates' analysis included the five bat species and the five bird species described previously. As for the first part of the study, we considered mean values per species for body mass, GA levels, maximum lifespan, as well as each species' diet and taxa (bats vs birds) (Table 4).

3. Statistical Analysis

All statistical analyses were carried in R (version 4.3.1) [256]. Because we wanted to account for the phylogenetic relationship between species in our analysis, we decided to use Phylogenetic Generalized Least Square (PGLS) models [257], using the *pgls* function from the *caper* package [258].

Two phylogenetic trees were built using the tree generator on Time Tree 5 [259] (Figures 13 and 14). Because we only wanted to account for the relationships among the taxa, and not the directionality of the evolutionary changes, we chose to not root our phylogenetic tree [260], freeing us from a potential bias that could have been caused by choosing a wrong or too distant outgroup [261,262].

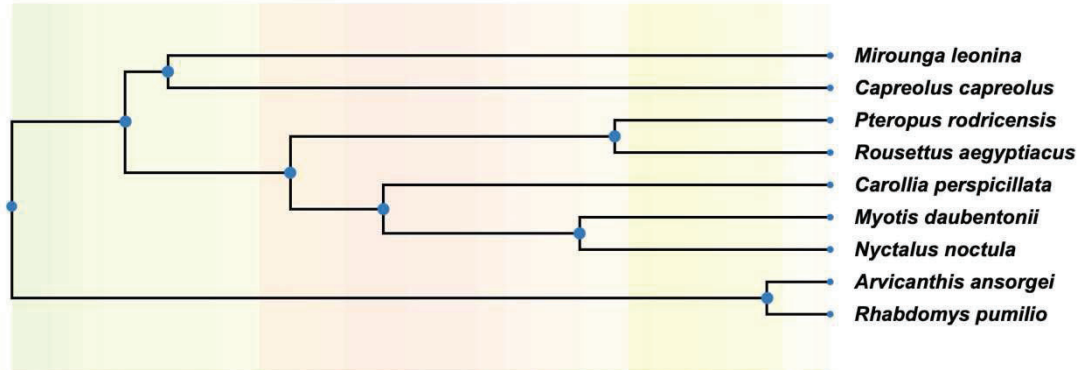


Figure 13. Phylogenetic tree for the nine mammal species used in the first part of this study

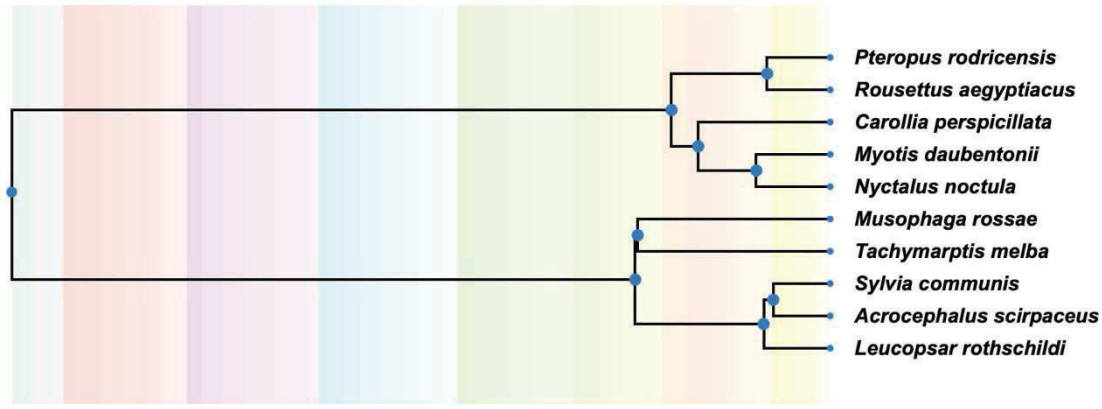


Figure 14. Phylogenetic tree for the bat and bird species used in first second of this study

We did not detect any glycated albumin in four-stripped grass mice. We however wanted to include this species in our analysis, because having a supplementary species would allow us to have more statistical power, and because we had data for glycemia and all life-history traits for this species. We therefore decided to impute the single GA missing value in *R. pumilio* through a PCA procedure thanks to the *missMDA* package [263].

For the analysis focusing on mammals only, a preliminary exploration of the data revealed that several life-history traits were highly inter-correlated. We thus conducted a Principal Component Analysis (PCA) on life-history traits and body mass, which additionally allowed us to reduce the number of variables to include in our model, thus avoiding overfitting. Glycemia was not included in the PCA, since it did not highly correlate with the other variables. We then retained the principal components (PCs) whose eigenvalues were greater than 1 to be included in our models.
Our first PGLS model (model 1, see Table 5) included glycemia as the dependant variable, and both PCs as explanatory variables, along with a "bat (yes/no)" factor.
In our second PGLS model (model 2, see Table 5), GA levels was the dependant variable, and both PCs and glycemia were entered as fixed factors, along with a "bat (yes/no)" factor.

For the second analysis on flying vertebrates, we conducted two PGLS models. The first model included glycemia as dependant variable, and diet, adult body mass and maximum lifespan as explanatory factors. Since we wanted to test for differences among bids and bats, we included the two-way interactions between a "taxa (bats/birds)" factor and diet and maximum lifespan. We did not include a *body mass x taxa* interaction, since we purposely choose species of similar masses in both taxa. Therefore, body mass was left as a single fixed factor in the model (model 3, see Table 5). The same model was then conducted with GA levels as independent variable, except that we added a third interaction (*Glycemia x taxa*) to the model, to test for differences between birds and bats in terms of relationships between GA and glycemia (model 4, see Table 5). Both full models then underwent a manual backward selection procedure.

The final models retained were the ones with all variables showing significance at a 5% level or a tendency at a 10% level. Additionally, we checked that among all models during the selection process (initial full one, in-between ones and last best one), the one which was being

retained had the best fit (evaluated through the adjusted R^2). We also had a look at the corrected Akaike Information Criterion (AICc) (which avoids data overfitting through correction for small sample sizes, [264] of all models, and made sure that the best-selected model was included among the competing models with an AICc difference below 2.
The relative effect of the phylogenetic tree on the PGLS model could be estimated as a λ parameter, ranging between 0 (covariation among species measurements is independent of co-ancestry) and 1 (covariance entirely explained by co-ancestry).

The normality and homeodasticity of residuals of all the retained models were verified through standard residual plot techniques along with a Shapiro-Wilk normality test, and the goodness-of-fit through calculating the conditional (total variance explained by the best model) and marginal (variance explained by fixed effects alone) R^2.

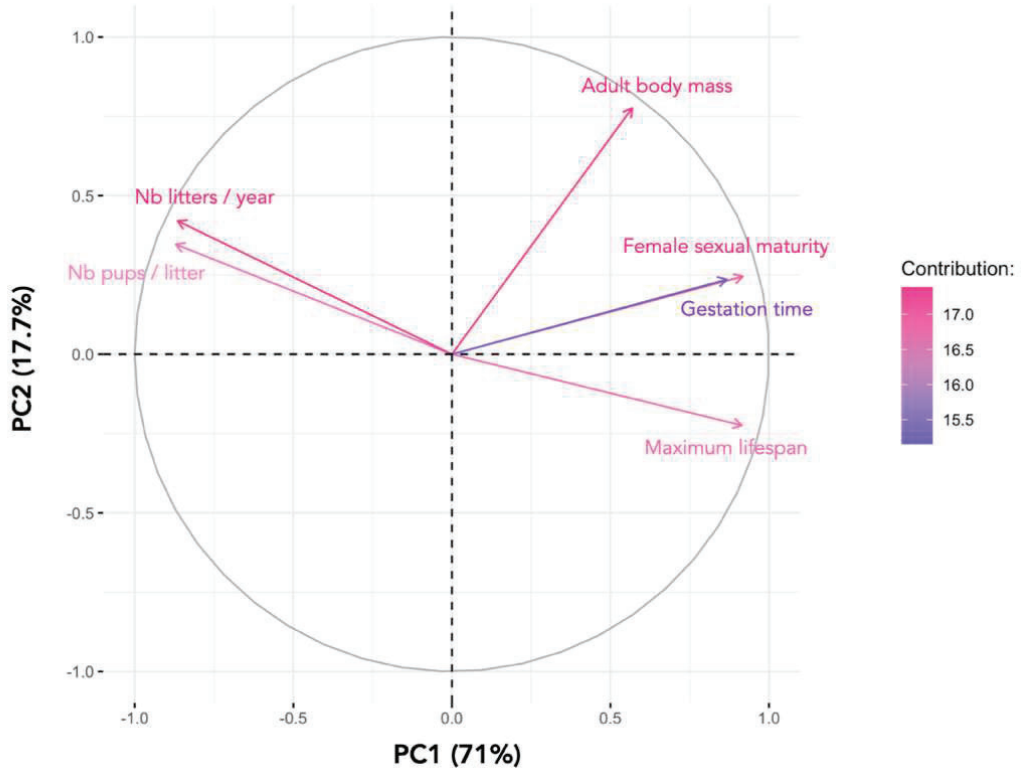
All initial models and their corresponding datasets are summarized in the following table (Table 5).

Table 5. List of initial full models (and their corresponding datasets) tested in this chapter.

	Initial full model	Dataset
Model 1	Glycemia ~ PC1 + PC2 + Bat (yes/no)	Dataset mammals
Model 2	GA ~ PC1 + PC2 + Glycemia + Bat (yes/no)	Dataset mammals
Model 3	Glycemia ~ Diet*Class + poly(Maximum lifespan,2)*Class + Body mass	Dataset flying vertebrates
Model 4	GA ~ Diet*Class + poly(Maximum lifespan,2)*Class + Body mass + Glycemia*Class	Dataset flying vertebrates

Poly(Maximum lifespan, 2) stands for a quadratic relationship.

(a)



(b)

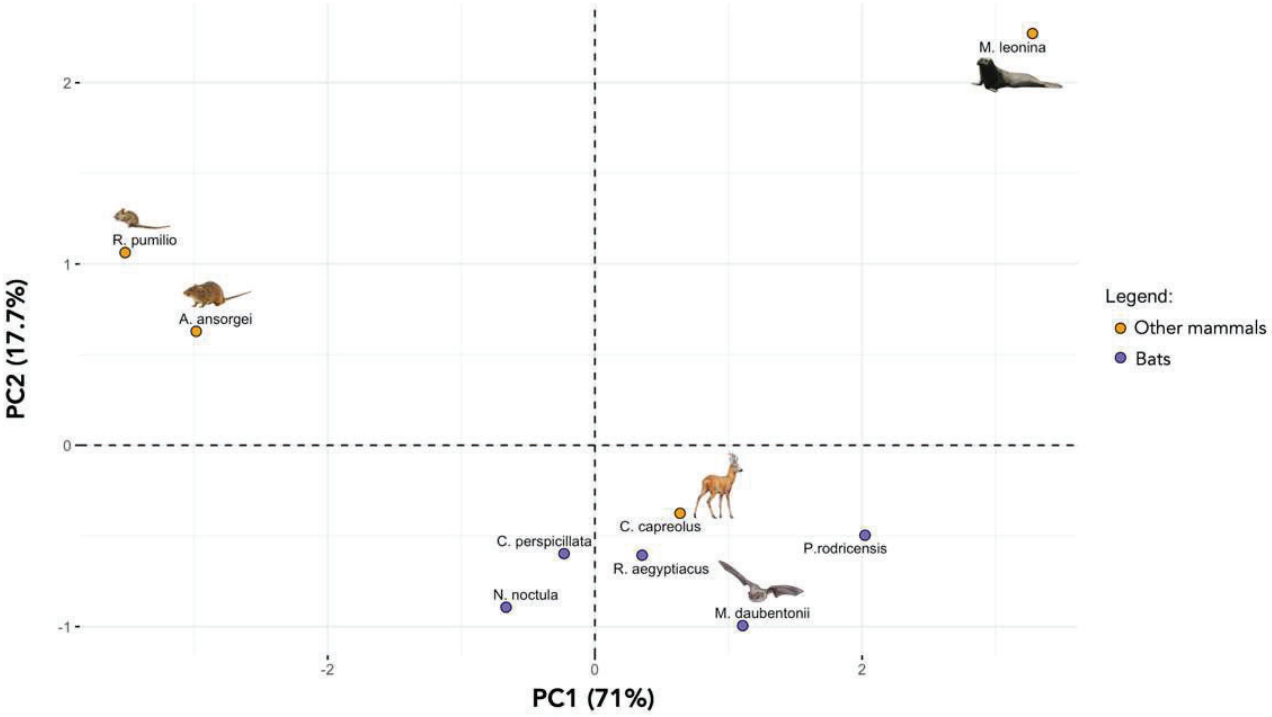


Figure 15. Principal Component Analysis for six life-history traits in nine mammal species – (a) Correlation circle showing the projection of all variables on principal components 1 (PC1) and 2 (PC2). (b) Projection of individuals on the first two principal component

III. Results

1. Links Between Glycated Albumin Levels, Pace-of-Life, Body Mass and Glycemia Among Mammals

a) Principal Component Analysis on Life-History Traits

The PCA carried out on the five life-history traits and body mass led us to retain the two first principal components PC1 and PC2, which respectively explained 70.99 % and 17.75 % of the total variance of the dataset. Female age at sexual maturity, maximum lifespan, number of litters per year, gestation time and number of pups per litter were the variables contributing most to PC1 (19.76%, 19.64%, 17.81%, 17.64% and 17.56%, respectively), while adult body mass (and number of pups per litter to a lesser extent) was the main contributor to PC2 with 56.57% (16.58% of contribution for number of pups per litter). Female age at sexual maturity, maximum lifespan and gestation time strongly positively correlated with PC1 (r= 0.92, 0.92 and 0.87 respectively), while number of pups per litter and number of litters per year strongly correlated negatively with this same component (-0.87 for both variables) (Figure 15.a.). PC1 therefore reflects the pace of life of species, independently of the body mass effect, with increasing positive values indicating a slower pace of life (longer lifespan and lower reproductive output) and increasing negative values pulling towards the fast end of the continuum (lower lifespan and increased reproductive output). On the other hand, adult body mass strongly positively correlated with PC2 (r=0.78), while number of pups per litter also correlated positively with PC2 but to a lesser extent (r=0.42). While it appears that PC2 is mainly driven by body mass, it also slightly considers the reproductive output of the species.

When looking at species' ranking along PC1 (Figure 15.b.), we can see that rodents and southern elephant seals are positioned at opposite extremes, the first ones exhibiting high negative values (< or = -3) highlighting a fast pace-of-life, and the second one having a high positive value (>3) corresponding to a slow pace-of-life. Bats are grouped rather around 0 with a tendency to positive values (ranging from -0.7 to 2), suggesting a rather moderate or slow pace-of-life. Interestingly, roe deers are positioned among the bat group, suggesting a comparable pace-of-life that pulls towards the slow end (0.7). While the southern elephant

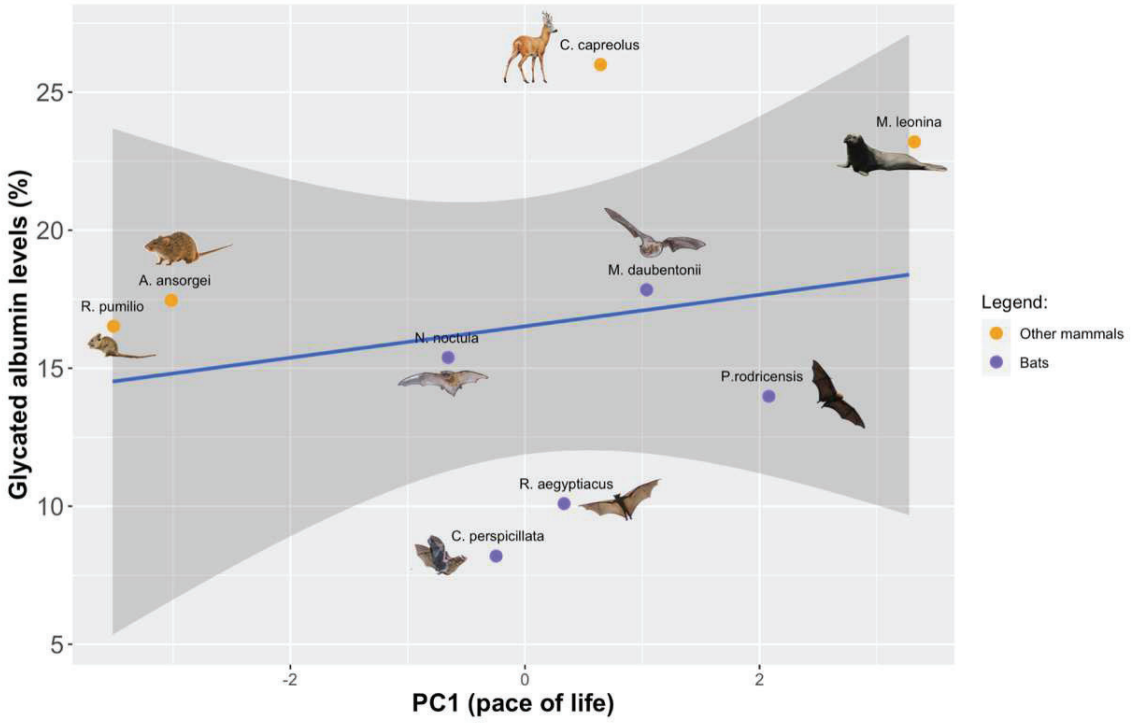


Figure 16. **Relationship between glycated albumin levels and PC1 (representing pace-of-life) in mammals** (n=9). Faster pace-of-lives are located to the left of the PC1 axis, while slower ones are situated to the right. Points represent species.

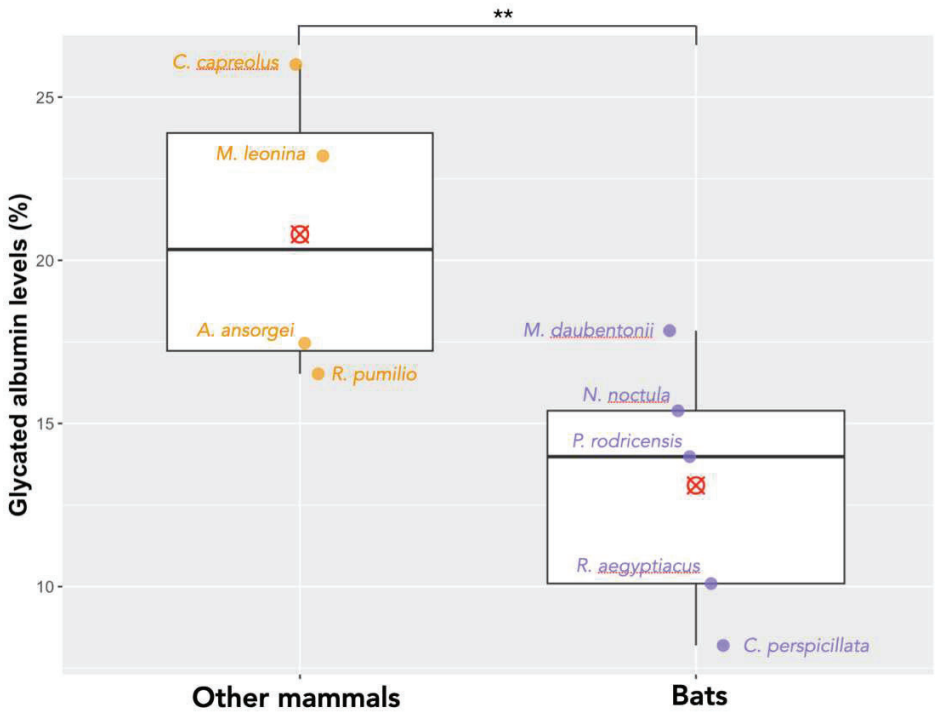


Figure 17. **Boxplot of glycated albumin in bats and other mammals**. Points represent species, red crossed circles represent mean GA level in bats and other mammals respectively.

seal exhibits the highest values on PC2 (>2), which is consistent with their mass being much greater than that of the other species in our dataset, we can see that rodents are ranked higher than bats and, more surprisingly, than the roe deer. Despite having a superior adult body mass (which is balanced by litter size), *C. capreolus* is again placed on a pair with bats on PC2.

b) Variation of Glycated Albumin Levels with Principal Components and Glycemia

The PGLS model testing for the effects of PC1, PC2 and glycemia on glycated albumin levels in our nine mammal species (model 2, see Table 5) revealed that, as we anticipated, bats had significantly less glycated albumin than other mammals (p=0.00773) (Figure 17). Contrary to our expectations, PC1 was found to show a slight positive correlation with GA levels (p=0.02944), suggesting that species pulling towards a slower pace-of-life also exhibit slightly higher GA levels than faster lived species (Figure 16). PC2 did not significantly explain glycation levels, but it however showed a tendency towards lighter species with lower reproductive output to exhibit more glycated albumin (p=0.09897). Finally, there was no correlation between GA levels and glycemia. λ was not significantly different from 0 or 1 (Table 6), thus not indicating us if the observed relationships are dependent or not of the phylogenetic links between our species. Concerning glycemia (model 1, see Table 5), the final selected model only retained the "bat (yes/no)" factor as explanatory variable but was found not significant (p= 0.5056055).

Table 6. Parameters of the selected PGLS model explaining glycated albumin levels in mammals (n=9)

	Estimate	SE	p-value	λ_{ML}	λ=0	λ=1
Intercept	24.66	2.12	8.27 $e^{-0.5}$ ***	0	p=1	p=0.48
PC1	1.56	0.52	0.03*			
PC2	-3.19	1.58	0.098 ·			
Bat [Yes]	-14.66	3.41	0.008 **			

Adjusted R^2 = 0.7434; P < 0.10; * p < 0.05; ** p < 0.01; *** p < 0.001
Lambda estimated by maximum likelihood (λ_{ML}) represents phylogenetic dependence level varying from 0 (total phylogenetic independence) to 1 (total phylogenetic dependence).

2. Comparison Among Flying Vertebrates

a) Birds Have Higher Glycemia Than Bats of Comparable Diet, Body Mass And Longevity

In testing for the differences between bats and birds in terms of the effects of maximum lifespan, adult body mass and diet on glycemia, it turned out that birds have higher glycemia compared to bats (p=0.0005337) (Figure 18), but that none of the other factors significantly explained glycemia in those two taxa. The model highlighted that this result was not fully explained by the phylogenetic relationships among the species, as suggested by λ which was significantly different from 1 (p=0.0035678), but not from 0 (Table 7).

Table 7. Parameters of the selected PGLS model explaining glycemia levels in flying vertebrates (n=10)

	Estimate	SE	p-value	λ_{ML}	λ=0	λ=1
Intercept	125.84	28.82	0.0024 **	0	p=1	p=0.0036
Taxa [Birds]	226.64	40.75	0.00053***			

Adjusted R^2 = 0.7688; ** p < 0.01; *** p < 0.001
Lambda estimated by maximum likelihood (λ_{ML}) represents phylogenetic dependence level varying from 0 (total phylogenetic independence) to 1 (total phylogenetic dependence).

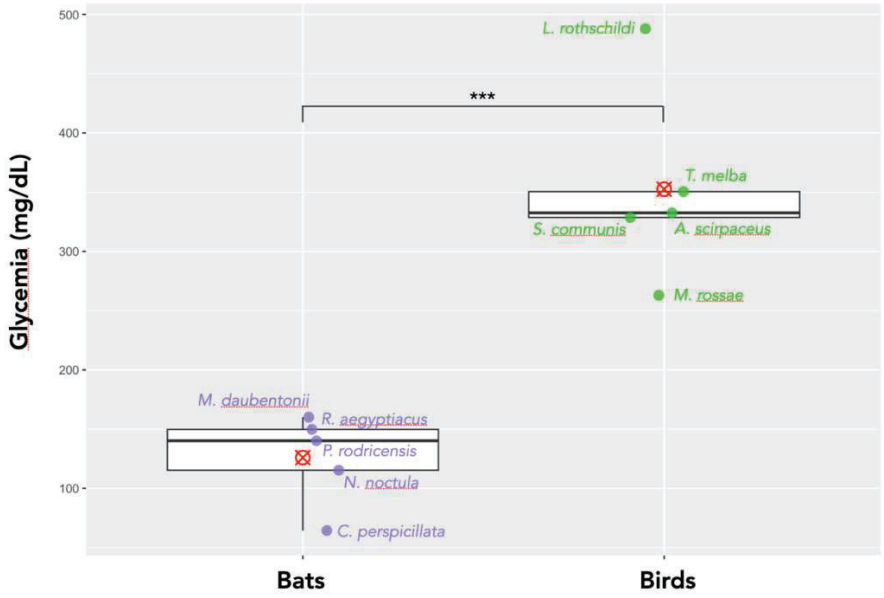


Figure 18. **Boxplot of glycemia in bats and birds**. Points represent species, red crossed circles represent mean GA levels for each class.

b) Glycated albumin levels comparison between birds and bats

Among all the variables tested to explain GA levels among flying vertebrates, only glycemia did not emerge from the model (Table 8). Insectivorous species had significantly more glycated albumin than frugivorous ones, both taxa together ($p=4.163e^{-05}$) (Figure 19). For both taxa combined, GA levels also appeared to be positively correlated with adult body mass (p=0.003671) (Figure 20). Lastly, the interaction between maximum lifespan and taxa stood out, showing that for a same maximum lifespan, birds had more GA than bats (Figure 21).

Table 8. Parameters of the selected PGLS model explaining glycated albumin levels in flying vertebrates (n=10).

	Estimate	SE	p-value	λ_{ML}	λ=0	λ=1
Intercept	4.78	0.79	0.004 **	0	p=1	p=0.015382
Diet [Insectivorous]	8.03	0.41	$4.16\ e^{-0.5}$ ***			
Adult body mass	0.0097	0.0016	0.0037 ** ·			
Maximum lifespan	0.19	0.036	0.0064 **			
Taxa [Birds]	-3.51	1.17	0.04 *			
Maximum lifespan x Taxa [Birds]	0.36	0.049	0.0018 **			

Adjusted R^2 = 0.9911; P < 0.10; * p < 0.05; ** p < 0.01; *** p < 0.001
Lambda estimated by maximum likelihood (λ_{ML}) represents phylogenetic dependence level varying from 0 (total phylogenetic independence) to 1 (total phylogenetic dependence).

Figure 19. **Boxplot of glycated albumin levels depending on the diet**. Points represent classes, red crossed circles represent mean GA levels for each diet.

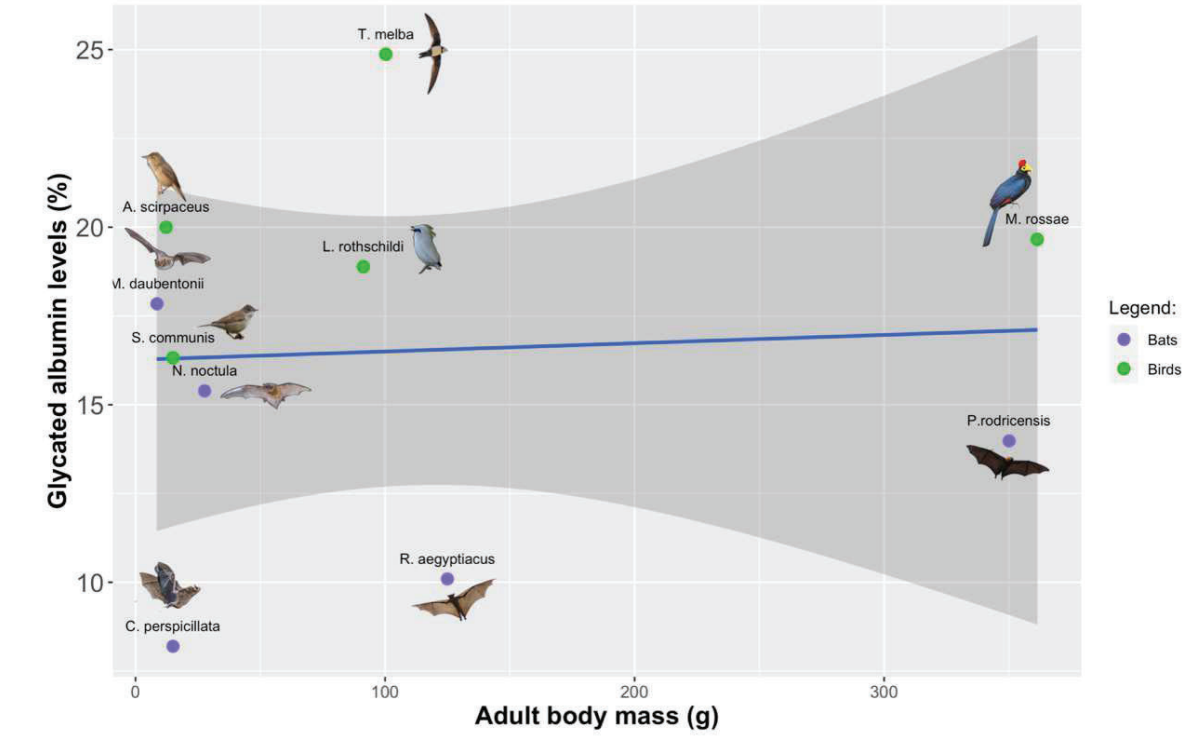


Figure 20. **Relationship between glycated albumin levels and adult body mass in flying vertebrates** (n=10). Points represent species.

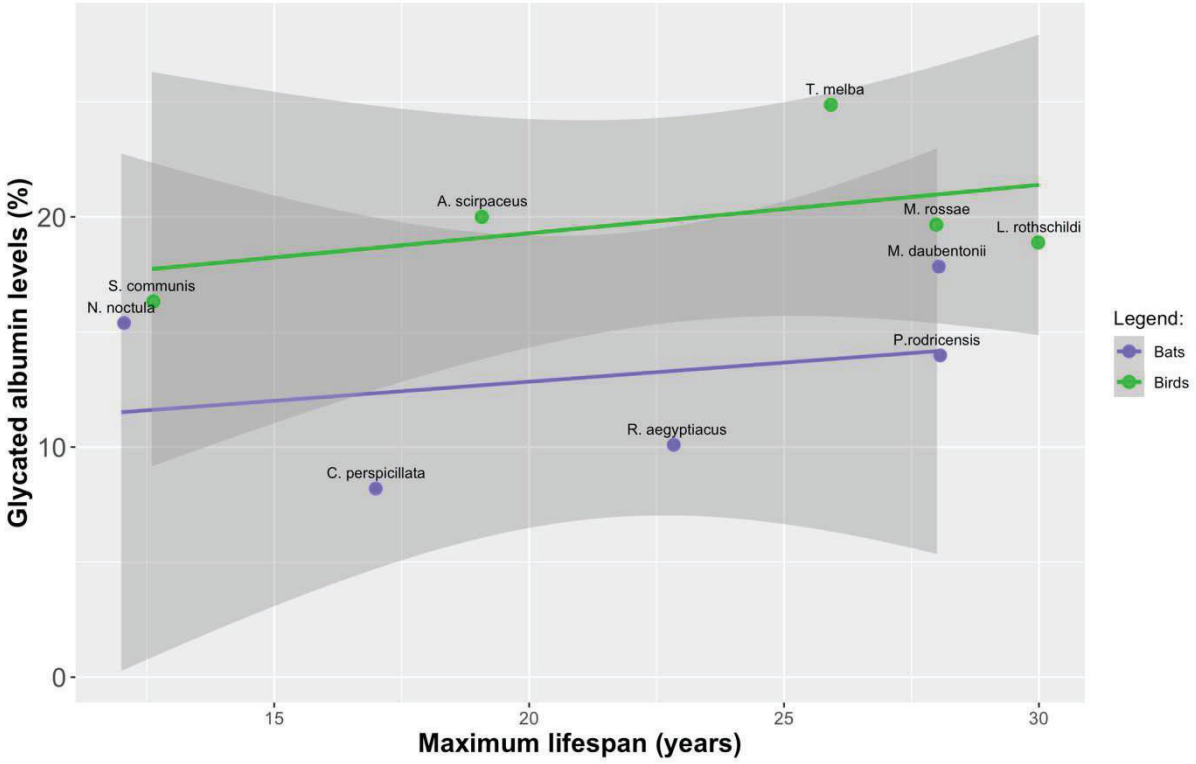


Figure 21. **Relationship between glycated albumin levels and maximum lifespan in each taxa** (n=10). Points represent species.

IV. Discussion

1. Comparative Analysis of Glycated Albumin Levels in Mammals

a) Variable Tolerance to Protein Glycation Among Mammal Species

We have shown that glycated albumin levels do not correlate with glycemia at the interspecific scale in mammals. This result may seem surprising at first sight, the glycation reaction being spontaneous and thus it should increase along with rising blood glucose levels. This is indeed the case in human. However, some studies highlighted that this relationship is not verified in some bird species, such as the zebra finch [87], or in our case in mammals, i.e., the roe deer (see Chapter 3) or bats (*R. aegyptiacus* and *R. rodricensis*, see Chapter 2). This absence of positive relationship at the interspecific scale thus suggests that all mammal species do not react in the same way to glucose and do not lead to the same levels of glycation. The correlate is that there are some species probably better protected than others against the adverse effects of glucose. Indeed, the absence of a systematic increase in glycated albumin levels with a higher glycemia in the context of a non-enzymatic reaction may suggest several possible scenarios. It may be that the initial stage of the reaction (attachment of sugar to protein) does not take place (which we will call “glycation resistance”), or that there are mechanisms for regulating and degrading glycation products once formed, which explain why their levels are not higher despite high blood glucose levels (which may be termed “glycation tolerance”). Glycation tolerance may also encompass species with high glycation levels, which form and accumulate glycation without any deleterious effect on at least their longevity. Potential explanations for this apparent resistance and/or tolerance at the species level may include a quick utilization or storage of circulating blood glucose right after ingestion, making it unable to be glycated [107,116]. Another possibility could be variations in the amino acid sequence or conformation of albumin, which could affect the accessibility of glycation sites (as previously shown in chicken [155,156]). Unfortunately, albumin sequences are not available for the species we included in our comparative analysis, apart from the Egyptian Rousette. We were therefore able to look at whether any notable sequence differences existed between the albumins of this fruit bat, human and chicken. This is presented in Chapter 2 of this manuscript, dedicated to bats. It could also be argued that some species present more efficient glycation

regulatory mechanisms, such as cleaning of early glycation products before they turn into non-degradable AGEs. Finally, glycation tolerance can also be attributed to a blockade of AGE pro-senescence signaling pathways, making the formation and/or accumulation of AGEs ultimately less harmful to the organism. The absence of AGE receptors is the main hypothesis for such a tolerance in birds ([177], but see [179,180]).

b) An Increased Protein Glycation Tolerance Correlates with a Slower Pace-Of-Life Among Mammals

Contrary to our expectations, our results show that species pulling towards the slow end of the pace-of-life continuum and exhibiting longer lifespans independently of body mass tend to have more glycated albumin. Generally speaking, mammals with slower pace-of-life and longer longevities experience senescence at a slower rate compared to faster-lived species [16,265]. Glycation products are known to contribute to the senescence process in many ways, and their levels are increased in humans with ageing-related pathologies such as diabetes or Alzheimer, those same diseases being known to alter lifespan. A study carried out on another glycation product, pentosidine, in several mammalian species was also in line with this: it showed that shorter-lived species accumulate pentosidine more quickly than longer-lived ones [100]. This is coherent with the hypothesis that glycation generally accelerates actuarial senescence (e.g., the increase in mortality with age), and therefore might contribute partly to a shorter lifespan. Our result thus contradicts the general idea of pro-ageing effects of glycation, at least when considering only Amadori compounds. In fact, we are not the first to show this absence of the expected negative correlation with maximum lifespan, since this is also the case for GA levels in birds [222], or between plasma AGEs and the age/maximum lifespan ratio in birds and mammals [94]. Altogether, these studies (including ours) rather suggest that species at the slower end of the pace-of-life point toward a better tolerance, rather than resistance, to protein glycation.

One might assume that species that have developed tolerance mechanisms to glycated proteins, enabling them to escape some of the latter's pro-aging effects, have seen their longevity increasing as a result. This would be coherent with the fact that species at the lower end of the pace of life usually exhibit longer longevities, which has been confirmed by the strong positive correlation between maximum lifespan and PC1 (standing for the fast-slow

continuum of the pace-of-life) found in our Principal Component Analysis. Overall, this would suggest that, on an interspecific scale in mammals, and restricted to our species, longevity was more related to the ability to tolerate the effects of glycated proteins, rather than preventing their formation.

c) Links Between Glycated Protein and Body Mass Appear to Be Species-Specific

The fact that the principal component mainly driven by body mass (PC2) did not significantly correlate with GA levels is coherent with previous interspecific studies, which did not highlight any correlation between body mass and several plasma AGEs across several species of mammals and birds [94], as well as more specifically in wild canids [95].

Still, studies at intraspecific scales reveal discrepant results. While an absence of correlation between body mass and GHb or total glycated plasma proteins was highlighted in two bird species, *Ficedula albicollis* and *Taeniopygia guttata* respectively [87,89], another study in Alpine swift (*Tachymarptis melba*) found a negative correlation between plasma AGEs and individual body mass [266]. In our case, a positive relationship between individual body mass and glycated albumin levels was highlighted in wild roe deer (see chapter 3). Taking these last two studies as an example, we know that in swifts, body mass is a marker of individual quality. Individuals with a better physiological state can be expected to have a better ability to regulate their protein glycation levels, which results in a negative correlation between body mass and glycation. On the other hand, if body mass rather reflects accessibility to food resources, as it does in roe deer, then we might expect that a higher intake of sugars would result in a greater amount of glycated protein, *i.e.,* a positive correlation between body mass and glycated protein levels. In that case, access to a larger energy resource may allow individuals to invest more in buffering AGEs' deleterious effects, through the production of RAGE antagonists, or through an active competition due to the soluble s-RAGEs form, which acts as a decoy receptor, binding RAGE ligands and thus blocking their interaction with membrane-bound RAGE. This may explain s-RAGE protective effect against AGEs in mice [267]. This suggests that the direction of correlations between body mass and glycated protein levels would in fact be dependent on and/or representative of what body mass actually reflects for each species.

d) Higher Resistance of Bats to Albumin Glycation Compared to Non-Flying Mammals

In line with our initial hypothesis, we have shown that bats do have lower levels of glycated albumin than other mammals. This is surprising at first sight, knowing that three of our five bat species are frugivorous, and that all other mammals in our dataset are not feeding on a carbohydrate-rich diet. The observed differences in glycation rates between bats and other mammals do not appear to be related to body mass nor glycemia (the interactions did not come out significantly in our models). Mean glycemia between bats and the other mammals did not significantly differ, and a study in nectarivorous bats even showed that they have fasting blood glucose levels comparable to those of healthy humans [107]. This supports the idea that bats have an ability to regulate glycemia in a very effective way. The most prominent distinguishing factor among bats and the other mammals in our dataset is flight. Previous studies in nectarivorous bats and birds already hinted that the evolution towards flight also constrained the evolution of a specific glucose metabolism [107,116,117,268]. Despite being fed with high quantities of sugar and reaching high post-prandial glycemic peaks (the highest post-prandial glucose peak was measured in the tiny *Glossophaga soricina*), frugivorous bats manage to regulate high post-prandial blood glucose levels in relatively short times, inversely related to the time they spent flying after eating [107]. One explanation lies in their ability to rapidly mobilize the ingested glucose to fuel this high energy-demanding activity that is flight [107,112,116]. Small nectarivorous bats (and birds) present a reduced intestinal surface area [138], and accordingly fewer glucose transporters, which they compensate with higher capacities of intestinal paracellular absorption [134–136,138,269]. It was also suggested that they do not rely much on insulin-dependent regulatory mechanisms [270]. However, most of the mechanisms by which bats could achieve this remains an open question.

2. Comparative Analysis Among Flying Vertebrates

a) For A Given Maximum Lifespan, Birds Exhibit Higher Glycated Albumin Levels Than Bats of Comparable Diet and Body Mass

Based on these findings about mammals, and the fact that a main point of divergence between bats and other mammals is flight, we investigated the differences between these same bats and the other major taxon of flying vertebrates, birds. Our results show that birds have higher glycemia compared to bats, regardless of diet or mass. Moreover, we show that birds exhibit higher levels of glycated albumin for a given maximum lifespan compared to bats, hereby highlighting that either 1) birds demonstrate a higher tolerance to glycation or 2) bats more efficiently regulate their glycated protein levels.

If flight has promoted a more efficient glucose metabolism and reduced glycation in both taxa, the link with longevity is not convergent, as glycation rates are lower in flying mammals than non-flying mammals, but higher in birds, whose longevity is comparable to that of bats. Then, the underlying proximal reasons could be multifaceted.

On the first hand, one could consider the evolutionary timeframe difference between these two taxa. Although mammals appeared before birds (a little before 200 million years ago [271] and between 140 and 160 million years ago [272], respectively), birds have had a longer evolutionary period to adapt to powered flight compared to bats. Indeed, the oldest bat fossil dates from approximately 52.5 million years ago [273], and it is known that among vertebrates, active flight appeared successively in pterosaurs, birds and bats [274]. This extended evolutionary timeframe may have allowed birds to develop more effective mechanisms for managing the energy sources that fuel their flight, in particular the use of glucose. Additionally, when considering frugivorous diets, evolutionary biologists agree that frugivory in mammals and birds evolved from carnivorous and/or insectivorous ancestor, respectively [275]. However, frugivory in bats appeared after its emergence in birds (between 38 and 24 million years ago and around 58 million years ago, respectively) [275]. Consequently, frugivorous birds had more time to develop adaptations to high-sugar diets compared to bats. An interesting fact is that, unlike birds, frugivory (and nectarivory) evolved independently multiple times in bats (4 times for frugivory[276]). This is even more remarkable given that this diet presents

several challenges, notably in terms of glycations, since a high-sugar diet should lead to a rise in blood sugar levels and consequently glycations. This suggests that glycation tolerance may also have evolved several times in bats, in conjunction with the evolution to a frugivorous diet.

On another hand, the GA levels differences observed between bats and birds could be explained by a variability in adaptations. While flight evolved independently in both taxa, this evolution was accompanied by similar physiological adaptations, particularly in small nectar-feeding species [74,117,269]. Nevertheless, differences remain in aspects such as rates of dietary sugar uptake and oxidation by muscles to fuel the metabolic demand of aerobic activities (known to differ between species and to plateau in most mammals) [277], or the speed at which these same dietary sugars will be utilized post-ingestion (personal communication, results not yet published). These differences suggest that each clade may have developed unique physiological, metabolic, or even behavioral adaptations to glycation tolerance, potentially explaining why birds, despite having higher glycation rates, can achieve comparable lifespans to bats.

b) Insectivorous Bats and Birds Have More Glycated Albumin Than Their Frugivorous Counterparts

Finally, we showed that insectivore bats and birds display higher GA levels than their frugivorous counterparts. Previous studies in different animal species feeding on low carbohydrate but high protein and fat diets showed that those species tend to have higher glycemia and reduced insulin sensitivity [250,278,279]. Another study conducted on bats of different diets highlighted a reduced glucose tolerance and a trend for insectivorous bats to exhibit post-ingestion higher glycemia compared to frugivorous ones [251]. Several studies, particularly in birds, have explored explanations for why insectivores exhibit higher glycemia rates compared to frugivores. Insectivory precedes frugivory evolutionally, and insectivorous ancestors likely developed specialized mechanisms to cope with a diet richer in sugars, potentially enabling them to better control their blood glucose levels (and glycation levels) compared to their insectivorous counterparts. Such mechanisms have been identified in nectar-feeding birds and bats, as mentioned earlier (fast glucose utilization after ingestion

[107,116], specific protein sequences [155,156] etc.). However, this alone does not account for the observed differences.

Insectivorous species in the wild naturally consume less sugar compared to frugivores, whose diets are rich in fruit sugars and nectar. The availability of these sugars, especially for immediate mobilization during flight, differs significantly between insectivores and frugivores. An additional consideration, already proposed for raptor birds, is gluconeogenesis, a pathway occurring in the liver that allows to synthesize glucose using non-carbohydrates precursors, such as amino acids from muscle protein degradation or lactate. It has for example been shown that black vultures (*Coragyps atratus*) are able to maintain their glycemia constant during 3 days of fasting, thanks to an increased neoglucogenesis (twice higher than chicken in similar conditions) [280]. Similarly, in the barn owl (*Tyto alba*), high rates of neoglucogenesis were noticed following a glucose challenge, associated with poor capacities to down-regulate this pathway due to reduced glucokinase activity [281].

3. Conclusion and Perspectives

In this chapter, we demonstrated that bats exhibit lower glycated albumin levels than non-flying mammals of varying body mass and lifespans. Interestingly, insectivorous species happened to have more albumin glycation than frugivorous ones.

Moreover, it appears that among mammals (flying and non-flying), species with a slower pace-of-life display a better tolerance towards albumin glycation compared to faster lived ones, suggesting that glycation tolerance might have played a role in the evolution of longer lifespans in mammals.

To refine the present findings, it could be useful to expand our sampling across a broader range of mammal species with varying life histories, longevities and diets. This broader phylogenetic framework would enhance statistical power and provide a more robust estimate of phylogeny dependence. Advanced methods such as MCMC approaches could also be explored, as they would allow us to calculate the phylogeny effect separately on each variable included in our models [282].

To find out more about the differences between bats and other mammals, it would also be beneficial to categorize non-flying mammals into specific groups to determine if bats diverge

more significantly from certain mammalian groups rather than from all other mammals in general. Investigating the differences between modes of locomotion and living environment (marine, terrestrial, flying, arboreal or subterranean species) could perhaps teach us more about the co-evolution of tolerance to glycation and longevity as a function of environmental constraints. Furthermore, in order to assess whether flight might indeed be the main explanation for the differences in glycated albumin levels found between bats and other mammals, it might be interesting to add species displaying non-powered flight, such as gliding marsupials.

While the links between diet and glycemia are often discussed, a closer look at the impact of diet on protein glycation, including diets such as herbivory, omnivory or even sanguinivory, in a wide range of mammals, could tell us more about evolutionary divergences between diets, and at the same time about the mechanisms by which certain species adapted to sugar-rich diets.

CHAPTER 2 - Study of The Links Between Glycation and Oxidative Stress in Two Species of Fruit Bats

Illustration by Eulalie Boucher

I. Introduction

In the previous chapter, we highlighted that glycations are not necessarily a marker of aging for all mammalian species, and that it is likely that the dynamics of glycations and tolerance to them are species-specific. The preceding study also showed that bats seem to stand out from other non-flying mammals, with lower levels of glycated albumin, despite the fact that they are relying on food resources with high glycemic index. In this second chapter, we therefore decided to narrow our scope from the interspecific to the intraspecific scale, by taking a closer look at the links between glycations and aging in two species of these special flying mammals.

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) (e.g., derived from mitochondrial activity as a by-product for oxygen-derived energy metabolism), and antioxidant defenses / repair mechanisms of oxidative damage to macromolecules. The severity of oxidative stress is determined by the degree of imbalance between pro-oxidant and antioxidant processes; when ROS production increases or antioxidant defenses decrease, oxidative stress escalates, which causes gradual damage to cellular components throughout life. This damage eventually leads to cellular dysfunction and apoptosis, forming the theoretical basis of the well-known free radical theory of aging. According to this theory, aging and aging-related diseases are (at least partially) attributable to the cumulative oxidative damage inflicted on biological macromolecules over time [283].

Several mechanisms have been proposed to explain how oxidative stress contributes to aging. One of the primary mechanisms involves disruption in protein homeostasis. Most amino acids are vulnerable to oxidation, and the oxidative modification of protein residues can impair their structure and function [284,285]. Chronic oxidative stress has also been shown to induce protein misfolding or unfolding, leading to impaired degradation of damaged proteins, as well as the formation of insoluble aggregates [286,287]. In addition to damage to proteins, oxidative stress is recognized as a major cause of DNA damage [288]. We know, for example, that it induces damage to guanine-rich sequences (T_2AG_3 telomeres), and could be one of the causes leading to accelerated telomere erosion [289,290].

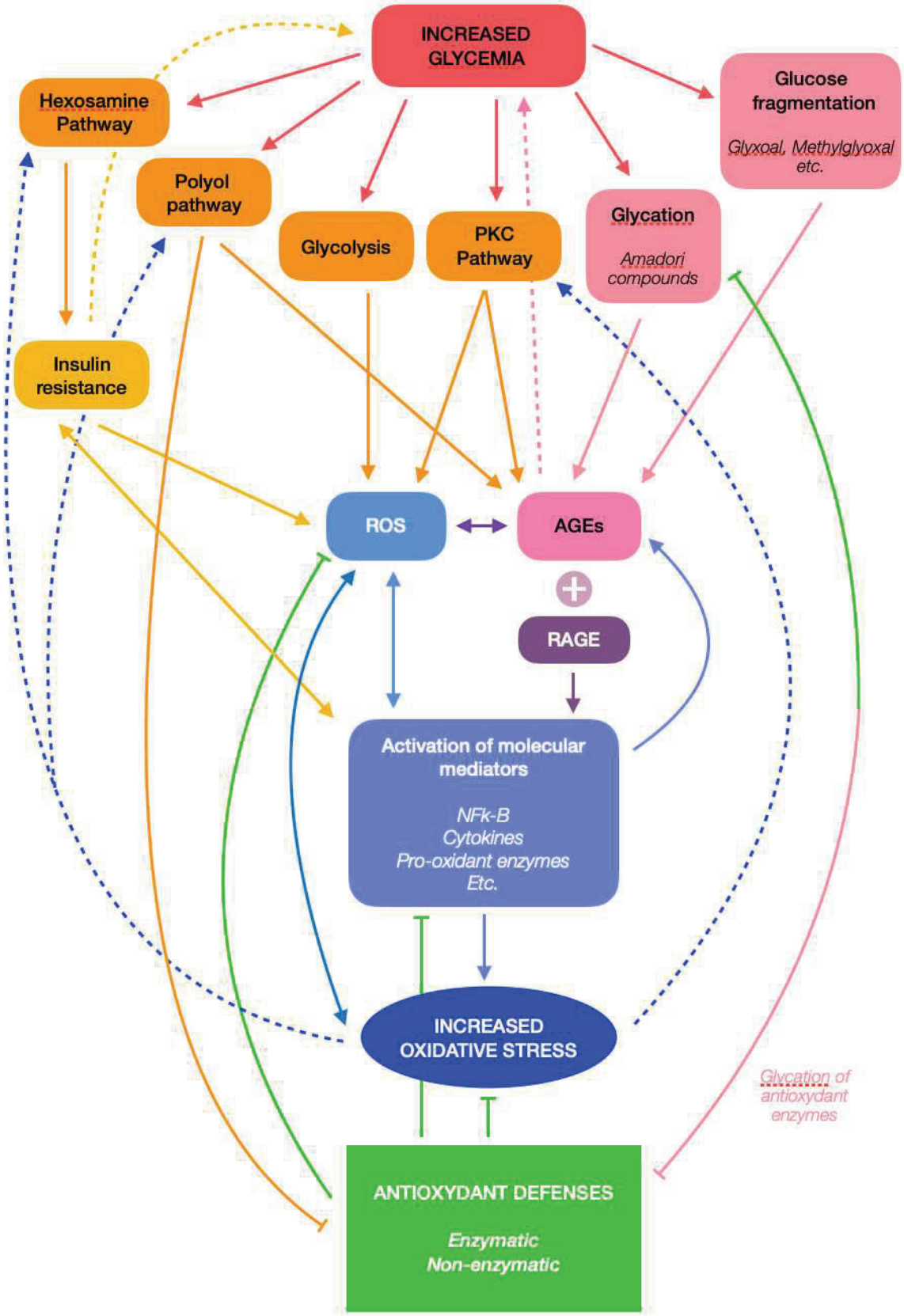


Figure 22. Pathways by which hyperglycemia, glycation and oxidative stress contribute to each other

DNA damage induced by oxidative stress therefore leads to the activation of DNA repair mechanisms such as telomerase. However, recent studies have shown that the generation of 8-hydroxy-2-deoxyguanosine (8-OHdG, a specific oxidative damage occurring on guanosine residues of DNA [291]) on telomeric sequences inhibits telomerase activity, hereby lowering DNA repair post-replication and accelerating telomere shortening [292].

Hyperglycemia is known to activate pathways that promote oxidative stress [293] (Figure 22). Among the various pathways through which glucose enhances ROS production—including glycolysis and mitochondrial respiration, the polyol pathway, and the hexosamine pathway—the spontaneous glycation reaction, which is exacerbated under hyperglycemic conditions, is a significant contributor to elevated oxidative stress.

It has been established that glycation can occur on specific antioxidant enzymes, leading to their inactivation [294]. For instance, glycation of the extracellular superoxide dismutase isoenzyme SOD3 impairs plasma antioxidant capacity [61]. Moreover, one of the major sources of oxidative stress is the accumulation of advanced glycation end-products, which bind to specific cellular receptors known as RAGE. The AGE-RAGE interaction activates a range of signal transduction pathways, including protein kinases (PKC, JAK, p38-MAPK, and ERK), GTPases, TGF-β, transcription factors (such as NF-κB, an essential pathway in the activation of inflammation), and pro-oxidant enzymes such as NADPH oxidase (NOX) and nitric oxide synthase (NOS), which collectively increase intracellular oxidative stress. Beyond exacerbating oxidative stress, these signaling pathways also activate other glucose metabolism pathways outside of glycolysis, creating a vicious cycle between glycation and oxidative stress (see Figure 22). As a result, AGEs serve as a reliable marker of plasma oxidative stress due to hyperglycemia [61]. Another pathway leading to AGE formation involves the spontaneous reactions of glucose that produce ketoaldehydes, such as methylglyoxal and glyoxal, which directly contribute to oxidative stress [295,296] (Figure 22).

Still, most of our knowledge on glycation and oxidative stress originates from studies conducted on humans or laboratory animal models. Evolution has produced a large panel of physiological adaptations, and, among the tree of life, there are organisms that are chronically exposed to high glucose levels, such as frugivorous and nectarivorous species. Given that most bat species are long lived animals, they must have acquired anti-ageing capacities. In that context, they are intriguing organisms to study in relation to both glycation and oxidative stress.

As mentioned in the previous chapters, several studies have indeed noted a hyperglycemia resistance in frugivorous bats [107,116,117]. As for oxidative stress, it has been highlighted that some species exhibit lower levels of oxidative damage compared to mammal species of similar body mass. A study on the little brown bat (*Myotis lucifugus*) indeed reported that H_2O_2 production (normalized by oxygen consumption) in tissues (heart, kidney, and brain) was 50% to 67% lower than in two rodent species [297].

However, this same study found no significant difference in enzymatic antioxidant activity (superoxide dismutase, SOD) between the three species. On the contrary, several studies have reported higher non-enzymatic antioxidant defenses in bats. For instance, a study on five South American bat species found that their tissues (liver, heart, kidney, and pectoral muscles) contained 1-2 times higher levels of alpha-tocopherol and beta-carotene compared to those typically found in rats and mice [298]. Another study in frugivorous bats reported simultaneously lower oxidative damage and higher levels of circulating antioxidants [245].

That said, it seems that the antioxidant defense mechanisms favored by bats differ according to their diet. When examining the brains of various bat species with different diets (insectivores, frugivores, nectarivores, and hematophages), it was found that insectivores exhibited higher SOD and fumarase activity, while hematophages had higher glutathione peroxidase activity, despite frugivores showing lower oxidative damage [299]. This appears coherent, given that frugivorous species will find large quantities of non-enzymatic antioxidants in fruit. Conversely, the diets of insectivorous or carnivorous species are less rich in them, so it seems logical that these species rely more on enzymatic defense mechanisms.

However, antioxidant defenses are not the only mechanisms that have been demonstrated to explain bats' tolerance to oxidative stress. Bats are indeed known to exhibit resistance to protein oxidation, as well as to maintain enhanced protein homeostasis despite their high metabolic rates and elevated oxygen consumption due to flight. For instance, bat proteins

demonstrate longer stability and increased resistance to oxidation under acute stress conditions (gamma irradiation), and their proteins are more resistant to urea-induced unfolding compared to those of mice [184]. A study in *Myotis lucifugus* skin fibroblasts also revealed resistance to various lethal stresses, including H_2O_2, compared to mice [300]. Moreover, it has been suggested that bats have a higher rate of protein turnover, which may aid in the removal of oxidation-damaged proteins [184]. Finally, certain factors related to the lifestyle of certain species have also been highlighted, such as hibernation. Interestingly, the activities of SOD and catalase, as well as blood glutathione levels, were higher in torpid bats compared to active ones, suggesting a daily modulation of enzymatic antioxidant capacity [298].

In this context, we aimed at exploring how glycations and oxidative stress might correlate in two species of fruit bats, namely the Egyptian fruit bat (*Rousettus aegyptiacus*) and the Rodrigues flying fox (*Pteropus rodricensis*).

From previous studies in fruit and nectar-eating bats, we know that these species have evolved specific physiological and cellular adaptations to tolerate high glycemia. However, the evolution of a species-wide tolerance does not in any way prevent the existence of variations between individuals, with supposedly some individuals being more tolerant than others. It is this inter-individual variation within these two bat species that we have particularly targeted in this study.

We first looked at how blood glucose levels evolve as a function of age, sex and individual mass in these two species. By doing so, we wanted to verify whether there is a dynamic of glucose tolerance over age (i.e., as a senescence marker) and/or if glucose is dependent on individual body condition.

We then looked at two Amadori products: glycated hemoglobin and glycated albumin. For each of them, and within each of the two species, we assessed whether interindividual variations in glycation levels could be explained by age, mass, sex and/or glycemia. Our hypothesis being that fruit bats are tolerant to glycations: as such they may either uncouple blood glucose from GHb and GA levels (i.e., resistance of proteins to glucose) or exhibit high GHb and GA levels without any ageing consequences (i.e., glycation and/or oxidative stress

insensibility). As ageing still happens in bats, we did, however, expect to find a slight tendency towards higher levels in older individuals, glycations accumulating over age.

To try to disentangle resistance to glycation and to oxidative stress, we addressed in a second part oxidative damage and the antioxidant capacities of plasma. We looked at how both of them correlate with age, sex and individual mass, but also with glycation levels and glycemia. Knowing that bats exhibit resistance mechanisms towards oxidative stress, we did not expect to find strong positive correlations with age or mass. Although we assume that bats exhibit a tolerance to glycations compared to other mammals, we still expected to find a positive correlation between GHb, GA and markers of oxidative status, as glycations may contribute to oxidative stress among individuals.

Finally, in mammals, as males tend to have a shorter lifespan than females [301,302], we hypothesized that males would show higher levels of both glycations and oxidative stress than females.

II. Materials & Methods

1. Bat Species Selection

Among the five bat species sampled in this study (see General Materials & Methods section, p. 35), we have focused here on two particular species, both from captive colonies maintained at the Zoo de la Palmyre: the Rodrigues Flying Fox (*Pteropus rodricensis*) and the Egyptian Rousette (*Rousettus aegyptiacus*).

Both species offer the advantage of being frugivorous, and thus are subjected to sugar-rich diets, which is particularly relevant for studies investigating potential glycation resistance in bats. Additionally, the *P. rodricensis* population provided the significant benefit of consisting of individuals with known ages.

As for the Egyptian Rousette, this bat species represents an especially valuable model due to its extensive study, particularly regarding glucose metabolism and oxidative stress [136,303,304]. Furthermore, it is one of the few bat species for which protein sequence data are available.

Further details on the husbandry conditions of these individuals are provided in the General Materials and Methods section.

2. Data Collection

a) Individual Characteristics

Regarding *P. rodricensis*, we sampled 15 individuals of varying ages (ranging from 4 months to almost 15 years old), with approximately equal proportions of males and females (n=8 and n=7, respectively).

For *R. aegyptiacus*, we did not know the chronological age of the individuals. However, the larger population allowed us to sample a sufficient number of individuals to create four homogeneous groups with the following characteristics: juvenile males (n=6), adult males (n=6), juvenile females (n=7), and adult females (n=6). All individuals were also weighed.

b) Glycation Measurements

For each individual from both species, we collected plasma and red blood cells. From these samples, we measured blood glucose and glycated albumin levels on the one hand, and glycated hemoglobin (GHb) levels on the other. The method for measuring glycation via LC-MS is detailed in Part II of the General Materials and Methods section of this manuscript (p. 47).

c) Oxidative Status Measurements

To evaluate oxidative status, we quantified two distinct markers of oxidative stress in plasma: 8-hydroxydeoxyguanosine (8OH-dG), a marker of DNA oxidative damage, and total plasmatic antioxidant capacity (OXY).

8OH-dG is a prominent product of DNA oxidation and is widely used as an indicator of cellular oxidative damage. Briefly, guanine damages caused by oxidative stress are excised from DNA through enzymatic repair processes, entering the bloodstream before being excreted in urine. Circulating levels of 8OH-dG are thus considered a reliable marker of systemic oxidative stress, as its plasma concentration reflects both the extent of DNA damage and the efficiency of DNA repair mechanisms. The concentration of 8OH-dG in plasma was measured using a competitive immunoassay (plasma diluted 1:20; DNA damage (8-OHdG) ELISA Kit, StressMarq Biosciences INC., Canada) according to the manufacturer's instructions, and results are expressed in ng of 8OH-dG/mL.

In ecophysiology and evolutionary biology, the plasma antioxidant defense marker OXY is widely used, as it has been shown to be linked to investments in activities that are costly in terms of metabolism and survival (growth, reproduction, immunity) [305–307] but see [308]. We assessed it using the OXY-Adsorbent Test (DIACRON INTERNATIONAL, Italy), following the provided protocol. Briefly, this assay evaluates the capacity of various non-enzymatic antioxidants (e.g., vitamins, carotenoids, flavonoids, and thiols) to neutralize a massive oxidative insult induced by hypochlorous acid (HClO). Plasma antioxidant capacity is expressed in µmol of neutralized HClO per mL. To ensure measurement reliability, all samples were analyzed in duplicates for OXY, and in triplicates for 8OH-dG. The intra-assay coefficient of

variation based on duplicates or triplicates measurements was 6.70 % for OXY and 14.01 % for 8OH-dG. Inter-plate variability was assessed using control samples repeatedly analyzed across plates and sessions. Inter-plate variation was 22.46 % for OXY and 21.92 % for 8OH-dG.

d) Albumin and Hemoglobin Sequences

Albumin sequences for Egyptian Rousette, chicken and human were obtained from the UniProt Knowledgebase (UniProtKB; *R. aegyptiacus*: A0A7J8E5X0; *G. gallus*: P19121; *H. sapiens*: P02768). For both chicken and human, sequences were issued from the SwissProt section of the UniprotKB, thus certifying a high level of reliability. Beta-globin sequences of Egyptian rousette and human were obtained from the SwissProt section of the UniProtKB (*R. aegyptiacus:* P02058*; H. sapiens:* P68871).

3. Statistical Analysis

All the analyses were performed using R version 4.3.1 [256].

a) Rodrigues Flying Fox

Since we had chronological age for the Rodrigues flying fox, we first aimed at finding the function that would give the best fit of the relationship between chronological age and glycemia, GA and GHb, respectively. We tested two different types of functions: linear and quadratic. Following Burnham & Anderson's recommendations [264], we selected the model with the lowest corrected Akaike Information Criterion (AICc) (which avoids data overfitting through correction for small sample sizes). For glycemia and GHb, the linear model happened to be the best one, contrary to GA, for which the quadratic function was the most appropriate. To assess changes in glycemia in relation to age, sex and body mass, we used a linear regression (function *lm* from the package *stats*). Glycemia was entered as the response variable, while the other variables were input as fixed factors, with all two-way interactions between them. Three-way interactions were omitted to avoid model over- fitting due to our reduced sample size (n=14 after the removal of one individual that was graphically identified as an outlier). To

select the best model, we used an automatic selection procedure (*dredge* function from the MuMIn package [309]), which is based on the AICc. As previously described, here too we retained the model with the lowest AICc, and between two competing models with an AIC difference below 2, we respected the parsimony rule [264]. The normality and homeodasticity of residuals were verified through standard residual plot techniques along with a Shapiro-Wilk normality test.

We then tested the same model but with GHb and GA as variable to be explained. For glycated albumin, age was included in a quadratic relationship, for the reasons explained above.

To study oxidative stress, we performed two models, one with 8OH-dG as response variable, and the other one with OXY as response variable. Missing values in both variables were handled through a PCA imputation procedure thanks to the *missMDA* package [263]. In the first model containing 8OH-dG as the variable to be explained, we included GA, GHb, glycemia, OXY, age, sex and mass as explanatory variables, along with their two-way interactions. The same model was then performed with OXY as response variable and 8OH-dG as fixed factor, to assess inter-individual variations in terms of antioxidant defenses. Best models were then selected through the same exact procedure as above, with the exception that, in the automatic selection procedure, we limited the number of explanatory factors to be tested in the models to 3, to avoid overfitting due to our reduced sample size.

All initial full models before selection are summarized in the following table (Table 9).

Table 9. List of initial full models tested for the Rodrigues flying fox

	Response variable	Explanatory variables
Model 1	Glycemia	(Age + Sex + Body mass)^2
Model 2	GHb	(Age + Sex + Body mass + Glycemia)^2
Model 3	GA	(poly(Age,2) + Sex + Body mass + Glycemia)^2
Model 4	8OH-dG	(OXY + GHb + GA + Glycemia + Age + Sex + Body Mass)^2
Model 5	OXY	(8OH-dG + GHb + GA + Glycemia + Age + Sex + Body Mass)^2

Poly(Age, 2) stands for a quadratic function; (…)^2 stands for the two-way interactions between each factor included in brackets.

b) Egyptian Rousette

Overall, we tested the same models for the Egyptian rousette, with the only difference being that we did not have the precise age of the individuals. We therefore replaced the age variable with the age category variable, with two modalities, juvenile and adult. Two individuals were removed from dataset, one for which we had no measurements of glycemia nor glycation, and another which, after graphic analysis, appeared to be an outlier. In the remaining 23 individuals, we did not detect glycated albumin in many individuals (see results below), and thus our sample size for this variable was very low (n=5). As such, GA was not included in our models. The missing values for the other variables, on the other hand, were relatively few, so we handled through a PCA imputation procedure with the *missMDA* package [263], just as for *P. rodricensis*. Best model selection was then carried out in the same way as for the Rodrigues flying fox. All full models tested on *R. aegyptiacus* are summarized in the following table (Table 10).

Amino acid sequences of Egyptian rousette were aligned with human chicken sequences using the Clustal Omega Program available through the UniProtKB website.

Table 10. List of initial full models tested for the Egyptian rousette

	Response variable	Explanatory variables
Model 1	Glycemia	(Age category + Sex + Body mass)^2
Model 2	GHb	(Age category + Sex + Body mass + Glycemia)^2
Model 3	8OH-dG	(OXY + GHb + Glycemia + Age category + Sex + Body Mass)^2
Model 4	OXY	(8OH-dG + GHb + Glycemia + Age category + Sex + Body Mass)^2

(…)^2 stands for the two-way interactions between each factor included in brackets.

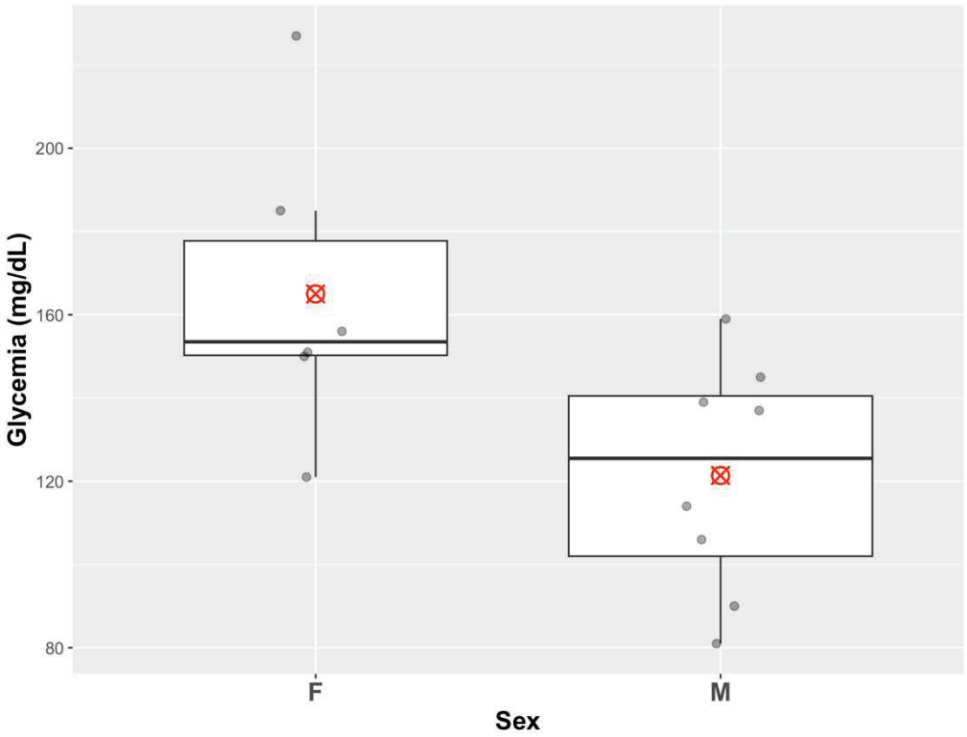


Figure 23. Glycemia differences between males and females in the Rodrigues flying fox (n=14). F and M stand for female and male, respectively. Grey points represent individuals, red crossed circles represent the mean glycemia in each group (see text for details).

Table 11. **Model selection table** (dredge function from the *MuMin* package, automatic selection procedure) with ΔAICc < 2 for the factors explaining glycated albumin in *R. aegyptiacus*.

Full model: Glycemia ~ (Age category + Sex + Body mass)^2. (n=14)								
	Intercept	**Age category**	**Body mass**	**Sex**	**k**	**AICc**	**ΔAICc**	**AICc weights**
Model 1	216.5		-0.6102		3	282.1	0.00	0.383
Model 2	160.6			-19.12	3	282.4	0.27	0.336
Model 3	154.0	-5.855			3	282.8	0.62	0.281

^2 stands for all two-way interactions between the variables entered in brackets. k: number of parameters in the model. AICc: corrected Akaike Information Criterion. ΔAICc: difference between the AICc of the candidate model and the AICc of the selected model. AICcw: AICc weight (measures the relative likelyhood that a given model is the best among all the fitted models). None of the models are significant.

III. Results

1. Glycemia and Glycation Levels In Relation to Age, Sex and Mass

a) Glycated Albumin and Glycated Hemoglobin Measurements

Glycemia ranged from 81 to 227 mg/dL (mean value of 140.07 +/- 37.87 mg/dL) and from 45 to 301 mg/dL (mean value of 149.65 (+/- 78) mg/dL) across all samples from *P. rodricensis* and *R. aegyptiacus*, respectively.

We were able to detect and quantify glycated albumin in all samples from the Rodrigues flying fox, while we did so only in 5 samples out of 25 in the Egyptian rousette. GA values in *P. rodricensis* ranged from 12.56 % to 17.75 %, with a mean of 13.98 +/- 1.31 % (glycated albumin levels expressed as percentage of total albumin), while GA values in *R. aegyptiacus* ranged from 9.13 to 11.34 % (mean value of 10.09 (+/- 0.95 %).

In both species, we were able to detect two haemoglobin chains, which we have correlated with the alpha and beta hemoglobin chains in humans (see Table 3 for molecular masses). However, in both species, we only detected glycation on the beta-like hemoglobin chain (as for humans). Hereafter, when we speak of GHb, we are referring to glycation values measured on the beta chain of hemoglobin, in analogy with the term HbA1c used in humans (which reflects glycation on the human beta chain). We detected glycated heamoglobin in 23 samples of *R. aegyptiacus* (out of a total of 25 samples), and in 14 (out of 15) samples of the *P. rodricenis* samples. GHb ranged from 2.42 to 5.14 % (mean value of 2.93 +/- 0.5 %) in the Egyptian roussette, and from 3.67 to 5.92 % (mean value of 4.6 +/- 0.57 %) in the Rodrigues flying fox, all values therefore being below the HbA1c cut point of 6.5 % recommended for diagnosing diabetes in humans [310].

b) Glycemia in Relation to Sex, Mass and Age in Both Bat Species

In the Rodrigues flying fox, after model selection, glycemia appeared to be best explained by sex (estimate: (sex-male), -43.62 +/- 17.16, p=0.02583, adjusted R^2= 0.2959, n=14), with males

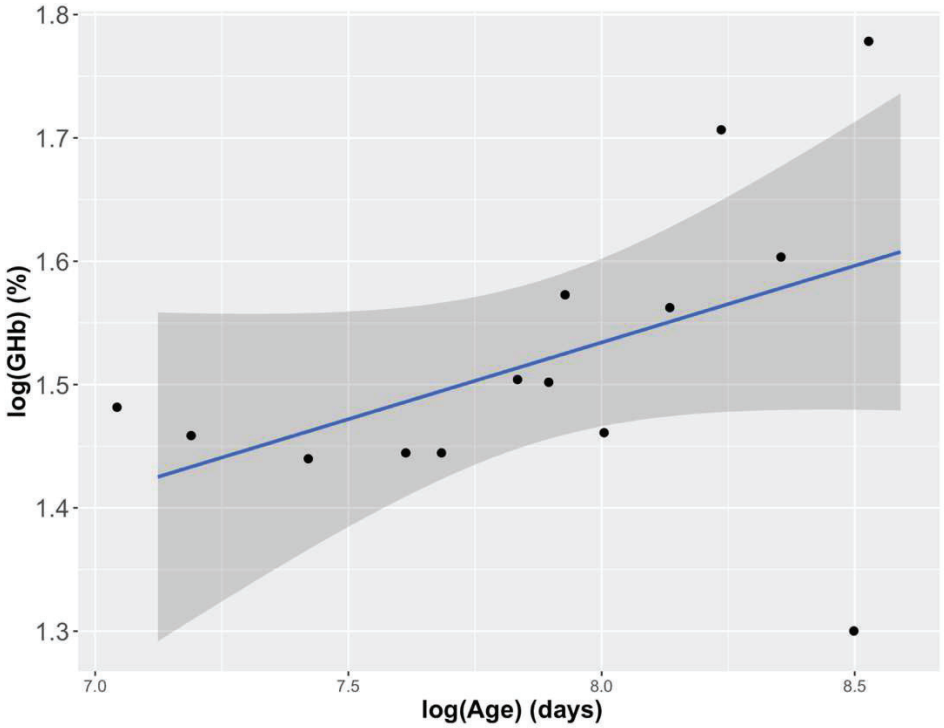


Figure 24. Correlation between glycated hemoglobin levels (GHb) and chronological age in the Rodrigues flying fox (n=14). Black points represent individuals, the blue line represents the linear regression model and the grey area the 95% confidence interval of the model. The relationship did not come out as significant (estimate= 0.12443 +/- 0.07107, p= 0.105)

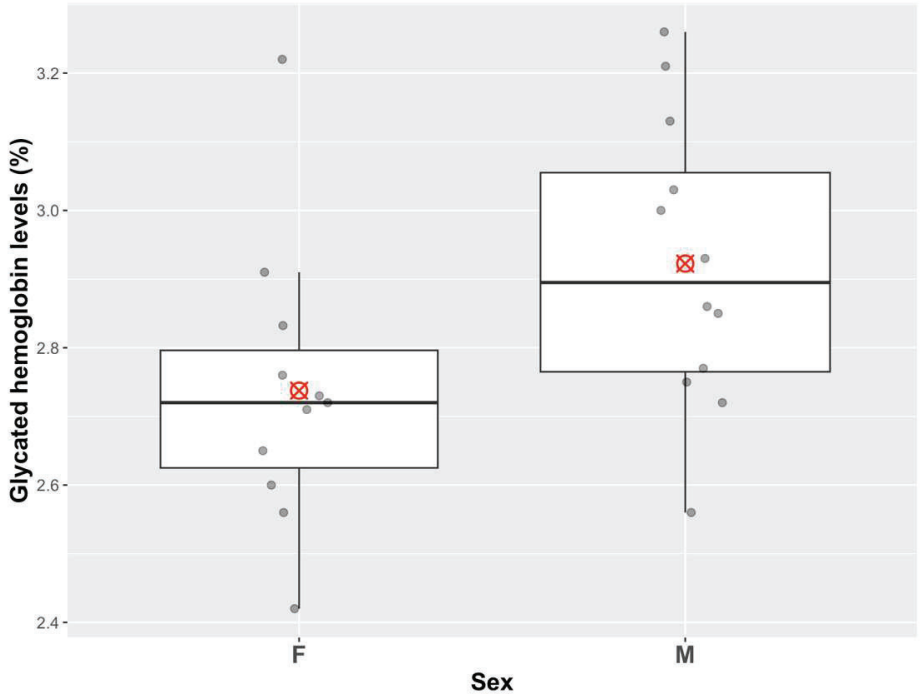


Figure 25. Glycated hemoglobin levels differences between males and females in the Egyptian Rousette (n=23). F and M stand for female and male, respectively. Grey points represent individuals, red crossed circles represent the mean glycemia in each group (male mean GHb levels = 2.92 +/- 0.21 % > female mean GHb levels = 2.73 +/- 0.22 % mg/dL).

exhibiting lower blood glucose than females (male mean glycemia =121.38 +/- 27.86 mg/dL < female mean glycemia = 165 +/- 36.56 mg/dL; Figure 23).

For the Egyptian Roussette, after model selection, three models with one explanatory variable each happened to have a ΔAICc <2 (see model selection table, Table 11): body mass, sex and age category. However, none of them was significant (p= 0.4439, p=0.5549 and p=0.857, respectively).

c) Glycated Heamoglobin Levels Variation with Sex, Mass, Age and Glycemia in Both Species

For the Rodrigues flying fox, glycated hemoglobin levels were best explained by chronological age (estimate= 0.0002566 +/- 0.0001175, p= 0.0496), however the residuals were not normal. To obtain the normality of the residuals, we log-transformed our two variables (dependent and explanatory). As a result, the age effect no longer appeared to be significant (estimate= 0.12443 +/- 0.07107, p= 0.105; Figure 24).

However, in the Egyptian Rousette, GHb levels were best explained by sex (estimate(sex-male= 0.18502 +/- 0.08755, p= 0.0467, adjusted R^2= 0.1361, n=23), with males exhibiting higher levels of glycated hemoglobin compared to females (male mean GHb levels = 2.92 +/- 0.21 % > female mean GHb levels = 2.73 +/- 0.22 %) (Figure 25).

d) Links Between Glycated Albumin Levels and Sex, Age, Mass and Glycemia in the Rodrigues Flying Fox

After model selection, three models with one explanatory variable each happened to have a ΔAICc <2 (see model selection table, Table 12): sex, glycemia and body mass. However, none of them was significant (p= 0.425, 0.522 and 0.678, respectively).

Table 12. **Model selection table** (dredge function from the *MuMin* package, automatic selection procedure) with ΔAICc < 2 for the factors explaining glycated albumin in *P. rodricensis*.

Full model: GA ~ (poly(Age,2) + Sex + Body mass + Glycemia)^2. (n=14)								
	Intercept	**Sex**	**Glycemia**	**Body mass**	**k**	**AICc**	**ΔAICc**	**AICc weights**
Model 1	13.92	-0.3713			3	40.9	0.00	0.381
Model 2	13.14		0.004062		3	41.1	0.27	0.332
Model 3	14.60			-0.002956	3	41.4	0.56	0.287

^2 stands for all two-way interactions between the variables entered in brackets. k: number of parameters in the model. AICc: corrected Akaike Information Criterion. ΔAICc: difference between the AICc of the candidate model and the AICc of the selected model. AICcw: AICc weight (measures the relative likelyhood that a given model is the best among all the fitted models). None of the models are significant.

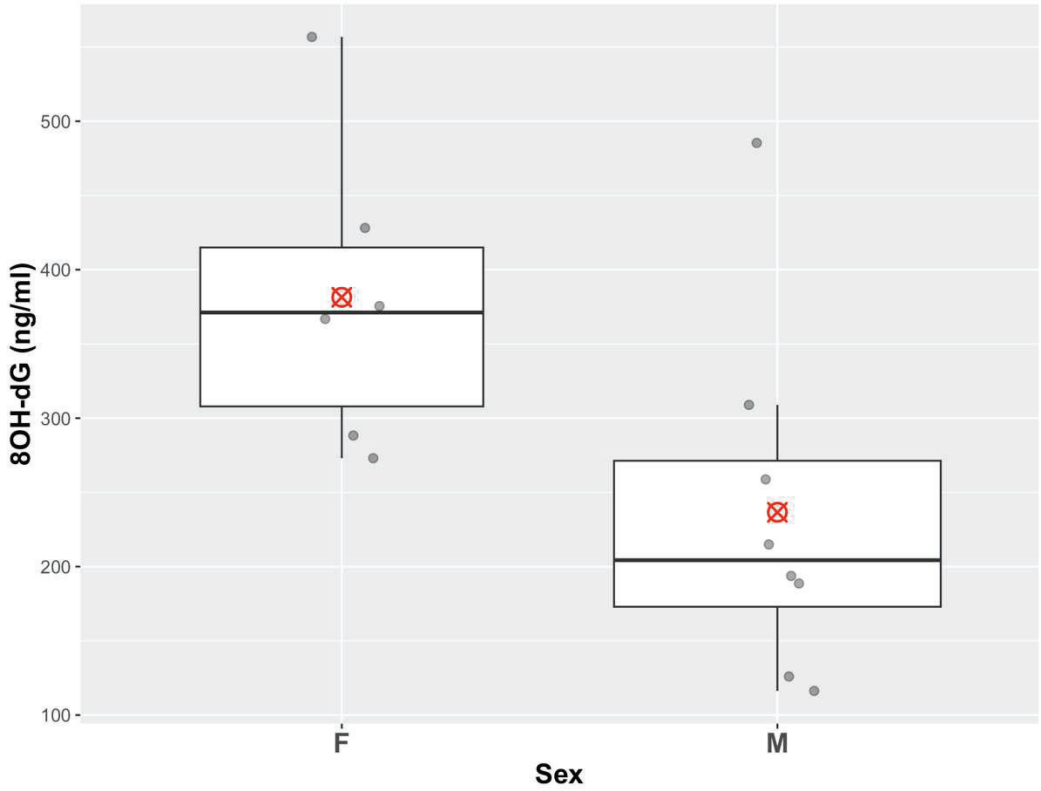


Figure 26. DNA oxidative damage (8OH-dG) differences between males and females in the Rodrigues flying fox (n=14). F and M stand for female and male, respectively. Grey points represent individuals, red crossed circles represent the mean glycemia in each group (female mean 8OH-dG concentration = 344.93 +/- 135 ng/mL > male mean 8OH-dG concentration = 243.43 +/- 126.69 ng/mL).

2. Oxidative Stress and its Relation With Glycation Levels In *P. Rodrincensis* And *R. Aegyptiacus*

a) Oxidative Damage

In the Rodrigues flying fox, after model selection procedure, it appeared that 8OH-dG concentrations were not explained by glycation levels. However, the best model highlighted that DNA oxidative damage significantly differed between males and females (estimate(sex-male)= -144.83 +/- 60.89, p= 0.0348, adjusted R^2= 0.2638, n=14), with males exhibiting significantly lower 8OH-dG concentrations compared to females (Figure 26).

In the Egyptian roussette, oxidative damage concentrations were also not significantly explained by glycation levels. However, the best model highlighted that 8OH-dG concentrations positively correlate with circulating antioxidant capacities (Table 13, Figure 27.a., and that oxidative damage was higher in juveniles compared to adults (Table 13, Figure 27.b.).

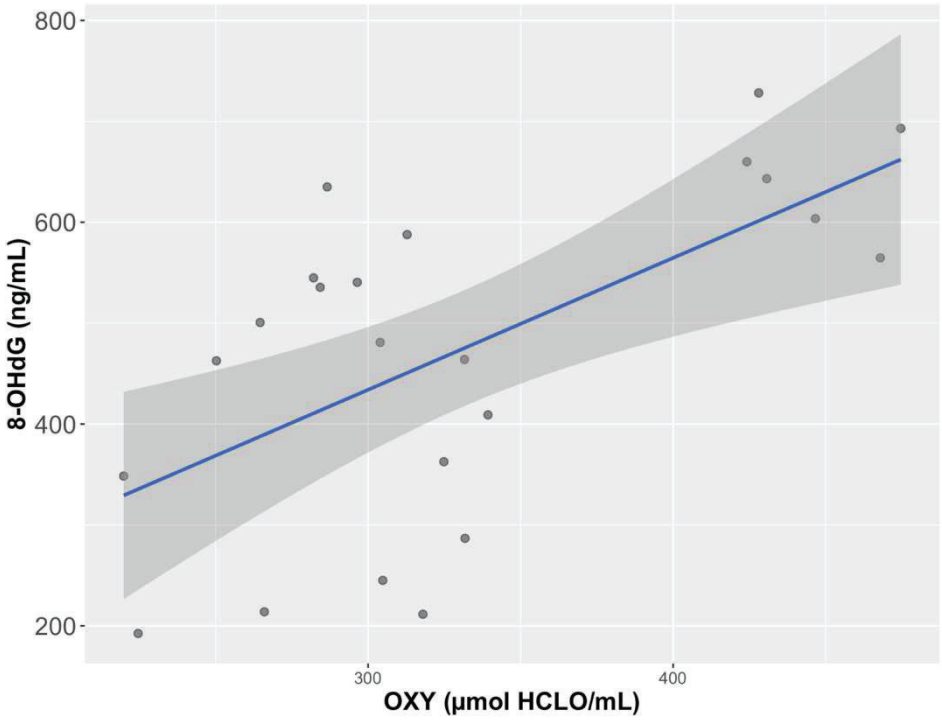
Table 13. Parameters of the selected model explaining DNA oxidative damage concentrations in the Egyptian Rousette (n=23; adjusted R^2 = 0.4984) (* p < 0.05; *** p < 0.001)

	Estimate	SE	p-value
Intercept	33.1138	109.4873	0.76544
Age category [Juvenile]	134.7934	49.4738	0.01306 *
OXY	1.1210	0.3296	0.00283 **

b) Antioxydant Capacities

For the Rodrigues flying fox, the best retained model highlighted that plasma's total antioxidant capacities significantly increased with increasing GHb levels (estimate = 72.120 +/- 26.266, p= 0.0177, adjusted R^2= 0.3347, n=14; Figure 28).

(a)



(b)

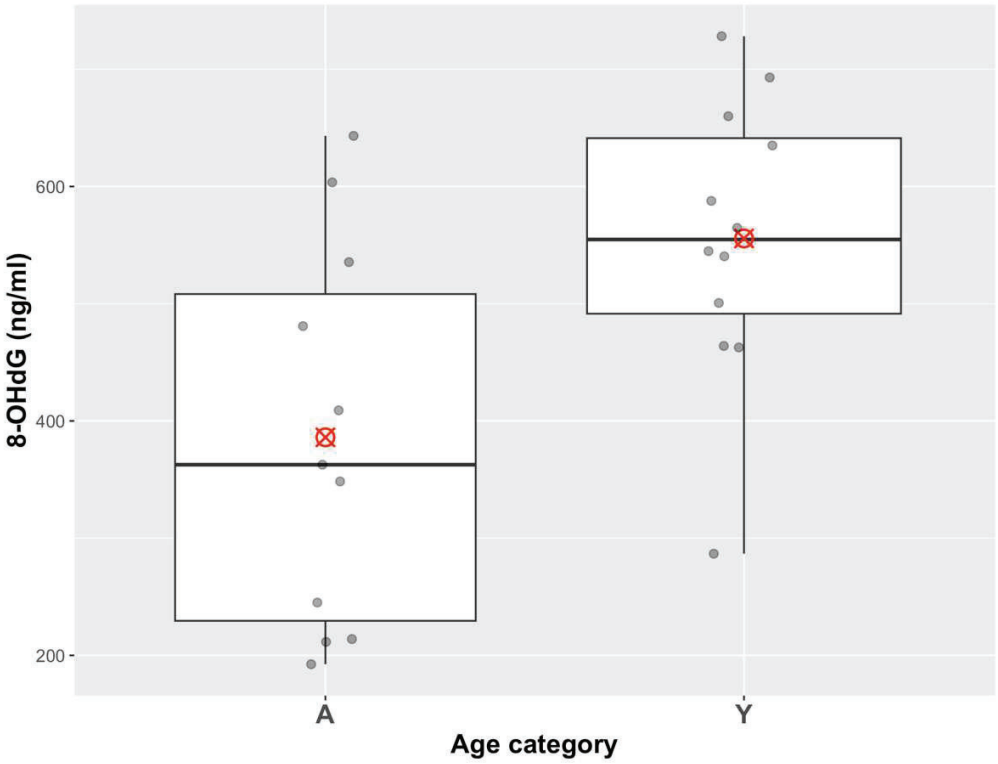


Figure 27. **Factors significantly explaining oxidative damage concentrations in the Egyptian Rousette.** (a) Correlation between 8OH-dG concentrations and circulating antioxidant capacities (OXY). (b) Boxplot of 8OH-dG concentrations in juvenile and adult Egyptian Rousettes. Grey points represent individuals, red crossed circles represent mean 8OH-dG concentrations of each age category.

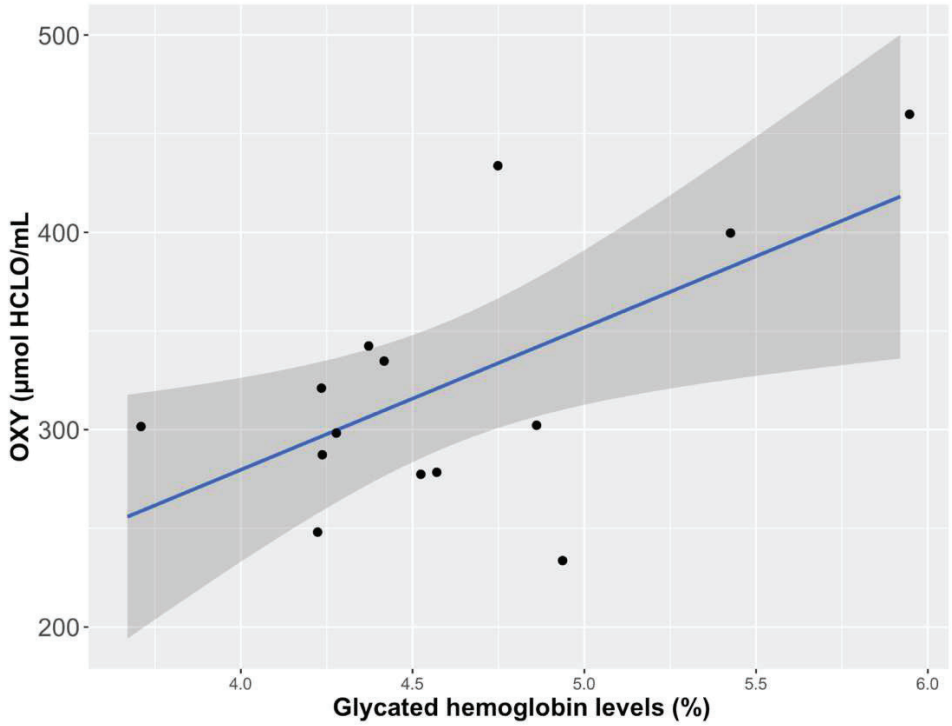


Figure 28: Evolution of plasma's antioxidant capacities with glycated hemoglobin levels in the Rodiguez flying fox (n=14). Black points represent individuals, the blue line represents the linear regression model and the grey area the 95% confidence interval of the model.

For the Egyptian Rousette, the antioxidant barrier was best explained by the interaction between sex and 8OH-dG concentrations (p= 0.0137, Table 14), with males exhibiting increasing antioxidant capacities with increasing DNA oxidative damage, contrary to females, for which OXY remained stable despite increasing 8OH-dG concentrations (Figure 29).

Table 14. Parameters of the selected model explaining circulating antioxidant capacities in the Egyptian Rousette (n=23; adjusted R^2 = 0,5472) (· p < 0,1; * p < 0.05; *** p < 0.001)

	Estimate	SE	p-value
Intercept	2.907e+02	4.870e+01	9.58e-06***
8OH-dG	5.623e-03	1.111e-01	0.9602
Sex [male]	-1.369e+02	7.123e+01	0.0697 ·
8OH-dG x Sex [male]	3.955e-01	1.457e-01	0.0137 *

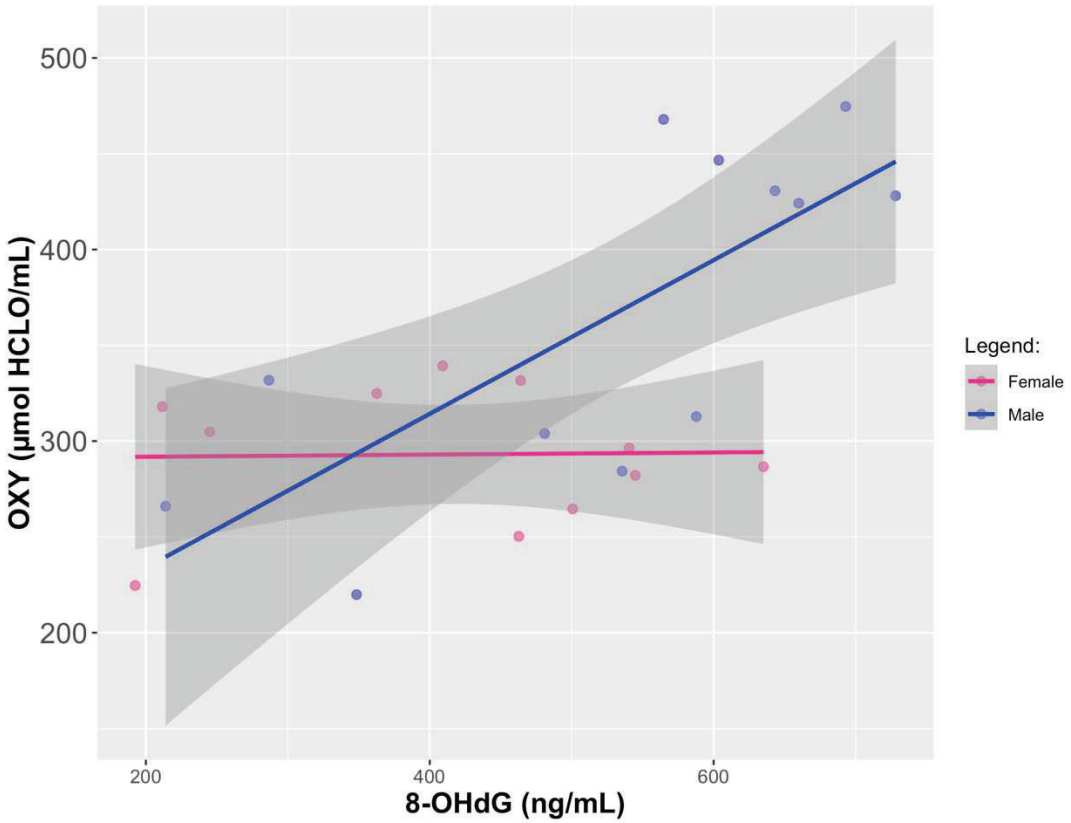


Figure 29. Evolution of plasma's antioxidant capacities with DNA oxidative damage males and females of *R. aegyptiacus* (n=23). Points represent individuals, lines represent the linear regression models and the grey areas the 95% confidence interval of the regressions.

3. Albumin Sequence Comparison Between Egyptian Flying Fox, Chicken and Human

Albumin sequences from Egyptian rousette and human have almost the same length (608 and 609 AA respectively), with 71.4 % sequence identity. In contrast, chicken albumin sequence exhibits a length of 618 AA, with only 45.1 and 47.5 % sequence identity with R. aegyptiacus and human homologues, respectively. Out of the 22 known glycation sites in human albumin, 18 appear to be conserved in the Egyptian Rousette, while only 6 are conserved in the chicken (Figure 30.a.).

Beta-globin chains in R. aegyptiacus and human appeared to also only have 1AA length difference (146 and 147 AA respectively), with 87 % sequence identity. All 6 known glycation sites in human beta-globin appear to be conserved in the Egyptian rousette (Figure 30.b.).

(a)

```
sp|P19121|ALBU_CHICK            MKWVTLISFIFLFSSATSRNLQRFARDAEHKSEIAHRYNDLKEETFKAVAMITFAQYLQR   60
sp|P02768|ALBU_HUMAN            MKWVTFISLLFLFSSAYSRGVF---RRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQ   57
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  MKWVTFISLLFLFSSAYSRGVF---RRDTHKSEIAHRYTDLGEEHFRELVLISFSQYLQN   57
                                *****:**::****** **.:    *   ****:***:.** ** *: :.:*:*:****.

sp|P19121|ALBU_CHICK            CSYEGLSKLVKDVVDLAQKCVANEDAPECSKPLPSIILDEICQVEKLRDSYGAMADCCSK   120
sp|P02768|ALBU_HUMAN            CPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAK   117
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  GPFDEFIKLTNEVNELAKTCVADESAANCDKSLHTIFGDKLCTVASARETYGEVADCCEK   117
                                  ::   **.::* ::*:.***:*.* :*.* * ::: *::* * . *::** :**** *

sp|P19121|ALBU_CHICK            ADPERNECFLSFKVSQPDFVQPYQRPASDVICQEYQDNRVSFLGHFIYSVARRHPFLYAP   180
sp|P02768|ALBU_HUMAN            QEPERNECFLQHKDDNPNLPR-LVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAP   176
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  QEPERNQCFLKNKDDDPNFPK-LVRPSPEVLCTAFQENDKKILDTYLYEISRRHPYFYAP   176
                                 :****:***. * .:*:: :   **  :*:*  :::*  .:*  ::*.::****::***

sp|P19121|ALBU_CHICK            AILSFAVDFEHALQSCCKESDVGACLDTKEIVMREKAKGVSVKQQYFCGILKQFGDRVFQ   240
sp|P02768|ALBU_HUMAN            ELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFK   236
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  ELLYYVKKYKEMLAECCQAADKAACLTPKADDLKSNLLASDAKQRYKCSSLEKFGERPLK   236
                                 :* :.  ::  : .**: :* .***  *   ::.:  . ..**:  *. *::**:* ::

sp|P19121|ALBU_CHICK            ARQLIYLSQKYPKAPFSEVSKFVHDSIGVHKECCEGDMVECMDDMARMMSNLCSQQDVFS   300
sp|P02768|ALBU_HUMAN            AWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSIS   296
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  AWTLAVLSQRYPKAEFMELSKLVTSIVKVQKECCHGDLLECADDREEVIKYMCEHQDSIS   296
                                *  :  ***::*** * *:**:* .   *:.***.**::** **   : . :*.:** :*

sp|P19121|ALBU_CHICK            GKIKDCCEKPIVERSQCIMEAEFDEKPADLPSLVEKYIEDKEVCKSFEAGHDAFMAEFVY   360
sp|P02768|ALBU_HUMAN            SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLY   356
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  DKLKACCDKPLLEKSHCILEMENDEKPNDLPSLTDDYVEDKEVCKNYKEAKDVFLGTFLY   356
                                .*:* **:**::*:*:** * * ** * *****. .::*.*:***.:  .:*.*:. *:*

sp|P19121|ALBU_CHICK            EYSRRHPEFSIQLIMRIAKGYESLLEKCCKTDNPAECYANAQEQLNQHIKETQDVVKTNC   420
sp|P02768|ALBU_HUMAN            EYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNC   416
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  EYSRRHPEYSVSMVLRIAKEYETTLERCCATDDPHACYAKVLDELQVIADEPQKLIKKKC   416
                                **:****::*: :::*:** **: **:** : :*  ***:. ::::   .* *.::* :*

sp|P19121|ALBU_CHICK            DLLHDHGEADFLKSILIRYTKKMPQVPTDLLLETGKKMTTIGTKCCQLGEDRRMACSEGY   480
sp|P02768|ALBU_HUMAN            ELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDY   476
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  DLYESLGEYRFQNALIIRYTKKLPLVSTPSILKFAKEVGNLGGRCCKRPESKRLACAEDY   476
                                :* .. **  * :::::*****:* * *  ::: .::: .:* :**:  * :*: *:*.*

sp|P19121|ALBU_CHICK            LSIVIHDTCRKQETTPINDNVSQCCSQLYANRRPCFTAMGVDTKYVPPPFNPDMFSFDEK   540
sp|P02768|ALBU_HUMAN            LSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHAD   536
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  MSMTLNRLCVLHEKTPVSDKITKCCSGSLVNRRPCFSALEADETYVPKEFNAETFTFHAD   536
                                :*:.::  *  :*.**:.*.:::**:   .******:*: .* .***  ** : *:*. .

sp|P19121|ALBU_CHICK            LCSAPAEEREVGQMKLLINLIKRKPQMTEEQIKTIADGFTAMVDKCCKQSDINTCFGEEG   600
sp|P02768|ALBU_HUMAN            ICTLSEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEG   596
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  VCTLPDDEKILKKQIALVELLKHKPKATEEQLKTVMENFSAFMQKCCTADDKEACFTEEG   596
                                :*:   .*: : :   *::*:*:**: *:**:*:: :.*:*:::***. .* ::** ***

sp|P19121|ALBU_CHICK            ANLIVQSRATLGIGA     615
sp|P02768|ALBU_HUMAN            KKLVAASQAALGL--     609
tr|A0A7J8E5X0|A0A7J8E5X0_ROUAE  PKLVASSQATLA---     608
                                 :*:. *:*:*.
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(b)

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sp|P68871|HBB_HUMAN       MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPK   60
sp|P02058|HBB_ROUAE       -VHLSGEEKAAVTALWGKVKVEEVGGEALGRLLVVYPWTQRFFDSFGDLSSASAVMSNPK   59
                           ***: ***:*********:*:********************:******: .***.***

sp|P68871|HBB_HUMAN       VKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFG   120
sp|P02058|HBB_ROUAE       VKAHGKKVLDSFSEGLQHLDSLKGTFAKLSELHCDKLHVDPENFRLLGNVLVCVLARHFG   119
                           *********.:**:** ***.******.****************************:***

sp|P68871|HBB_HUMAN       KEFTPPVQAAYQKVVAGVANALAHKYH    147
sp|P02058|HBB_ROUAE       KEFTPQVQAAYQKVVAGVATALAHKYH    146
                           ***** *************.*******
```

Figure 30. Amino acid sequences comparison of chicken, human and Egyptian rousette of (a) albumin and (b) beta-globin. (*): positions that have a single and fully conserved residue. (:): conservation between groups of strongly similar properties (with a score higher than 0.5 in the Gonnet PAM 250 matrix). (.): conservation between groups of weakly similar properties (with a score lower or equal to 0.5 in the Gonnet PAM 250 matrix). AAs highlighted in purple indicate the known glycation sites of human albumin and whether they appear to be present in the Egyptian rousette and the chicken.

Table 15. Summary table of correlations found in this chapter

Species	Glycemia	GHb	GA	DNA damage	Antioxidants
Rodrigues Flying Fox	(-) males	(+) Age	No relation	(-) males	(+) GHb
Egyptian fruit bat	No relation	(+) males	NA	(+) antioxidants (-) old individuals	(+) DNA damage in males

(+) and (-) indicate a positive and a negative correlation, respectively. NA means that we have not conducted an analysis on this specific glycation marker (GA in the Egyptian Fruit Bat).

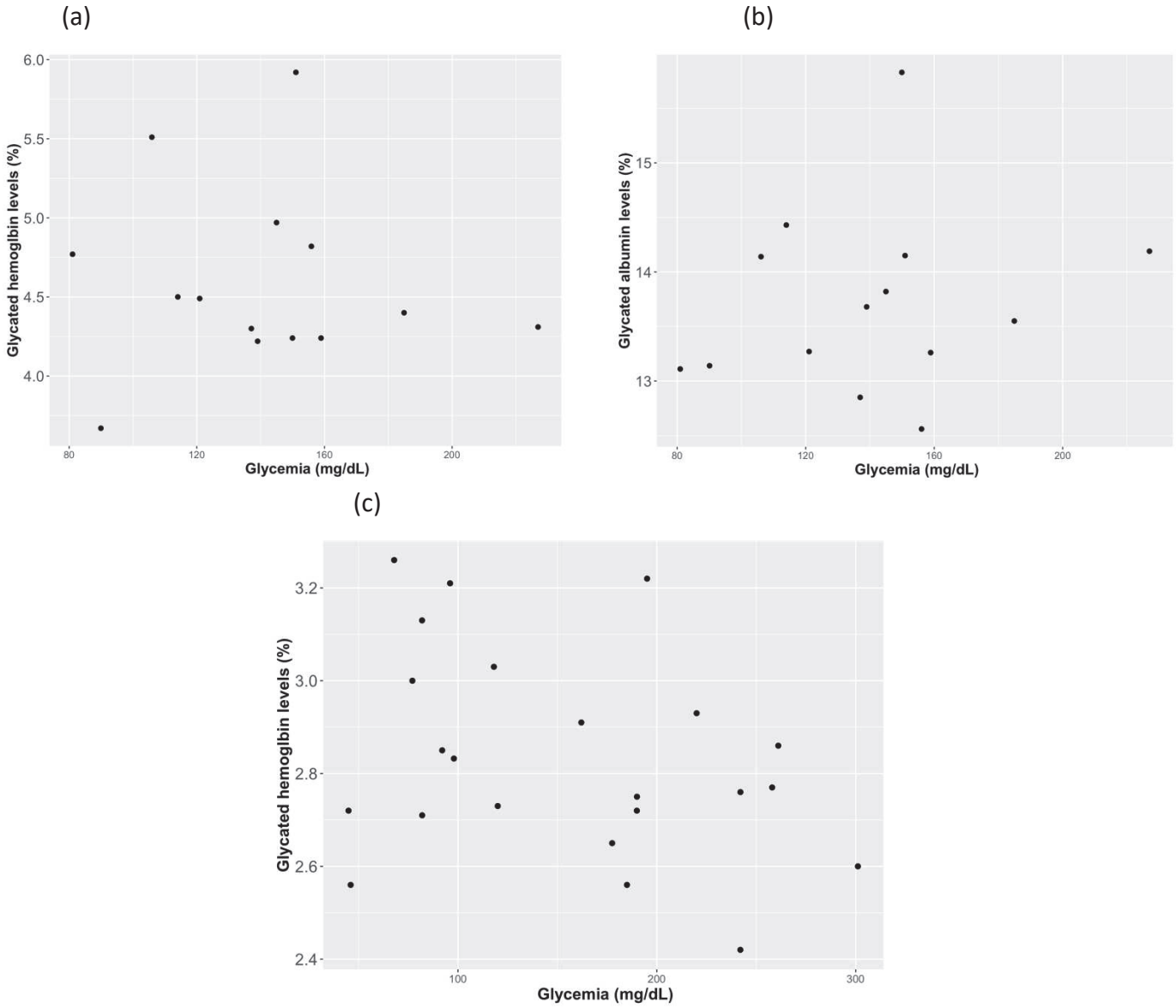

Figure 31. Intraspecific relationships between glycemia and (a) glycated albumin and (b) glycated hemoglobin levels in the Rodrigues flying fox (*Pteropus rodricensis*), and between (c) glycemia and glycated hemoglobin levels in the Egyptian fruit bat.

IV. Discussion

1. Glycated Albumin and Glycated Hemoglobin Levels as Markers of Individual Quality in Two Species of Fruit Bats

In this chapter, we have shown that there is no link between glycated protein levels and glycemia in the two species of fruit bats *Rousettus aegyptiacus* and *Pteropus rodricensis*, whatever the glycation marker considered (glycated hemoglobin or glycated albumin). As the glycation reaction is non-enzymatic, a positive correlation would be expected. This lack of relationship thus suggests a decoupling of blood glucose and glycation in these two species.

a) No Apparent Structural Resistance of Albumin and Beta-Globin at the Species Level in the Egyptian Fruit Bat

A first hypothesis to explain this lack of correlation could be the existence of resistance to protein glycations in these species. This is one of the suggestions that also emerged from Chapter 1, where bats overall had lower glycated albumin levels than other mammals. To test this hypothesis, we aimed to compare the protein sequences of our bats with those of humans, as well as with chicken in the case of albumin. Although we did not have these sequences for the Rodrigues flying fox, a comparison of both hemoglobin and albumin sequences from Egyptian rousette with human ones revealed that they were very similar, and that most known glycation sites in humans are conserved in bats. Furthermore, it appeared that chicken albumin sequences were very different from those of *R. aegyptiacus* and human. At first glance, it therefore seems that there is no specific structural resistance to glycation of albumin or beta-globin in the Egyptian fruit bat, based on amino acid sequences. However, to really conclude on this subject, we need to look at the 3D conformation of these two proteins. Taking the example of chicken albumin, we know that their lysine residues are less exposed to circulating plasma compared to bovine serum albumin [155]. We might have a similar explanation for the Egyptian fruit bat, which has most of the known glycation sites in humans, yet shows slightly lower glycation levels.

b) Existence of Variability in Individual Tolerance to Glycation Within The Rodrigues Flying Fox and the Egyptian Rousette Suggests That Glycated Protein Levels are Likely to Be a Marker of Individual Quality

To gain insights on the absence of correlation between glycated protein levels and glycemia in our two bat species, given that there does not seem to be a structural resistance of proteins to glycation at the species level, we thus had a look at the graphs showing the evolution of glycated albumin and glycated hemoglobin levels as a function of blood glucose within the two species (Figure 31). It can be clearly seen that, for both species and whatever the glycation marker considered, some individuals with high blood sugar levels do not present high glycation levels, while others, conversely, have high blood sugar levels and high glycation levels. It appears that some individuals are better able to manage the trade-off between high energy intake (beneficial for flight) and the potential adverse effects this can have (protein glycation). This inter-individual variability suggests that glycated protein levels are more likely to be a marker of individual quality within the Rodrigues flying fox and the Egyptian fruit bat. We are therefore going to look at what might explain this difference in individual quality (intraspecific scale) in terms of protein glycations.

Among the parameters frequently studied and known to reflect individual quality we have body mass. In most mammalian species, greater body mass means better body condition, better access to resources and better health. However, our analysis did not highlight the existence of any significant correlation between GHb and body mass in both bat species (or GA levels in the Rodrigues flying fox). As presented in the general introduction of this manuscript, the results of intraspecific studies linking glycated protein levels with body mass are quite discrepant, with some species exhibiting positive correlation between the two parameters (such as with GA in the European roe deer, see Chapter 3), while others display an absence of correlation (such as in *F. albicollis* when considering GHb [89]). It is nevertheless interesting to note that in the Rodrigues flying fox, glycated hemoglobin levels significantly increased with age. A larger sample size is needed to test whether this may be attributed to an altered body condition in old individuals, or on the contrary to a positive selection of individuals with higher GHb levels, that may reflect altered glucose metabolism but also better

access to food resources of individuals living longer. We therefore considered a second parameter known to reflect individual quality differences in many species: oxidative stress.

2. Contrasted Relationship Between Oxidative Stress and Glycation in Two Species of Fruit Bats

As presented in the introduction to this chapter, glycations contribute through various mechanisms to an increase in oxidative stress. However, we have also mentioned the existence of studies suggesting that bats are tolerant to oxidative stress. We might therefore assume that tolerance to glycations in bats is achieved through, among other things, tolerance to oxidative stress. Furthermore, we know that oxidative stress also reflects the quality of an individual. We therefore looked at how levels of glycated proteins and oxidative stress (both the amount of oxidative DNA damage and the capacity of the antioxidant barrier) correlate on an intraspecific scale in the robin.

a) Glycated Hemoglobin Levels and Oxidative Stress Do Correlate in The Rodrigues Flying Fox

In our study on *P. rodricensis*, we found a positive correlation between antioxidant capacities and glycated hemoglobin, but no significant association between 8OH-dG and GHb. This result suggests a link between glycation and oxidative stress, at least in the blood. Indeed, as exposed in the introduction, glycation is known to contribute to oxidative stress through various mechanisms. It seems therefore coherent that increased glycation levels are associated with elevated antioxidant capacity to mitigate oxidative stress.

The absence of a relationship with 8OH-dG despite the positive correlation with OXY may thus indicate that we actually did not assess the appropriate marker to quantify oxidative damage. It is possible that glycated hemoglobin does not contribute to increased DNA oxidative damage but instead affects other types of cellular damage. Bats are known to possess efficient DNA repair mechanisms. For instance, a study on the little brown bat demonstrated enhanced DNA repair following gamma-induced DNA lesions [230]. As a consequence, 8OH-dG may not be a

valuable stress marker in *P. rodricensis,* or in bat species more generally, even if the examination of alternative damage markers, such as reactive oxygen metabolites concentrations in plasma (ROMs), could provide additional insights about the relation between glycation and oxidative damages. Alternatively, our results may suggest that glycation is rather the stress marker of importance that needs to be buffered in priority in these bats, given the expected positive link with antioxidant capacity.

b) Absence of Correlations Between Oxidative Stress and Glycated Hemoglobin in The Egyptian Fruit Bat: A Fact, or a Wrong Targeting of the Markers Studied?

Interestingly, in *R. aegyptiacus*, we did not observe any correlations between oxidative damage and glycation levels, nor between antioxidant defenses and glycations. Several hypotheses could explain these findings: (1) glycation and oxidative stress may not be interconnected at all in this species or (2) this species might experience low levels of oxidative damage overall, thus preventing any correlation with increasing glycation levels.

Several studies have indicated that *R. aegyptiacus* exhibits reduced oxidative stress compared to other mammals. One study demonstrated that even under experimentally induced bacterial infection, various markers of oxidative status (both damage and defenses) did not significantly increase, while inflammation did [303]. Previous research on fruit bats have shown that they possess higher levels of circulating antioxidants than omnivorous or insectivorous/carnivorous species, resulting in lower oxidative damage [245,298]. It has been suggested that these elevated antioxidant levels may originate from their fruit-based diet (as fruits are rich in antioxidants), and that these could be stored in tissues like the liver, allowing them to better manage oxidative bursts generated during normal physiological processes.

Interestingly, some authors have suggested that the direct contribution of Amadori products—specifically glycated hemoglobin HbA1c in humans—to plasma oxidative stress is likely minimal compared to other glycation products such as AGEs or glucose-derived reactive intermediates like methylglyoxal (which ultimately lead to AGE formation) [61]. Thus, the absence of a link between glycated hemoglobin and oxidative stress in the Egyptian fruit bat could be explained

by our choice of glycation marker, which might not be the most relevant in relation to oxidative stress. Additionally, in bats, oxidative damage may not be best evaluated in plasma, as a study on five South American bat species showed that levels of SOD and catalase—two key antioxidant enzymes—were higher in blood compared to other tissues [298]. Therefore, it may be beneficial to explore oxidative damage in other tissues.

3. Sex Differences in Resistance Towards Glycation and Oxidative Stress

We did find differences between males and females, both in terms of glycated protein levels and oxidative stress. It seems that females are more tolerant to both phenomena than males. What could explain this apparent greater tolerance on the part of females?

a) Higher Resistance Towards Protein Glycation in Females

In the Egyptian Rousette, no significant difference in glycemia was observed between males and females. However, males exhibited significantly higher glycation of hemoglobin compared to females. This supports our initial hypothesis that males would present higher glycation levels compared to females, suggesting that males may have a lower resistance to hemoglobin glycation.

In mammals, males generally have shorter lifespans than females [301,302,311], and one may thus suspect that selection pressure has led to physiological differences between sexes. A reduced resistance to protein glycation in males may indeed represent one such physiological divergence. Evolutionary pressures, particularly in mammal species with slower life histories such as bats or human, may have favored longevity in females through the selection of specific physiological mechanisms such as glycation resistance mechanisms.

In the Rodrigues flying fox, females displayed significantly higher glycemia than males, but no significant differences were found between sexes in terms of GHb or GA levels. If both sexes exhibited equal resistance to glycation, we would have expected females, with their higher

glycemia, to also show higher glycation levels. The fact that females did not exhibit increased glycation, despite elevated glycemia, suggests that they may possess enhanced protective mechanisms against glycation of hemoglobin and albumin, further highlighting the potential increased vulnerability of males to glycation. Still males have lower DNA damage levels (see below), which would suggest that the expected glycemia-induced oxidative stress may be induced by alternative pathways not implying glycation processes, at least according to the glycation markers we used here (see Figure 1).

The question arises of why females should be more resistant to protein glycation than males. Recently, a hypothesis concerning the links between oxidative stress and reproduction in females has been attracting increasing attention: the shielding hypothesis. It postulates that it would be deleterious for the mother to transmit oxidative damage to her offspring, and that consequently females would produce more antioxidants at the time of reproduction and before lactation, in order to mitigate their oxidative stress and thus protect their offspring [312]. This theory has been verified and validated in several mammalian species, such as the Columbian ground squirrel (*Urocitellus* columbianus) [313] or the banded mangoose (*Mungos mungo*) [314], explaining why at the time of reproduction, females present lower rates of oxidative damage than males. It is conceivable that a similar mechanism exists for glycations. It could be that, at the time of reproduction, females develop increased resistance to protein glycations (more competitive inhibitors, more production of deglycating enzymes, etc.), thus preventing the transmission of glycated proteins to their offspring. This hypothesis could be tested in our two bat species by longitudinal monitoring of females. We could measure the amount of glycated proteins and AGEs in different tissues of females (as well as in the milk during lactation) and see how these levels vary according to the female's reproductive stage.

Studies on the links between glycation product levels and sex in non-conventional animal models remain scarce. No association between glycated hemoglobin and sex was found in domestic dogs and in two species of birds (*Falco sparverius* and *Ficedulla albicollis*), nor between total plasma glycated proteins and sex in the zebra finch or between GA and sex in wild roe deers (see Chapter 3). However, we have a greater number of studies on the subject in humans. Our results in bats are indeed coherent with previous studies, where it was shown that women had slightly lower HbA1c levels compared to men [315,316]. However, studies in

diseased patients, such as type 2 diabetes, highlighted the opposite, with women having a poorer glycemic control and higher levels of HbA1c compared to men [317]. These seemingly opposite results should perhaps be seen in the light of other studies. Carrera et al. [98] indeed showed that young women had lower HbA1c levels compared to young man, and that this difference between sex tended to be reduced while aging, even reversing the trend in the over-70 age group. This suggest that there may well be a difference in glycation tolerance between sexes, whose direction may also be age dependent. Although we did not find any interactions between sex and age in our two bat species, our sample size remains relatively small, and one cannot rule out that this interaction could emerge with a larger sample size. So it would also be interesting to look at how protein levels differ between males and females of different age groups, more specific than just “juveniles” or “adults” (as we have done here, and which did not point out any particular differences).

b) Sex Differences in Oxidative Stress

We also observed sex differences in oxidative stress between males and females in both species. In the Rodrigues flying fox, females exhibited higher oxidative damage than males, but antioxidant capacities did not differ between sexes. Similar results have been shown in human, with women having more reactive oxygen metabolites (ROMs) than men, while no difference was noted in the antioxidant defenses [318]. On the contrary, in Wistar rats and *Drosophila melanogaster*, two species in which females typically outlive males, higher antioxidant defenses and lower reactive oxygen species (ROS) were found in females [319,320].

As for the Egyptian rousette, whereas no difference in 8OH-dG concentrations were noted between sexes, the correlation between oxidative damage and antioxidant barrier however appeared to differ between males and females. Female antioxidant capacities remained stable despite increasing DNA oxidative damage, unlike in males, where a strong positive correlation appeared.

In view of all that has been said so far, we can put forward two hypotheses to explain these observations: either the females have a higher tolerance to the deleterious effects of oxidative stress, or we are missing information linked to the choice of our oxidative stress markers. Indeed, we have only looked at the non-enzymatic component of antioxidant defenses, but we

could perhaps detect other correlations by focusing on other components of antioxidant defenses.

Many studies have highlighted the existence of sex-differences in lifespan in animal species, however, sex-differences in aging rates and senescence-related mechanisms have been much less studied in non-conventional animal models. An extensive study on sex differences in lifespan and aging rates among mammals highlighted that the longer lifespans observed in females were not necessarily due to lower aging rates compared to males, but more likely to complex interactions between environmental conditions and sex-specific costs [311]. Once again, it seems that the differences observed between the sexes, particularly with regard to senescence rates, are species-specific. Indeed, a previous study in the greater sac-winged bat (*Saccopteryx bilineata*) highlighted that senescence was sex-biased in this species, with males exhibiting higher rates of senescence in annual individual fitness and in reproduction than females [321]. Thus, the fact that we observe differences in oxidative stress between the sexes in our two species could well also be due to a senescence rate that differ between males and females. That said, studies evaluating average longevities in males and females of these two species are still needed, as well as more studies on other parameters relevant to senescence.

4. Conclusion

To our knowledge, this is the first intraspecific exploratory study of glycations in bats.

Inter-individual differences in tolerance and/or resistance to glycations appear to exist within the two species, indicating that glycated protein levels may be a marker of individual quality in these bats. This would be consistent with similar deductions made within other wild animal species, such as Alpine swifts (ref) or roe deers (Chapter 3), for which it has been suggested that glycated albumin would reflect an individual body condition.

For at least one of the two fruit bat species, namely the Rodrigyez flying fox, glycation tolerance seems to be (at least partly) due to a higher antioxidant barrier. The differences observed between these two fruit bat species regarding the relationships between glycation and oxidative stress suggest significant interspecies variation in both glycation metabolism and oxidative stress tolerance. Indeed, several studies have highlighted substantial differences in oxidative damage and antioxidant defenses between bat species [299,322].

To further investigate these results, it would be interesting to carry out similar studies in these two species using other glycation markers (such as AGEs), as well as other oxidative status markers, in tissues rather than blood (that we have collected in the Flying fox). Finally, a deeper understanding of the differences in longevity and senescence rates between males and females in bats would enable us to shed a new light on these results

Chapter 3 - Glycated albumin levels in roe deer: a marker of body condition which is influenced by environmental quality

Juvenile European Roe Deer Capreolus capreolus (photo by Paul Revelli)

This chapter is the subject of an article currently in preparation, soon to be ready for submission. This is what is presented below, with some slight modifications to better suit the thesis structure.

Glycated Albumin Levels in Roe Deer: a Marker of Body Condition Which Is Influenced by Environment Quality

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[Article in Preparation]

I. Introduction

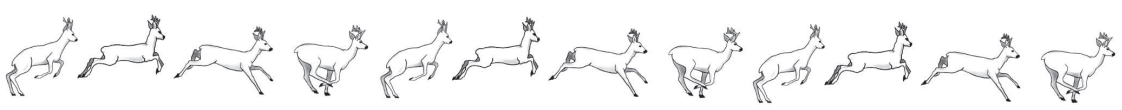
Senescence is defined as the progressive deterioration of the structural integrity and function of cells and tissues with age, ultimately leading to a decline in fertility and survival (reproductive and actuarial senescence, respectively) [5]. Senescence patterns vary considerably in terms of age of onset and rate from one species to another, as well as between individuals of a given species [16]. Such variability has been explained by sex [17], as well as by various genetic [323,324], epigenetic [325] or environmental factors (e.g., sociality level) [326]. Recently, the number of studies aimed at identifying the physiological processes underlying senescence in wild animal populations has risen sharply, involving, for example, mitochondrial dysfunction, oxidative stress and telomere attrition [30,327]. Glycation, a spontaneous non-enzymatic reaction of free reducing sugars with free amino groups of proteins, DNA, and lipids has been recognized as an aging reaction [328], which is now also receiving particular attention in senescence studies.

The binding of one or more reducing sugars to proteins, originally known as the Maillard reaction [31] and referred to here as protein glycation, leads to the formation of so-called Amadori compounds. Some Amadori compounds are essential biomarkers in human medicine, such as glycated haemoglobin HbA1c and glycated albumin (GA), which reflect blood glucose levels over the previous two weeks and one month, respectively. HbA1c and GA are therefore used to monitor diabetes in humans [38,329]. Importantly, Amadori compounds can undergo further irreversible reactions to finally form stable advanced glycation end-products (AGEs) [159,330], which tend to accumulate in cells and tissues over time [331].

When subjected to glycation, proteins undergo conformational changes [58], leading reduced functionality and subsequent alterations in various signalling and metabolic pathways. As a result, glycation products have been proven to contribute to several aging-related diseases, such as Alzheimer, Parkinson or diabetes complications, to name a few [332]. There is also an increasing body of evidence supporting the role of glycation products in the process of cellular senescence [54,63]. AGEs and Amadori compounds have notably been shown to interfere with different physiological and cell mechanisms such as oxidative stress [331], chronic inflammation [62] or telomere attrition [333].

To date, the vast majority of research on glycation has only been carried out in humans or in certain laboratory animal models. As recently reviewed [221], glycation and its link to senescence has hardly been studied in non-conventional animal models under natural conditions. However, this could provide key mechanistic information for our understanding of the variability of senescence patterns. The relevance of such studies would be furher enhanced by the fact that the influence of glucose and glycation on certain life-history traits, e.g., clutch size [89] and growth rate [90], has already been documented in several bird species. Interestingly, pentosidine (a cross-linking AGE) levels correlated positively with chronological age among several mammal species [44], but this was not the case of GA levels in the zebra finch (*Taeniopygia guttata*) [87] or of glycated hemoglobin (GHb) levels in the collared flycatcher (*Ficedula albicolis*) [89] and the domestic dog [99].

Bearing in mind that glycation studies in mammals remain scarce and studies in the wild are expected to open the way to innovative treatments for diseases such as diabetes in humans [221], we investigated whether protein glycation (assessed through glycated albumin and glycated haemoglobin) could be related to senescence in two contrasted wild populations of roe deer (*Capreolus Capreolus*), where reproductive [201] and actuarial senescence [334,335] have both been documented. We notably tested whether glycation profiles could be explained by environmental factors (living conditions and sampling year) and individual factors (sex, body mass, glycemia and age). We hypothesized that, if glycations contribute to senescence in roe deer in the same way as they do in humans, then glycated protein levels would be higher in older individuals. Because glycation is a spontaneous reaction often described as being positively correlated with glycemia [51,52], we performed the same tests on glycemia, in a hope to gain insights on the glycation results. As with most mammals, males live shorter and senesce faster than females in roe deer [311,336]. If protein glycation underpins the senescence process in roe deer, we would expect males to have higher levels of glycation products than females (for a same age), as has already been observed in human patients suffering from age-related diseases [104] or in Sprague–Dawley and stroke-prone rats [106]. Moreover, while no studies to date have looked at the impact of the environment on glycation levels, we do know that blood glucose levels can be influenced by environmental factors such as ambient temperature, day length or food availability, the latter being positively correlated to glycemia in stripped mice [81]. Since Amadori compounds are expected to positively correlate with blood glucose levels [80,86], we also expect glycation levels to vary between



individuals facing different environmental conditions. We thus expected that in roe deer, the population living in the environment of highest quality (more food resources, less parasite pressure, milder climatic conditions, etc.), where individuals display higher markers of body and health condition for a given age (eg. body mass [337]; telomere length, [338]), have developed some sort of resistance towards glycation (that we define as the mechanisms which prevent proteins to be glycated or allow their fast elimination before transforming into AGEs), and therefore present lower rates of glycation. In these two roe deer populations, year quality is assessed on the basis of annual survival data for juveniles born that same year (which is considered to be an integrative estimator of the overall quality of the year) [339], particularly in terms of food resources. Thus, a poor quality year will result in low juvenile survival rates, as opposed to a good year which will see higher survival rates. Therefore, in the same mindset as for habitat quality, we expected that better years (= higher juveniles survival rates) would allow individuals to have an overall better body and health condition, which would result in lower glycation levels. In addition, we tested whether glycated albumin levels co-vary with several haematological parameters known to decline with age and reflect health status and body condition [340,341].

II. Materials & Methods

1. Study Populations

We focused on two wild populations of roe deer *(Capreolus capreolus)* displaying contrasted environmental conditions and managed by the Office Français de la Biodiversité (OFB, formely Office National de la Chasse et de la Faune Sauvage). The "Territoire d'Etudes et d'Experimentation de Trois-Fontaines", which is located in the North-East France (48°430N, 4°550E), benefits from a continental climate, with cold winters and warm, rainy summers. Rich soils guarantee abundant and diverse food resources, resulting in a high-quality habitat for roe deer. On the other hand, the "Réserve Biologique de Chizé", located in western France (46°050N, 0°250W), has a temperate oceanic climate with Mediterranean influences, resulting in mild winters and frequent summer droughts [342]. The result is poor soil quality, limited food resources and a generally poor quality habitat for roe deer.

2. Data Collection

The Trois-Fontaines (TF) and Chizé (C) populations have been intensively monitored by capture-mark-recapture (CMR) since 1975 and 1977, respectively. Captures take place over 10–12 days each year, from mid-December to mid-march March [201]. All captured individuals are fitted with individually recognizable eartags and/or collars (either numbered, VHF or GPS collars), given a unique identifier and closely monitored throughout their lives by subsequent winter captures and field observations. Once captured (using a drive netting procedure), the identity, sex, body mass (±50 g) and age (see below) are recorded. We then collect biological samples from individuals whose exact age we know, as they were captured either as newborns in the spring (see [343] for more details) or at around 8 months of age during winter captures, when they still have their deciduous teeth (most often incisors and always premolars, [344]).
We focused here on samples from two capture sessions (2021 and 2022) and for each study site, we selected an equal number of males and females of known age. We ended up with a total of 48 samples, corresponding to 46 individuals (two were sampled in both 2021 and

2022). The age ranges of the different groups are as follows: TF-females: 1-10 years old (mean ± SD = 5.1 ± 3.5); TF-males: 1-7 years old (mean ± SD = 5.1 ± 3.4); C-females: 1-15 years old (mean ± SD = 5.7 ± 4.1); C-males: 1-10 years old (mean ± SD = 5.7 ±3 .9). Blood samples (1 mL/kg to a maximum of 20mL) were collected from the jugular vein in 20 mL syringes attached with sterile hypodermic needles (gauge 19, 1.1 x 38mm). The blood was then distributed either into tubes containing a coagulation activator, which were used to prepare serum for immuno-hematology assays, or into heparin-lithium collection tubes (Vacutainer, BD Medical) to prepare plasma for subsequent assays of glucose concentration and protein glycation rates. Within 30 min of sampling, the tubes were centrifuged at 3000 *g* for 10 min and the serum and plasma were transferred into cryogenic vials. Pellets from the heparin-lithium collection tubes containing red blood cells (RBCs) and leucocytes were then washed with an equivalent volume of 0.9% w/v NaCl solution. After a second centrifugation, we removed the buffy coat intermediate layer, mainly composed of leukocytes, and kept the washed RBCs. All the samples were immediately frozen on site at −80°C in a portable freezer (Telstar SF 8025) until analyses. Serum proteins were separated and quantified thanks to an automatic gel electrophoresis procedure (HYDRASYS, Sebia, Evry, France), which enabled us to obtain albumin (ALB), alpha-1-, alpha-2-, beta- and gammaglobulin data (expressed in g/L). Creatinine (CREA, in µmol/L) was measured by enzymatic photometric determination at 540 nm (reagents: creatinine (enzymatic) 98 18 45, Thermo Electron SAS) [340,341].

3. Glycemia and Protein Glycation Rate Measurements

Plasma glucose concentration was measured using a Contour Plus® 115 glucometer (Ascensia diabetes solutions, Basel, Switzerland), while protein glycation levels (for albumin in plasma and hemoglobin in RBCs) were assessed by liquid chromatography-mass spectrometry (LC-MS) analysis, as previously described [87] (see General Materials & Methods).

4. Statistical Analysis

All the analyses were performed using R version 4.3.1 [256]. We did not correct body mass by capture date in juveniles nor adults, since we did not detect any correlation between capture date (in Julian days) and body mass (p=0.595). We first aimed at identifying the best function describing the association between glycemia and chronological age. Following a classical approach in senescence studies [345], we fitted four different models characterized by (1) an absence of association with age (i.e., constant model), (2) a linear effect of age, (3) a quadratic effect of age and (4) a linear effect of age upon a threshold value using segmented function from the package *segmented* [346]). We used Akaike Information Criterion corrected for small sample size (AICc) to retain the most parsimonious model. We selected the model with the lowest AICc, but when the difference in AICc (denoted ΔAICc) between two competing models was less than two units, we retained the simplest model in accordance with parsimony rules [264].

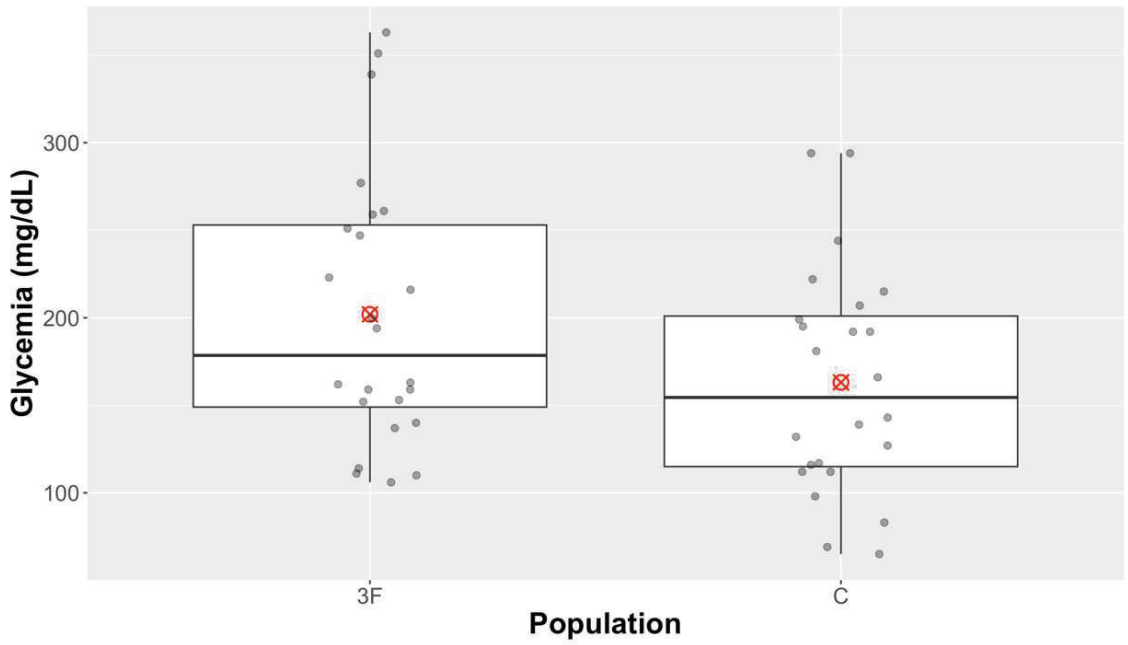
We did not detect any glycated haemoglobin in roe deers' blood samples (see results section below), so we were only able to carry out analyses on glycated albumin To assess changes in GA levels in relation to population, sex, body mass and year of capture, we used a linear model. GA was entered as response variable, while the other variables were input as fixed effects, with all two-way interactions between them (age was not included in the set of independent variables, as the above-mentioned analysis revealed no age effect on GA levels, see results below and supplementary material in Appendix n°4). Because we know that glycation levels positively correlate with glycemia in humans, we decided to include the latter as an additional covariate in our model. Three-way interactions were omitted to avoid model over-fitting. To select the best model, we used an automatic selection procedure (*dredge* function from the MuMIn package [309]). To avoid fitting over-parameterized models, we limited the number of variables to be tested in the models to 4, which ensured a ratio (sample size n / number of independent factors) greater than 10. We selected the best model on the basis of the AICc following the procedure described above [264]. The normality and homeodasticity of residuals were verified through standard residual plot techniques along with a Shapiro-Wilk normality test, and the goodness-of-fit through calculating the conditional (total variance explained by the best model) and marginal (variance explained by fixed effects alone) R^2.

We followed the exact same two-step procedure for the glycemia analyses (again age was omitted from the full initial model due an absence of relationship with glycemia, see supplementary material in Appendix n°4). As before, all two-way interactions were included.

For the second part of this study aiming at assessing the relationships between glycation levels and haematological parameters previously proven to undergo senescence in roe deer [340,341], we first conducted a Principal Component Analysis (PCA) on those parameters, in order to reduce the number of variables to include in our model (thus avoiding overfitting). We then included the two principal components (PCs) retained (eigenvalues greater than 1) (see results below) as fixed factors in a linear regression model, with glycated albumin as the response variable. Because we wanted to investigate if this relationship might differ between populations, we added the two-way interactions between population and the respective retained PCs. The best model was selected in the same way as for the first part of the analysis described above.

In our dataset, two individuals were sampled twice. To account for the pseudo-replication issue and test the robustness of our models, we ran all selected models with the individual as a random factor. If the results were unchanged (see supplementary material in Appendix n°4), we present the selected models without the individual random factor for simplicity.

(a)



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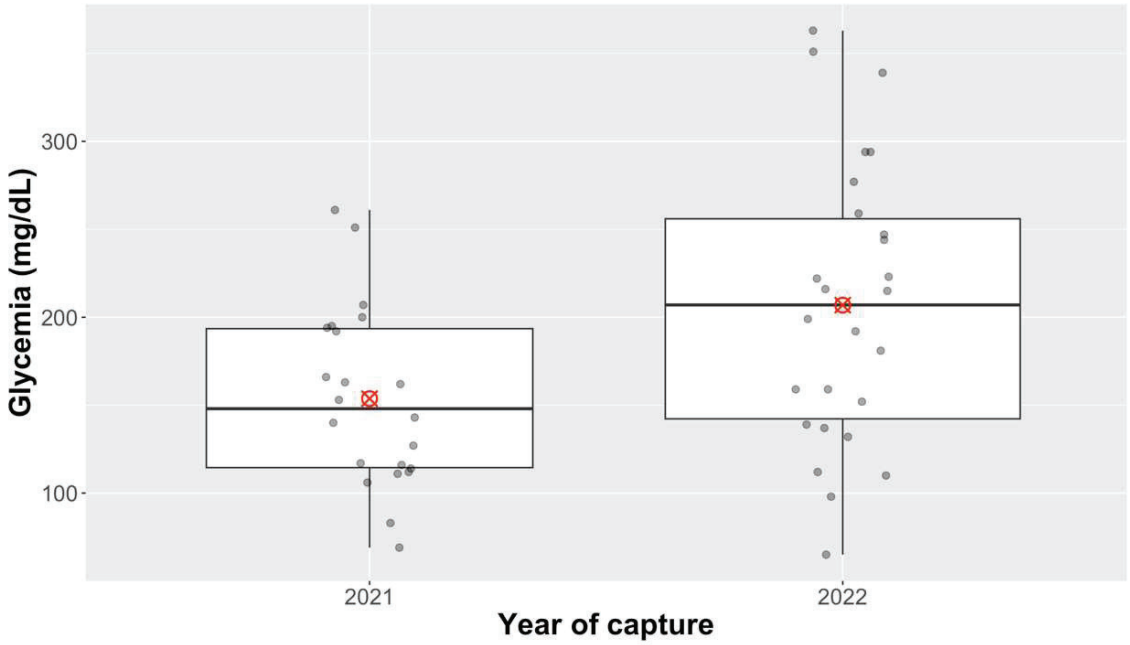


Figure 32. **Factors significantly explaining glycemia in roe deers.** (a) Difference in glycemia level between the two roe deer populations (Trois-Fontaines and Chizé). (b) Difference in glycemia level between the two year of sampling (2021 and 2022). Grey points represent samples, red crossed circles represent the mean glycemia for each population (a) and year (b).

III. Results

1. Blood Glucose and Protein Glycation Levels Do Not Correlate with Age

Glycemia ranged from 65 mg/dL to 363 mg/dL across our all our samples, with a mean value ± SD of 182.5 ± 10.5 mg/dL. For GA, we detected albumin forms only bearing a single glucose molecule. GA levels (expressed as a percentage of total albumin) ranged from 22.3 % to 28.6 %, with a mean ± SD of 25.7 ± 0.2 %. Interestingly, we did not detect glycated haemoglobin in any of our samples. Contrary to our predictions, we did not detect any effect of age on both glycemia and glycated albumin (i.e., the constant model was selected in both cases, Table S1 and Table S2).

2. Factors Influencing Glycemia and Glycated Albumin Levels

When testing for the effect of population, sex, body mass and sampling year of capture (and their two-way interactions) on glycemia, both the population (Figure 32.a.) and year of capture (Figure 32.b.) factors were retained in the best model (p = 0.005529; Table S3), with individuals from Trois-Fontaines, i.e., leaving in a high quality habitat, exhibiting significantly higher glycemia (201.96 ± 77.42 mg/dL) than those from Chizé, i.e., leaving in a poor quality habitat (163.08 ± 64.32 mg/dL). However, when adding "individual" as a random factor to account for the fact that two individuals were sampled twice in our dataset, the effect of the population becomes marginally-significant (p=0.062) (Table 16) .

Table 16. Parameters of the selected model explaining glycemia in wild roe deer (n[samples] =48) Marginal R^2/ Conditionnal R^2 = 0.183/0.610) (· p < 0,1 ; * p < 0.05).

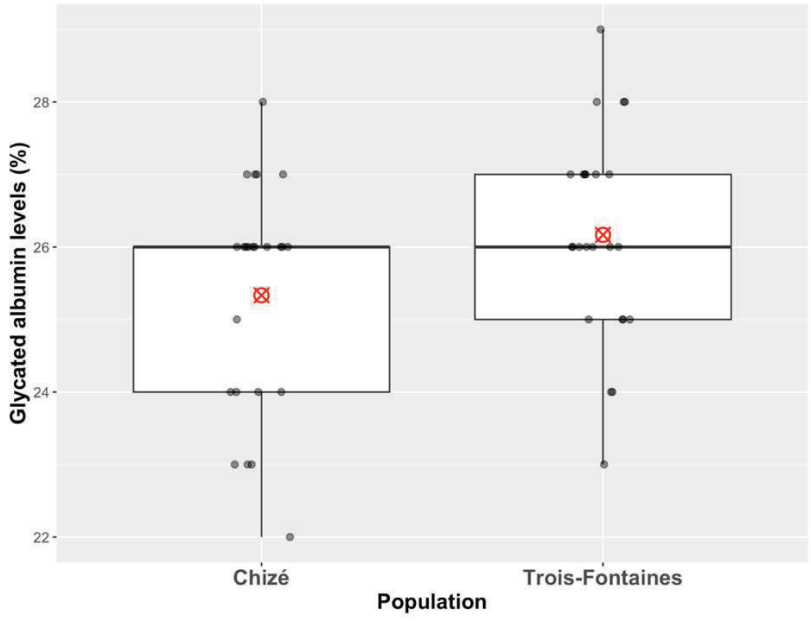
Fixed effets	Estimate	SE	t
Intercept	175.34	17.12	10.243 *
Population [Chizé]	-37.90	19.58	-1.935 ·
Year of capture [2022]	49.97	18.54	2.696 *
Random effect	**Variance**	**SD**	
ID (N=46)	2334	48.32	
Residuals	2136	46.21	

Differences in GA levels among individuals were significantly explained by the interaction population x year of capture (Table 17, Figure 33, Table S4). Among the population of Chizé, individuals exhibited markedly more GA in 2022 (24.5 ± 1.6 %) than in 2021 (26.0 ± 1.3 %,). In 2021 however, individuals from Trois-Fontaines showed higher GA levels than individuals from Chizé (26.7 ± 1.3 % and 24.5 ± 1.6 %, respectively). When considering both years, roe deers in Trois-Fontaines had more GA than those from Chizé (26.2 ± 1.4 % and 25.3 ± 1.6). GA levels also showed a trend towards a positive correlation with body mass (Table 17 and Figure 33). However, GA levels did not significantly correlate with glycemia. These relationships were still significant after adding individual as a random factor (Table S5).

Table 17. Parameters of the selected model explaining glycated albumin levels in wild roe deer (n=48 ; R^2 = 0.3009) (· p < 0,1 ; ** p < 0.01 ; *** p < 0.001)

Predictor	Estimate	SE	t	p-value
Intercept	22.45723	1.16884	19.213	<2e-16 ***
Body Mass	0.10936	0.05735	1.907	0.06322 ·
Population [Chizé]	-1.88307	0.59881	-3.145	0.00301 **
Year of capture [2022]	1.39180	0.55625	2.502	0.01623 *
Population [Chizé] x Year of capture [2022]	2.47930	0.78530	3.157	0.00291 **

(a)



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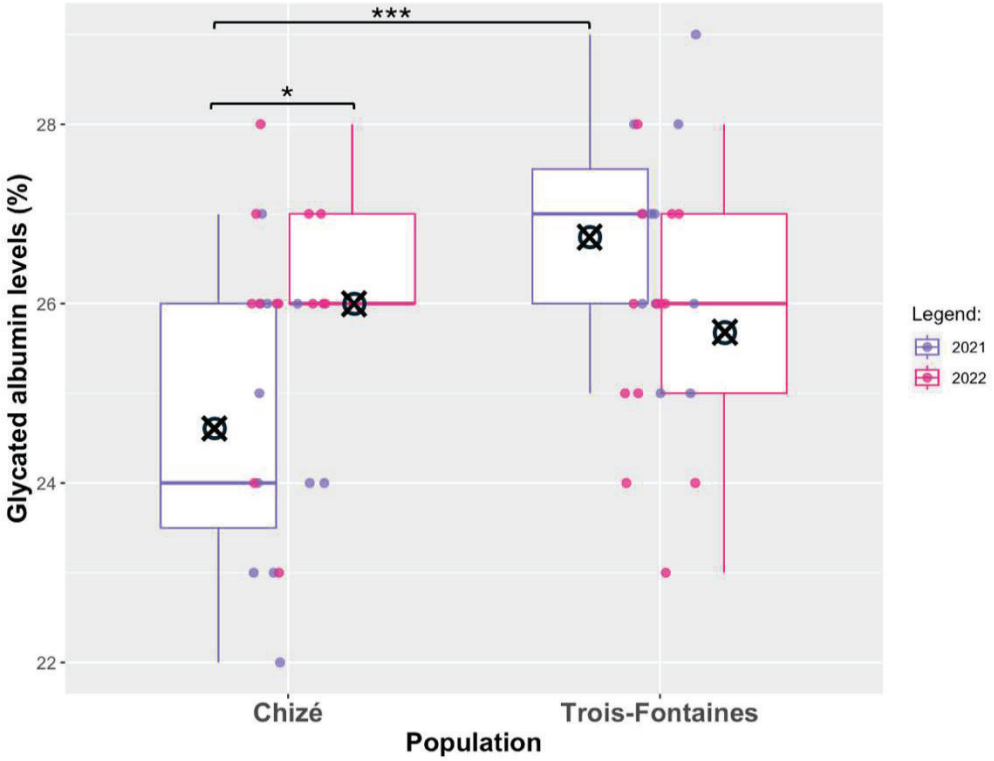

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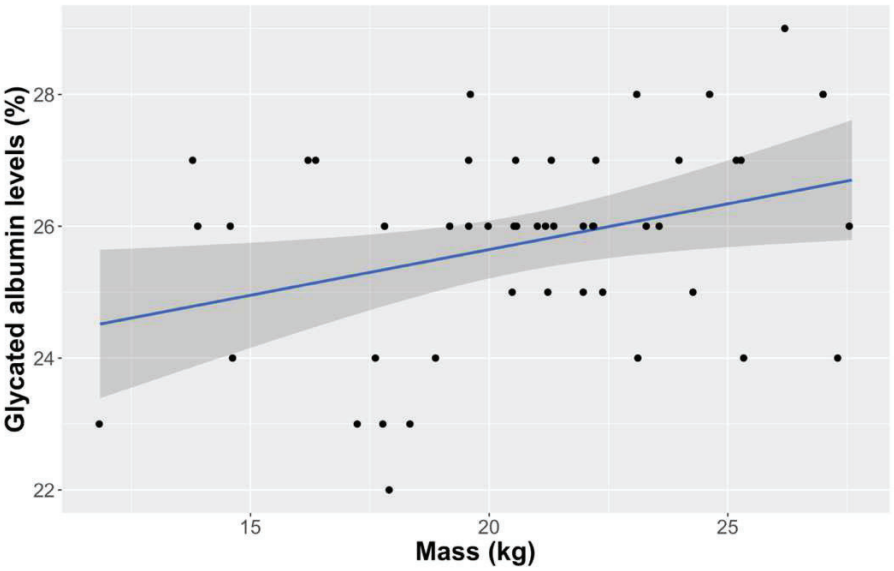


Figure 33. **Factors explaining GA levels in roe deers.** (a) Differences in GA levels between the two roe deer populations of Trois-Fontaines and Chizé. Only significant differences between groups are shown, with * = $p < 0.05$ and *** = $p < 0.001$. Red crossed circles represent the mean GA for each population, and grey points represent individuals. (b) Boxplot of the correlation between GA levels and the interaction population x year of capture. Black crossed circles represent the mean GA for each population. (b) Relationship between glycated albumin levels and body mass in wild roe deers (n=48 samples, R^2= 0,091).

Table 18. Parameters of the selected model explaining glycated albumin levels in relation to haematological parameters (n=44 samples; Marginal R^2/ Conditionnal R^2 = 0.220/0.162) (* p < 0.05 ; *** p < 0.001)

Fixed factor	Estimate	SE	t	p
Intercept	26.14882	0.30209	86.559	<2e-16 ***
PC1	0.06435	0.20193	0.319	0.7516
Population [Chizé]	-0.60576	0.43680	-1.387	0.1732
PC1:Population [Chizé]	-0.58227	0.27154	-2.144	0.0381 *

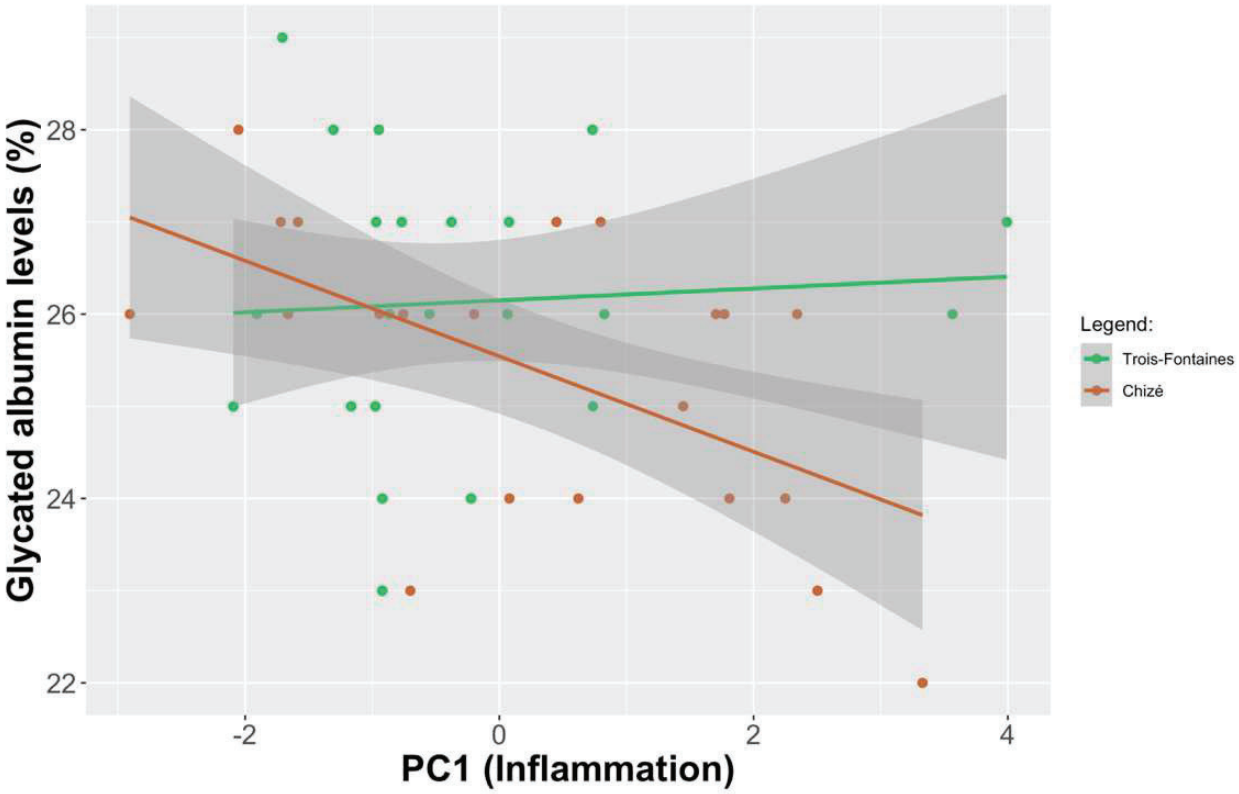
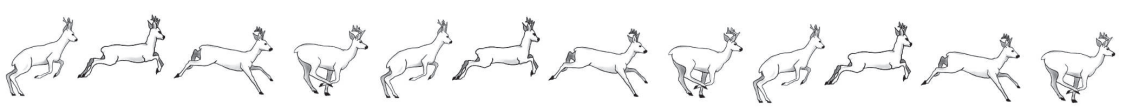


Figure 34. **Co-variation between glycated albumin levels and inflammatory markers (PC1) in the two roe deer populations (Chizé and Trois-Fontaines; n=44 samples)**. Black points represent samples

3. Glycated Albumin Levels Reflect the Inflammatory States According to the Population

The PCA carried out on the six haematological and inflammation markers led us to retain the two first principal components PC1 and PC2, which respectively explained 43.5% and 17.4% of the total variance of the dataset. Beta-, alpha-1- and gamma-globulins concentrations were the variable contributing most to PC1 (31.12%, 20.4% and 19.9% respectively), while creatinine (and gamma-globulin to a lesser extent) was the main contributor to PC2 with 62.6% (21.4% of contribution for gamma globulin). All the variables mentioned were strongly positively correlated with their respective PCs (PC1: r= 0.90 for beta-globulin, r= 0.73 for alpha-1-globulin, r=0.72 for gamma-globulin; PC2: r = 0.81 for creatinine and r=0.47 for gamma-globulin). PC1 therefore gives an indication of the inflammatory state of individuals, primarily liver inflammation as indicated by the beta-gamma block (high values of PC1 correspond to potential liver inflammation, and general inflammation and infection), while PC2 is more likely to be a marker of renal state, with high values corresponding to a diminished efficiency of renal filtration.

Our initial model included glycated albumin as a dependant variable, while PC1, PC2 and population were entered as fixed factors, along with the two-way interactions PC1 x population and PC2 x population. After selection and according to the parsimony rule, the interaction between the PC1 inflammatory axis and the population best explained the variations in GA levels (p=0.01802; Table 18; Table S6), with a negative association between GA levels and PC1 in Chizé, and inversely in Trois-Fontaines (Figure 34). Adding ID as a random factor did not change the model, as it explained virtually no variance (Table S7). Figure 3b shows the co-variation of glycated albumin levels with the three variables contributing most to PC1 (that are, alpha-1-, beta- and gamma-globulin), depending on the population.



IV. Discussion

In the present study, we note that plasma glycated albumin levels vary in free-ranging roe deer, and we explored the possibility that this could be a marker of senescence, potentially affected by environmental conditions, individual health status and sex. Only some of our predictions proved to be correct, suggesting overall that glycated albumin levels should be considered more as a marker of body condition and health in roe deer, rather than as a marker of senescence.

Glycated albumin levels were not associated with glycemia, age or sex. However, they differed between the two populations when considering the year of capture, and they also showed a trend towards a positive correlation with body mass.

The absence of correlation between GA and glycemia might seem surprising given that glycation is an inevitable chemical reaction [347] (Figure 35). However, previous intra-specific studies in bats and birds highlighted similar results, showing that a positive relationship between glycemia and albumin glycation is not systematic [87,266]. This absence of relationship between glycemia and GA levels could be attributed to the distinct effects glycemia and glycation may have on individual fitness [77], which could result in different selection pressures on their respective regulation mechanisms, and thus end with no link between both. Another explanation could lie in the fact that the formation of compounds such as glycated albumin takes several days, and albumin's half-life being between 14 and 21 days (depending on the species), it therefore reflects blood glucose levels over a previous period of 2-3 weeks [39], which could explain the lack of correlation between our two measurements. One way of checking this would be to measure glycemia at time t and measure glycated albumin at time t+14 and t+21, and see if there is still no correlation. We do know, however, that blood glucose levels are affected by short-term stressful events, such as the capture of individuals, which results in a rapid rise in post-capture glycemia [348–350]. Such a short, one-off event would thus be detectable in the blood glucose measurement but would not be included in the GA measurement (which incorporates a longer time scale), and could again result in a lack of correlation between the two measurements. It is interesting to note, however, that the Trois-Fontaines population exhibits higher average blood glucose and GA

levels than the Chizé population. Interestingly, in 2022, individuals from both populations combined also had higher glycemia than in 2021. Again, this yearly variation is also true for GA when looking at the populations separately. Those co-variations actually call for further study, to determine the underlying physiological explanations.

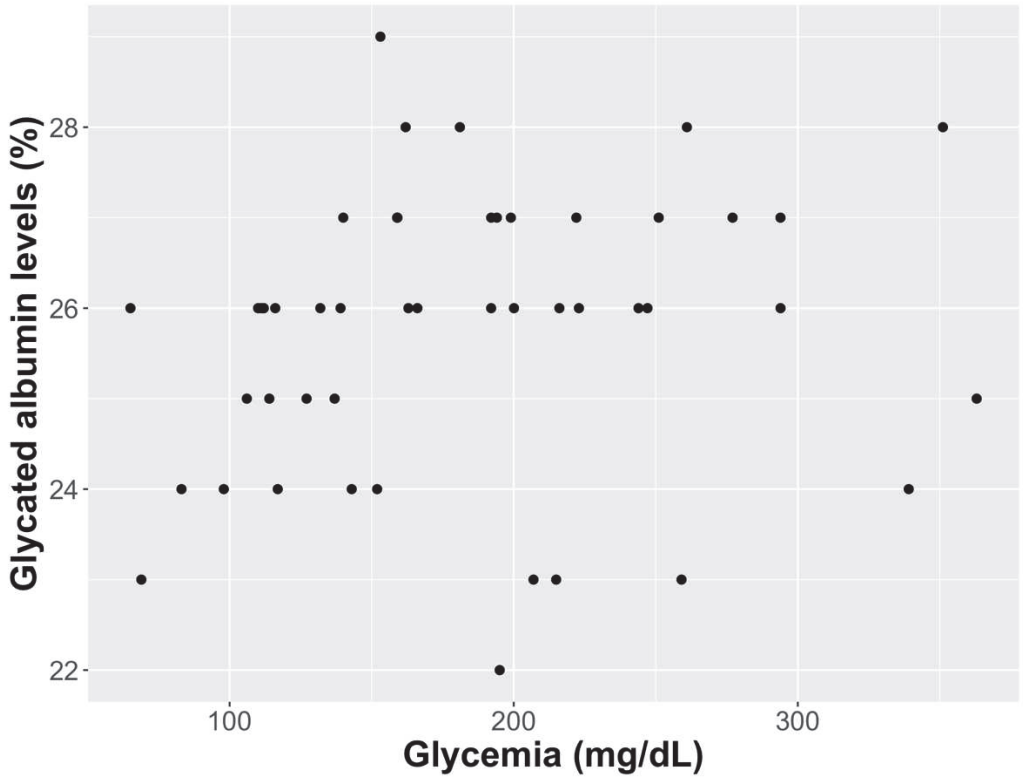


Figure 35. Co-variation of glycemia and GA levels in the European roe deer (both populations cofounded)

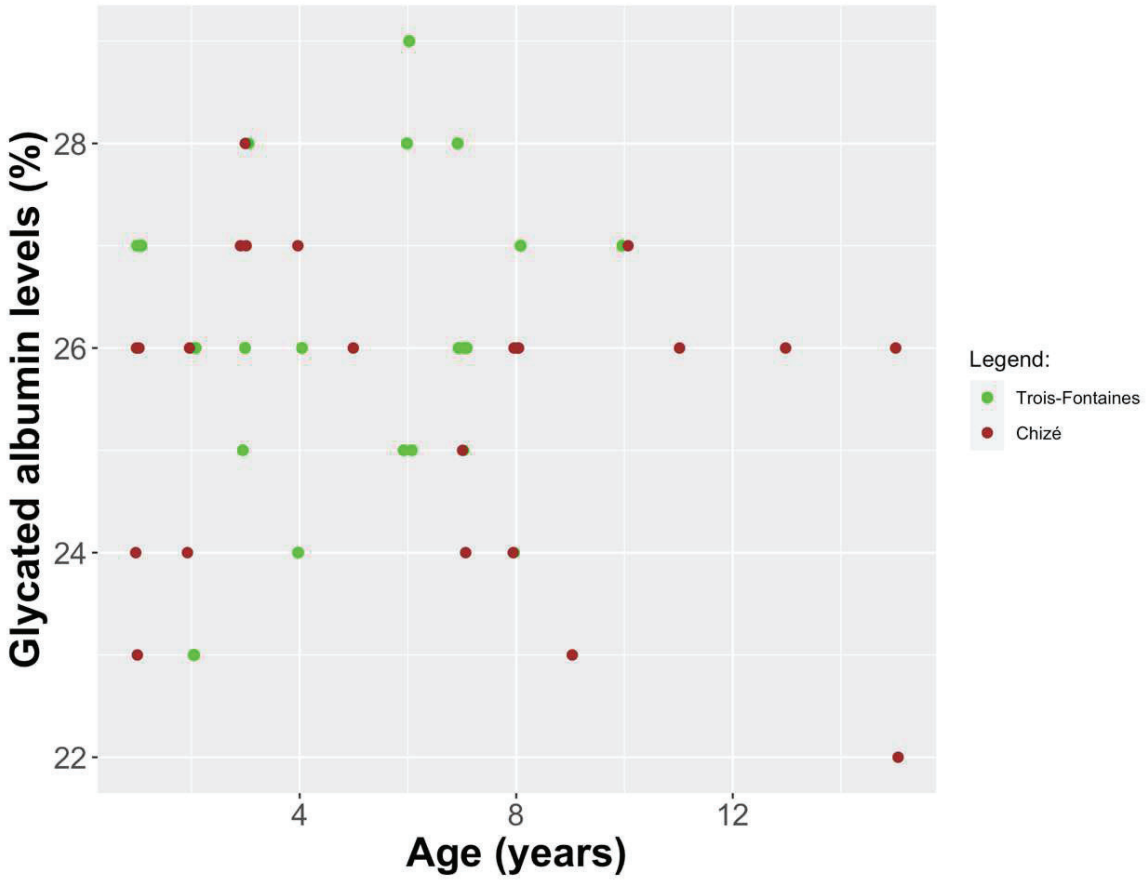
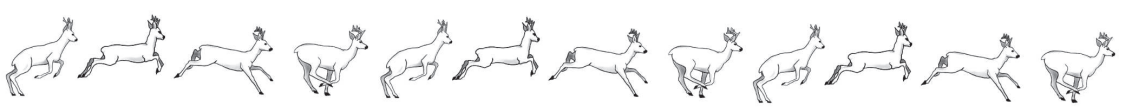


Figure 36. Relationships between age and GA levels in the European roe deer (*Capreolus capreolus*).

When looking at the co-variation between GA levels and age (Figure 36), we see that some old individuals exhibit high levels of GA, while others show lower levels. This highlight that some individual are able to live old while having high levels of GA, thus exhibiting a better tolerance towards their pro-aging effects than other individuals. Our interpretation is that roe deer (at the species level) may be tolerant to the deleterious effects of glycated albumin in some ways. Furthermore, we know that in both populations, males show faster senescence than females. However, in the present study, males did not exhibit higher levels of glycated albumin, suggesting no link between senescence rate and glycated albumin levels. These results indicate that glycated albumin cannot, so far, be considered as a marker of senescence in this species. This supposed tolerance to glycation in roe deer may be based on lower rates of transformation of Amadori's products into AGEs and/or on the mitigation of the pro-senescent effect of AGEs [351]. Still, we cannot exclude a certain resistance of other proteins to glycation, since we were unable to detect glycated haemoglobin. This absence of detection might either reflect an extremely low level of glycated hemoglobin (below our low limit of detection, knowing that in other species using the same LC-MS instrumentation, we were able to detect glycated haemoglobin rates as low as 1.4 ± 0.03% [87]) or an effective complet absence of glycated hemoglobin in this species. As elevated levels of glycated haemoglobin reflect a malfunction in glycemic control over 3-6 months in humans [352], this could suggest that roe deer are resistant to protein glycation in multiple ways (e.g., glycation of protein, cleaning of glycated proteins, inhibited AGEs pathways). Our hypothesis must be explored in parallel with comparable studies carried out on other animal species that are long-lived (as the roe deer), relying on high-sugar-diets and nevertheless exhibiting a comparable ability to resist to glucotoxicity (i.e., birds and bats, [353,354]).

Glycated albumin levels showed a trend towards a positive correlation with body mass. This is in contradiction with the few published data so far, which determined a negative relationship at the intra-specific level (Alpine swift, *Tachymarptis melba*, [222], see also [355] for a similar link with body mass index in humans). On the contrary, other studies happened to be unsuccessful in finding any significant relationship between GA levels and body mass (such as in *Pteropus rodricensis*, see Chapter 2). This discrepancy is therefore not attributable to methodological bias of detection, but may rather rely on the biology or physiology of the

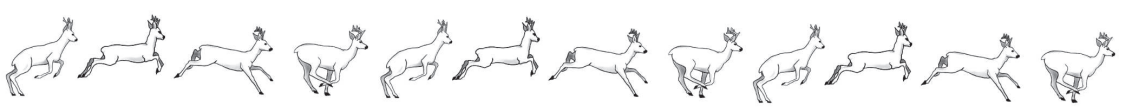


biological model under study. In our case, individuals with the highest body mass had higher glycation levels,

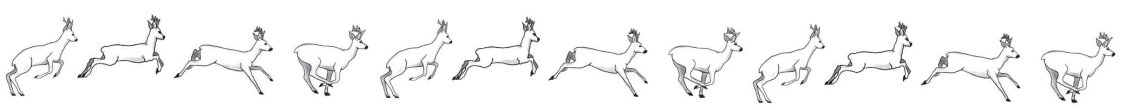
regardless of sex, population (i.e., environmental quality), glycemia and age. As in most ungulates, larger and heavier male and female roe deer beneficiate from a better fitness, and body mass stands as an indicator of phenotypic quality [356]. If roe deer have evolved some mechanisms that protect them from the deleterious effects of high levels of albumin glycation on fitness, and if glycated albumin simply covaries with body mass, it is not surprising to find a negative association between the two variables.

GA levels did vary between populations in 2021, with individuals from Trois-Fontaines (the high-quality habitat) exhibiting higher glycation levels than Chizé. Conversely, no significant difference between populations was highlighted in 2022. The fact that individuals from Trois-Fontaines show higher glycation levels compared to Chizé, while also experiencing a slower senescence rate [337] and a better body condition suggests that this population might better tolerate the pro-aging effects of glycation. Moreover, when focusing solely on the Chizé population, we showed that individuals exhibited higher levels of GA levels in 2022 compared to 2021. In Chizé, annual survival of juveniles was higer in 2021 (63.9 %) than in 2022 (25.3 %), highlighting that 2021 was overall a better year (notably in terms of food resources) than 2022. On the contrary, in Trois-Fontaines, it seems that 2022 was a better year than 2021 (67.5 % and 44.8%, respectively). No significant difference between years was seen in the Trois-Fontaines population, although Figure 2b shows that GA levels appear to be slightly higher in 2021 than in 2022. So, it seems that variations in GA levels reflect the quality of the year, and in a significant way in the population of Chizé. This might suggest that the population living in the poorest habitat, and also consequently exhibiting the lowest body condition (evaluated through body mass and senescence rate) might be more easily impacted by environmental variations, which appear to be reflected glycated protein levels. One could assume that a lower body condition might impact individuals' ability to efficiently regulate glycation levels, either through less efficient regulatory mechanisms and/or through a disabled glycation resistance (defined as the ability of a protein to escape glycation).

Specificaly in the Chizé population, when considering both years, glycated albumin levels were negatively related to inflammatory principal component axis (Figure 37), whereas no significant relationship was highlighted in Trois-Fontaines, where individuals seem the GA



levels remain stable with increasing inflammation. At first sight, this result might seem surprising and counter-intuitive, considering that in humans, increased glycation levels are positively associated with increased inflammation [62,70,165,352]. One explanation could be that in roe deer, there is a decoupling between inflammation and glycation levels, potentially via protective mechanisms specifically targeting the pathways linking glycation products and inflammation. However, this result could also be interpreted as follows: populations exhibiting different body and health conditions might regulate both their GA levels and inflammatory response in different ways, which would result in a different correlation between the two parameters. Under this light, it appears that low levels of glycated albumin could reflect an unfavorable environment when individuals suffer from inflammation or infection. The most parsimonious explanation relies on the fact that proinflammatory status has been previously associated to an increase in protein turnover in humans, which is particularly reflected by a decrease in circulating glycated albumin [357]. Interestingly, glycated albumin has pro-inflammatory effects by stimulating pro-inflammatory cytokines such as TNF-α, IL-6 and IL-8 in humans [358]. Hence, in roe deer, the negative relationship between glycated albumin levels and inflammation proxies in the Chizé population might reflect an adaptive response to mitigate inflammation. This would support the hypothesis that this species has evolved buffering mechanisms to tone down the pro-ageing signaling pathways that have been characterized in humans [351]. Moreover, the Chizé population was previously reported to have corticosterone-driven higher inflammatory status [359]. Also, early life conditions seem to prime immunosenescence trajectories, through glucocorticoid mediation in our populations, which may also putatively explain the population-specific relationship we outlined in the present study [360]. Therefore, how food resources, stress, glycation and inflammation are intertwined over different life stages constitute a clear next objective. Immunosenescence [340] and senescence patterns in other several traits (such as creatinine and albumin levels [341] or antlers length [345]) have been previously reported in our two populations. Due to our experimental design (transversal), which focuses on a relatively small sample size and over a large range of ages (1-15 years), we unable to adequately address the hypothesis that protein glycation levels may be associated with longevity at the individual level, as well as with actuarial senescence. A crucial next step will be to conduct longitudinal studies of individuals in both populations, to determine the precise dynamics of glycation levels over life. Still, this study already allowed us to demonstrate that 1) glycated albumin



levels are likely to be a marker of body condition and health in the European roe deer, reflecting the environmental conditons individuals are facing, and 2) that glycated albumin levels vary between populations facing different environmental conditions (habitat and year quality).

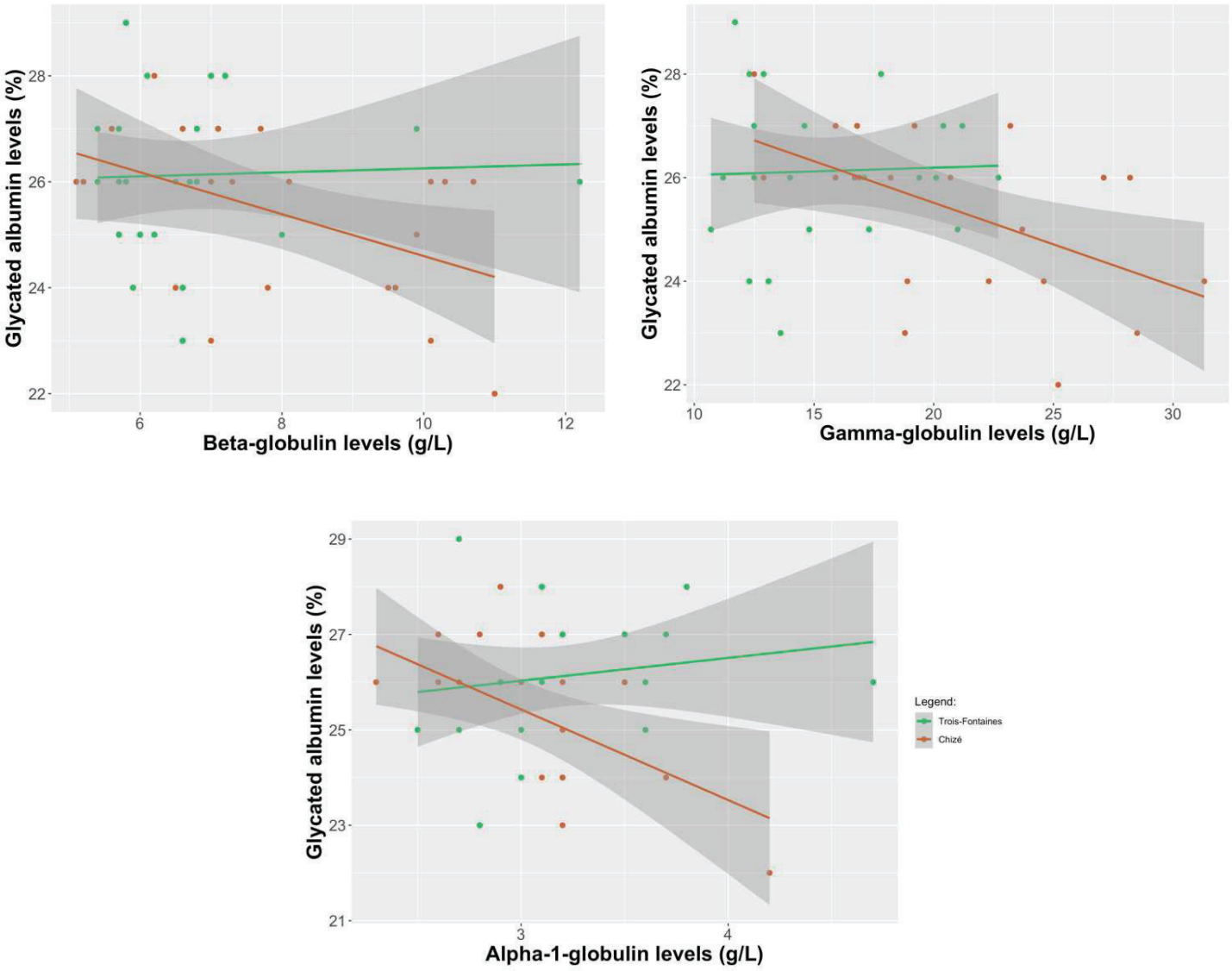


Figure 37. **Co-variation between glycated albumin levels with the three inflammatory markers contributing the most to PC1 in the two roe deer populations** (Chizé and Trois-Fontaines; n=44 samples)

GENERAL CONCLUSION

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While the study of glycation plays a central role in research on aging and associated pathologies in humans, it is evident that this field remains in its infancy for other mammalian species. To our knowledge, this thesis is one of the first exploratory studies on glycation in non-conventional wild mammalian species, producing two essential takeaways messages.

First, the entirety of this work suggests that, unlike in humans, **protein glycation may not systematically serve as a marker of senescence** across all mammalian species. Second, and contrary to our initial expectations, it appears that **mammalian species with a slower pace of life and greater longevity do not exhibit lower levels of protein glycation** compared to shorter-lived species with faster life histories (regardless of whether the species is flying or non-flying). The extension of this finding is that, although some of these species are exposed to diets that induce chronic glucose overload, they have at the same time developed an adaptive response that apparently mitigate the harmful effects of glucose (resistance) or glycation (tolerance). Such adaptations could lead to a new mechanistic hypothesis explaining animal variance in senescence and longevity.

Our intraspecific studies have revealed that **glycation might act more as a marker of individual quality in certain species**, such as the European roe deer, the Egyptian fruit bat, and the Rodrigues flying fox. In fruit bats, the observed inter-individual variability in glycation tolerance seems to be at least partly explained by individual differences in the mitigation of oxidative stress. In roe deer, protein glycation levels appear to vary depending on the living environment, particularly in relation to resource availability and to the individual's health status (*e.g.*, inflammation). The characterization of this inter-individual variability within a species has an evolutionary interest, as it is one of the three key conditions in evolutionary biology for a trait to be subject to natural selection.

Finally, we also demonstrated that our **bat species exhibit lower glycated albumin levels than non-flying mammals**, regardless of their body mass, and that **insectivorous bat species were found to have higher glycation levels than frugivorous species,** despite similar glycemia levels. This greater resistance to deleterious glucose effects was not attributed to a specific amino acid sequence of albumin (i.e., number of sites potentially exposed to glycation) and remained unexplained. In this concluding section, we aim to emphasize the exciting research prospects that these results open regarding the study of glycation in non-conventional species.

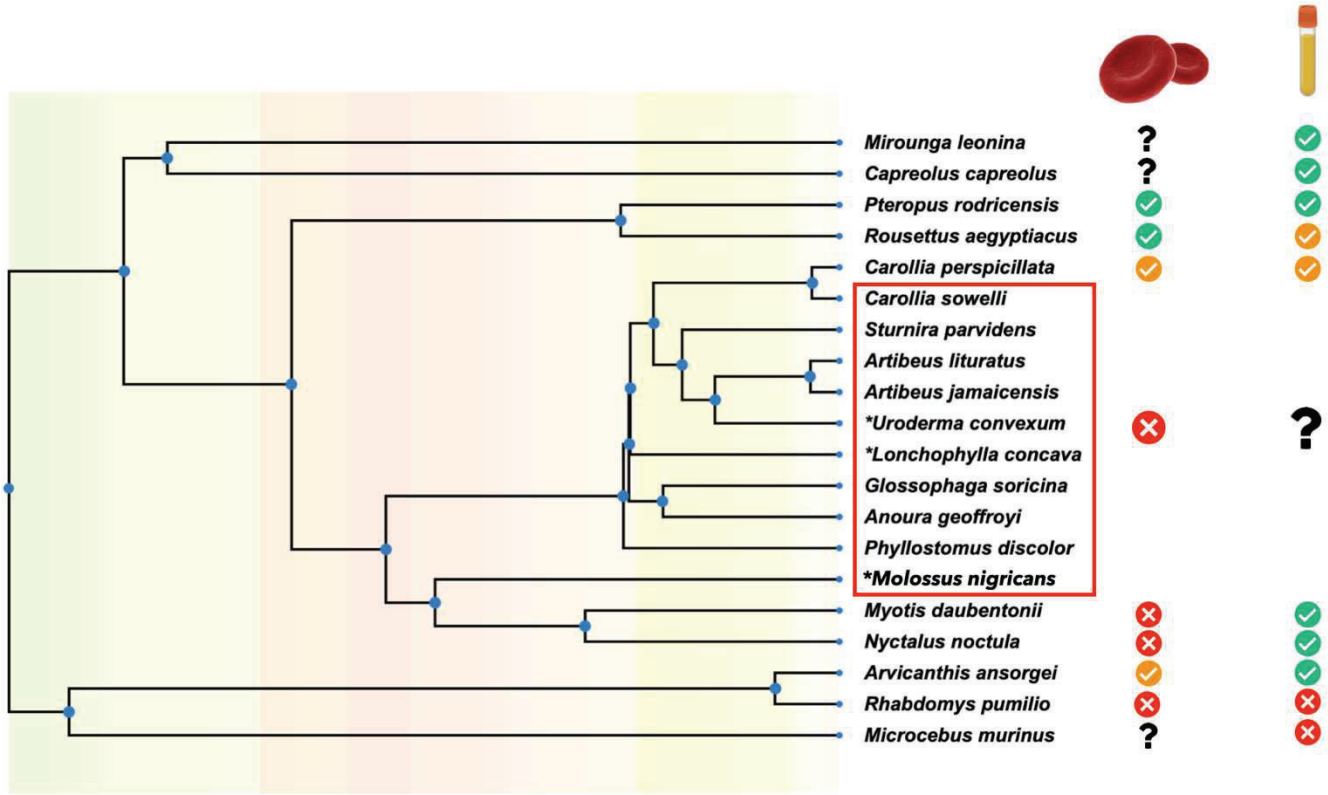

Figure 38. Phylogenetic tree including all species sampled during this thesis and the absence or presence of glycated hemoglobin (GHb) and glycated albumin (GA) detections. Red crosses represent absence of GHb and GA detection, respectively. Question marks indicate that we did not sample plasma and/or red blood cells for the concerned species. Green ticks indicate detection of GHb and/or GA in more than 50% of the sampled individuals; orange ticks indicate detection of GHb or GA in less than 50 % of the sampled individuals. Species marked with an asterisk indicate species that have only recently been distinguished from other closely related species, and whose new taxonomy is therefore unknown to NCBI (the reference database used by TimeTree to build its phylogenetic trees). These species have therefore been placed in this phylogenetic tree on the basis of their closest related species (those from which they have been distinguished). Thus, *Uroderma convexum* is placed according to *U. bilobatum*, *Lonchophylla concava* according to *L. handleyi* and *Molossus nigricans* according to *M. rufus*. The species *Glossophaga s. mutica* that we sampled being a sub-species of *Glossophaga soricina*, only the latter's name is written on the tree.

I. Non-Detection of Glycated Proteins in Various Individuals and Species: Hypotheses and Challenges

As outlined in the General Materials and Methods section of this manuscript, we sampled several species where no glycated proteins (glycated albumin and/or glycated hemoglobin) were detected. These include the grey mouse lemur (*Microcebus murinus*), the African striped mouse (*Rhabdomys pumilio*), and 10 species of wild South American bats (see Figure 38). Additionally, within certain species, such as the Egyptian fruit bat (*Rousettus aegyptiacus*) and the Soudanian grass rat (*Arvicanthis ansorgei*), we observed a lack of glycated protein detection in a non-negligible subset of individuals.

The main questions that arise from these observations is whether this absence of detection indicates (1) a true lack of glycated proteins in the blood of these species, possibly suggesting resistance to glycation processes, (2) glycated protein levels below the detection threshold of our mass spectrometer, or (3) a methodological bias in our analysis?

Previous studies conducted on zebra finches (*Taeniopygia* guttata) using the same measurement instrument and protocol [87] detected glycated hemoglobin at levels as low as 1.4 %. Therefore, for species like the Egyptian fruit bat, where we detected hemoglobin glycation levels of 2.4 % in certain individuals, an absence of detection may reflect glycation levels below the 1.4 % threshold rather than a complete absence of glycation, especially if we consider that a similar level of noise was found for the analysis of samples from different species. In species where a large number of individuals were sampled, and no glycation was detected, this same reasoning may apply, although the possibility of complete glycation absence cannot be entirely ruled out. However, for species such as *A. ansorgei*, this explanation seems less plausible. In the case of glycated albumin, for instance, we detected average levels of 17.5 % in many individuals, yet none in some others. At first sight, given that we carefully selected non-diabetic individuals, this substantial variation between the detection threshold and the actual levels observed in certain individuals suggests at first sight that the non-detection in some cases might be due to unexplained analytical issues rather than a true absence of glycation. It is known, for instance, that high lipid levels in plasma can interfere with the analysis, leading to a failure in detecting glycated proteins. The case of the grey mouse lemur highlights another potential methodological bias. The samples were frozen several years

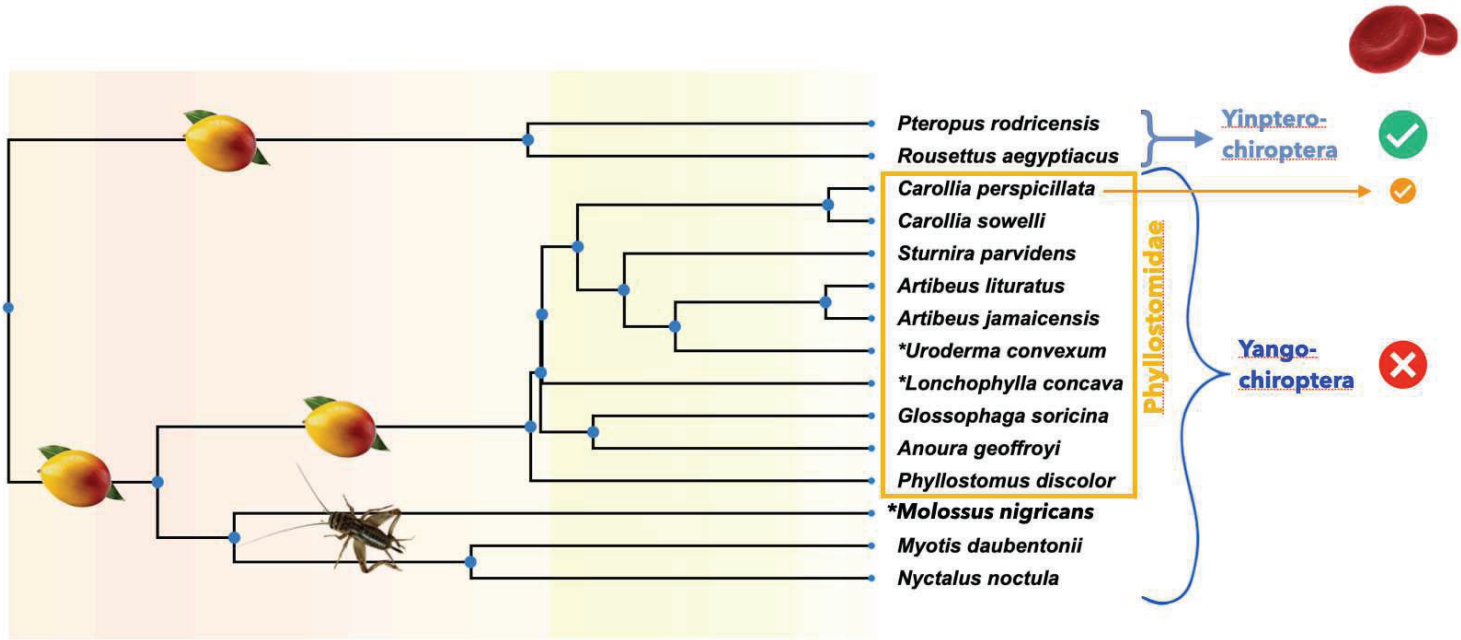

Figure 39. Phylogenetic tree of all bat species sampled during this thesis. The red cross represents an absence of GHb detection, while the green tick indicates a detection of GHb in almost all of the sampled individuals. The orange tick for *C. perspicillata* indicates that GHb was detected in only 2 individuals out of 24. Species marked with an asterisk indicate species that have only recently been distinguished from other closely related species (see Figure 1 legend for more details). The fruit indicates that the species are mostly frugivorous and/or nectarivorous (at least 60% of their diet). *M. nigricans*, *M. daubentonii* and *N. noctula* belong to different families but are all insectivorous.

ago during a previous experiment, and multiple freeze-thaw cycles may have occurred. Controlled tests on frozen samples have shown that repeated freeze-thaw cycles can degrade sample quality, affecting the LC-MS analysis [361] and resulting in a failure to detect glycated proteins even when present originally. An explanation for these non-detections may thus depend on sample composition or on storage duration and/or conditions. Nevertheless, we cannot be completely sure that these explanations are true in the case of *A. ansorgei* and *M. murinus*, and if by any chance it turns out that the results we have obtained reflect a biological truth, it would be more than wise to seek to understand them better in future studies, as they are likely to hold an important key to detoxifying glycations or avoiding them. For the grey mouse lemur, to verify whether this lack of detection is a species-specific trait, it would be necessary to collect new samples from multiple individuals and analyze them immediately, avoiding any freeze-thaw cycles. Additionally, it could be valuable to quantify the lipid content in the plasma of each species analyzed and investigate the relationship between lipid concentrations and the presence or absence of glycated protein detection (e.g, lipid threshold effect on glycation detection). This analysis could be performed both across and within species to better explain the non-detection issue.

The case of non-detection in South American bats may reflect yet another scenario. We had limited samples available for these species, which is a common limitation when working with wild non-conventional species. Consequently, with the knowledge we acquired on non-detection of glycation, the presence of glycated hemoglobin was tested only in one individual *per* species. Thus, we cannot exclude that, having analyzed all the samples, we might have detected glycated hemoglobin in some of these species. However, when examining the phylogenetic tree of all our bat species (Figure 39), alternative hypotheses can be proposed to explain the absence of glycated hemoglobin (GHb) detection in our 10 South American species. It appears that 9 of these 10 species belong to the same family, the Phyllostomidae that are predominantly frugivorous or nectarivorous. Interestingly, all of these species are part of the bat suborder Yangochiroptera (previously known as Microchiroptera), which also includes *M. nigricans*, a South American insectivorous species from the Molossidae family, as well as *M. daubentonii* and *N. nyctalus*, insectivorous European species from the Vespertilionidae family. No GHb has been detected in this wholegroup, with the exception of *Carollia perspicillata* (a frugivorous species, for which we sampled 24 individuals at the Papiliorama in Switzerland).

However, only 2 of these 24 sampled individuals exhibited glycated hemoglobin, and at very low levels. It is noteworthy that all individuals of this species originated from a captive population, unlike the other Yangochiroptera species, which were sampled from wild populations. Therefore, the very low detection rate in *C. perspicillata* could be attributed to captivity, which implies an exclusively frugivorous diet (whereas in its natural habitat, this species may also feed on insects depending on food availability), with fruits that differ from those typically found in its natural environment. Waiting for future studies analyzing protein glycation rates in wild individuals of *C. perspicillata*, it can be hypothesized that the absence of GHb could be a shared trait among all Yangochiroptera species.

Yangochiroptera should be distinguished from the other bat suborder, Yinpterochiroptera, which includes the formerly termed megachiropterans (such as our two species of Pteropodidae: the Rodrigues flying fox and the Egyptian fruit bat, both of which exhibited GHb in almost all individuals), as well as five families of the formerly termed microchiropterans. Examining the phylogenetic tree reveals that the Pteropodidae diverged early from other species and are both frugivorous. In contrast, within Yangochiroptera, insectivory seems to have appeared later. This divergence in evolutionary timing between the two groups, along with dietary shifts, could provide an initial avenue for exploring the differences in GHb detection.

Additionally, these two suborders differ in other aspects, such as their geographical distribution, species diversity, and size variation. Yinpterochiroptera are predominantly found in Africa, Asia, and Oceania, in tropical and subtropical regions. They generally include larger species (such as flying foxes) and are primarily frugivorous. In contrast, Yangochiroptera are distributed across all continents, in a variety of climates, with some non-tropical species undergoing hibernation. They are generally smaller in size (compared to Pteropodidae) and predominantly insectivorous, except for the Phyllostomidae. Characteristics such as geographical location and related physiological adaptations, like hibernation, could be relevant to further studies on glycation dynamics.

II. Glycation in Mammals: Perspectives and Future Avenues of Research

1. Testing Protein Resistance to Glycation

We have demonstrated that albumin in certain bat species exhibits greater resistance to glycation compared to that in other mammals, as well as a tolerance to its effects (long lifespans). Based on these findings, it would be interesting to investigate the resistance of hemoglobin and albumin to the chemical glycation reaction, particularly in bats, but also in species showing multiple instances of non-detection, such as the frugivorous grey mouse lemur, as this could put us on the trail to new treatments for hyperglycaemia related diseases such as diabetes in humans.

One approach would be to perform *in vitro* forced glycation tests, incubating plasma or red blood cell proteins with varying concentrations of glucose (or fructose), as was previously done with zebra finches [87]. Glycation levels could then be measured at different incubation times (e.g., at baseline, day1, day 3, day 5, week 1 and more, depending of the half-life of the studied protein). Beside testing the resistance of proteins to glycation, this method could also allow comparison of glycation rates and kinetics across different species, thereby notifying us of differences in glycation tolerance. This, in turn, may inform us on coevolution of glycation resistance and tolerance mechanisms, such as circulant and intracellular anti-glycation effectors or specific protein sequences.

Following this, another approach could involve studying the 2D and 3D structural conformation of hemoglobin and albumin proteins in these various species. Inspired by previous work in chickens [155], an *in silico* approach could be employed, using available protein sequences to reconstruct the three-dimensional conformation of the target protein. This would allow us to calculate the solvent-exposed percentage of glycation-prone residues, such as lysine and arginine.

Specifically for hemoglobin, it might be worth exploring its quaternary structure through native mass spectrometry (nMS) combined with native top-down mass spectrometry (nTD-MS). This technique has been successfully used in our laboratory to study zebra finch hemoglobin [87], revealing the presence of several isoforms: three different tetramers, as well as populations of dimers and monomers. It has been suggested that some of these isoforms

may exhibit greater resistance to glycation compared to others, supposedly linked to more or less dense conformations that hinder access to glycatable amino acids.

2. Expanding The Number and Diversity of Species

Our interspecific study demonstrated significant differences in tolerance to glycated albumin across species, with higher tolerance observed in longer-lived species. Variations related to the pace-of-life were also noted, along with an increased resistance and tolerance in bats compared to non-flying mammals.

As suggested in the discussion of Chapter 1, it is essential to expand the focus to a larger number of species with diverse modes of life, diets and life histories. This would not only provide a more comprehensive view of glycation dynamics in mammals but also allow for a more precise evaluation of the interspecies variation attributable to phylogenetic relationships. Certain species present particularly interesting characteristics relevant to our subject, such as the naked mole rat (a subterranean mammal with remarkable longevity for its body mass [362]) which seems to show no signs of senescence. Non-bat hibernating species have been shown to exhibit multiple tolerances to aging mechanisms, such as oxidative stress and telomere shortening [363–365]. Investigating the dynamics of glycation between hibernating and non-hibernating species represents a further promising area of research.

Another aspect that merits further investigation on an interspecific scale is dietary habits. Several studies have highlighted, through glucose tolerance tests, that frugivorous species have evolved mechanisms of resistance to hyperglycemia, in contrast to carnivorous and/or insectivorous species, a pattern corroborated by our results in flying vertebrates. Beyond the interest in species with low-sugar diets, one might ask whether, taking humans as an example, glycations pose a greater threat primarily to omnivorous species. Unlike carnivorous or insectivorous species, which in the wild are generally exposed to lower amounts of sugar intake from their food, omnivores consume a wider variety of food depending on availability. However, since they are not exclusively frugivorous, it can be hypothesized that they have not developed the necessary adaptations to cope with constant hyperglycemia, potentially leading

to the deleterious effects of glycation observed in humans. The case of humans could further illustrate this point, since the onset of the modern era has been accompanied by an increase in the amount of carbohydrates in the diet as a result of the industrialization of the food supply. It is likely that this rise in sugar consumption has exceeded the body's natural regulatory capacities, which would explain the deleterious effects and pathologies that ensued. The case of domesticated animals supports this hypothesis as well. It is known that rats, mice, dogs, cats or even parrots can develop diabetes. Rats and mice are omnivores, and domestic dogs and cats, while carnivorous by nature, are often fed industrial foods rich in carbohydrates, which is not aligned with their ancestral diets. Pushing this idea further, one could question whether glycations might serve as a marker of senescence only in omnivorous and carnivorous/insectivorous species, that come under conditions of domestication or captivity involving a modification of their original diet.

3. Studying The Links Between Glycation and Fitness at an Intra-Specific Scale

Following the aforementioned points, the question of glycation as a marker of senescence could be substantiated by studying the evolution of glycated protein levels in relation to chronological age at the level of a single individual. This would necessitate conducting longitudinal studies on multiple individuals within the same species. Indeed, in cross-sectional studies on the scale of populations such as ours, we cannot rule out the possibility that there is a selective disappearance of the least resistant or tolerant individuals to glycations, as shown with glycated hemoglobin in the collared flycatcher [91], which would explain the absence of a significant positive correlation between glycated proteins and age in our species. If we assume that only the most resistant (not tolerant) individuals survive, we could even imagine the existence of a negative link. Longitudinal tracking would also allow for a more comprehensive understanding of glycation in relation to fitness. One could for example imagine that if glycation levels increase at a young age, a selective appearance of individuals taking longer to reach their sexual maturity could happen. But this can only be verified in the presence of longitudinal studies with follow-up over the entire life of individuals.

As discussed in the introduction, for a trait to be subject to natural selection, it must meet three criteria: (i) variability in its expression within a population, (ii) heritability and transmissibility between generations and (iii) impact on fitness. Our intraspecific studies in *Rousettus aegyptiacus*, *Pteropus rodricensis* and *Capreolus capreolus* have demonstrated that glycated protein levels within these species fulfill the first criterion. As for the heritability and transmissibility criterion, we could imagine testing it on non-conventional species from zoos, provided the animals' pedigree has been established. The impact of glycations on fitness (3rd criterion) has been tested and validated at the intraspecific scale in certain bird species [89–91], and our interspecific study on mammals revealed variability in glycated albumin levels with different fitness related traits, such as gestation time, number of litters per year and pups *per* litter, female sexual maturation or maximum lifespan. However, this remains to be demonstrated on an intra-specific scale in various mammal species. Investigating the correlations between glycated protein rates and proxies of fitness and survival within species is fundamental to understand whether tolerance or resistance to glycation may have undergone evolutionary selection.

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APPENDIXES

Stylized vampire bat *Desmondus rotundus* (Illustration by Cyrielle Duval)

Appendix n° 1: Plasma and Red Blood Cell Sampling Protocol for Glycation Study

Protocol for glycations

Sampling protocol:

Step 1: draw blood on heparin tubes/capillaries.
Step 2: Centrifugation at 4°C, 10 min, 2500 x g
Step 3: Collect the plasma, freeze immediately (aliquots: 3 x 20 µl if enough material, otherwise 3 x 5 µl), then store at -80°C*
Step 4: Wash the blood cell pellet using PBS (add a large volume of PBS to the cells then centrifuge at 4°C, 10 min, 2500 x g then discard the supernatant)
Step 5: Repeat Step 4 if the discarded supernatant is not "clear"
Step 6: Freeze the blood cells for storage at -80°C (3 x 20 µl if enough material; otherwise 3x5 µl)*

* If there is no possibility to quickly freeze samples in liquid nitrogen, dry ice or a -80°C or -20°C freezer, then samples may be stored at 4°C before freezing. It is best to minimize the duration at 4°C and record it in case the samples are treated differently from the others. The sooner the samples are prepared then frozen after collection the better the analyses.

Additional comments: recording of variables such as age or life stage (immature, reproductive adult…), body mass or a proxy for the body condition would be a plus.

Appendix n° 2: Food Ration Composition of *R. aegyptiacus* and P*. rodricensis* at The Zoo de la Palmyre

RATION ROUSSETTES ZOO DE LA PALMYRE

Pour environ 300 Roussettes d'Égypte et 77 Roussettes de Rodrigues :

•Ration pour la journée :
Entre 8 et 11 seaux (10L) de fruits mélangés selon l'appétit
Pomme : De 19kg760 à 27kg170
Banane : De 4kg328 à 5kg951
Poire : De 11kg504 à 15kg818
Tomate : De 10kg064 à 13kg838
Kiwi : De 7kg576 à 10kg417
Mangue : De 1kg à 1kg 375

•La quantité totale de la journée est donnée en quatre fois dans la journée en proportions égales. Une distribution en début de matinée, une en fin de matinée, une en première partie d'après-midi et la dernière en en fin d'après-midi
•Plusieurs points de distributions: gouttières suspendues, gamelles. En fonction de la quantité à distribuer privilégier les gouttières suspendues

•Poudre MAZURI MP (E) F. Ground : 50g par seau (10L), à mélanger avec les fruits
•Certiselen E : 40mL par jour, à mélanger avec les fruits de la première distribution du matin

Appendix n°3: Red Blood Cell Reduction Protocol

Hémolysats de Roussettes d'Égypte

But : Repérer la présence ou l'absence de glycations sur les chaînes d'hémoglobines de Roussette d'Egypte. Regarder si cela varie en fonction de l'âge et du sexe de l'animal.

Echantillons :
24 hémolysats de Roussettes d'Égypte (individus 1 à 3 et 5 à 25, échantillonnés en décembre 2021 au Zoo de la Palmyre)

Pré-préparation manip :
Annoter : 24 epp. 0.5 mL de 1 à 3 et de 5 à 25. Le faire 3 fois. (Total : 72 tubes)
Peser : 1000 mg Guanidine → eppendorf de 5 mL → laisser à T ambiante + alu
80 mg TCEP → eppendorf 1.5 mL → alu + conserver au frigo
Préremplir 24 epp. 0.5 mL avec 99 µL d'eau milliQ.
Préremplir 1 epp. 5mL avec 3.8mL d'eau milliQ.

Préparation GR :

- **Centrifugation** : 12 000g pendant 10min
  → Récupérer 20 µL de surnageant → dans eppendorfs de 0,5mL.

- **Solutions tampon :**
  - **Tris 50mM** (conservé à T° ambiante) (dans un eppendorf de 5mL) : 0.2mL de Tris-HCl 1M (pH= 7.5) + 3.8 mL d'eau milliQ. Vortexer.

  - **Tampon Guanidine 3.5M** (placard F + 20°C) : (dans un eppendorf de 5 mL) : 1000 mg (ici : ***1000.15 mg***) de Guanidine Chloride (en poudre= pur) + 2 mL de Tris. Vortexer + agitateur jusqu'à dissolution totale des cristaux.

  - **Tampon TCEP 56 mM** (au frigo) : (dans eppendorf de 1.5 mL) : 80mg (***ici : 80.71 mg***) de TCEP (en poudre= pur) + 500 µL d'eau milliQ. Vortexer et couvrir avec alu.

- **Dilution échantillon :**
  Verser 99µL d'eau milliQ dans 24 eppendorfs de 0.5mL. Ajouter 1µL d'hémolysat. Vortexer.

- **Réduction :**
  Dans un eppendorf de 0.5mL : 65µL Guanidine + 25µL d'échantillon + 10µL de tampon TCEP
  → Laisser incuber 60min à 57°C (plaque chauffante avec légère agitation).

- **Arrêt réaction :**
  Ajout 1% TFA (1µL) pour stopper la réaction (acide, à manipuler sous hotte).

LC-MS :

Instrument : HPLC quat Agilent 1100 + q-TOF MaXis II
Colonne Vydac 208 MS 5µ 250 x 2.1mm

Calibration : CSI 0.33ppm 99.75%
Test de performance : Myoglobine → OK (1.5×10^5)

Préparation échantillons : injection volume complet (25µL d'échantillon dans 75µL de tampon)

Appendix n°4: Supplementary Material for Chapter 3 : "Glycated Albumin Levels in Roe Deer: a Marker of Body Condition Which Is Influenced by Environmental Quality"

Model	k	AICc	ΔAICc	AICc weights
Constant	**2**	**551.49**	**0.00**	**0.40**
Linear	3	553.12	1.63	0.18
Quadratic	4	554.60	3.11	0.09
Segmented (threshold = 3 years old)	3	551.89	0.39	0.33

Table S1. Model selection procedure to test for the relationship between glycemia and chronological age in all individuals of the dataset (n=48). The selected best model is highlighted in bold. k : number of parameters in the model. AICc : corrected Akaike Information Criterion. ΔAICc : difference between the AICc of the candidate model and the AICc of the selected model. AICcw: AICc weight (measures the relative likelyhood that a given model is the best among all the fitted models).

Model	k	AICc	ΔAICc	AICc weights
Constant	**2**	**174.47**	**0.00**	**0.56**
Linear	3	176.47	1.99	0.21
Quadratic	4	176.83	2.36	0.17
Segmented (Threshold = 4.8 years old)	3	179.00	4.53	0.06

Table S2. Model selection procedure to test for the relationship between glycated albumin and chronological age in all individuals of the dataset (n=48). The selected best model is highlighted in bold. k : number of parameters in the model. AICc : corrected Akaike Information Criterion. ΔAICc : difference between the AICc of the candidate model and the AICc of the selected model. AICcw: AICc weight (measures the relative likelyhood that a given model is the best among all the fitted models).

Global model: Glycemia ~ (Population + Sex + Body mass + Year of capture)^2					
	Independent variables	**k**	**AICc**	**ΔAICc**	**AICc weights**
Model 1	**Population + Year of capture**	**4**	**545.1**	**0.00**	**0.191**
Model 2	Population + Sex + Year of capture	5	545.2	0.17	0.176
Model 3	Mass + Year of capture	4	545.6	0.57	0.144
Model 4	Mass + Population + Year of capture	5	545.9	0.85	0.125
Model 5	Mass + Sex + Year of capture	5	546.3	1.22	0.104
Model 6	Mass + Population + Sex + Year of capture	6	546.5	1.39	0.095
Model 7	Mass + Year of capture + Mass:Year of capture	5	546.7	1.60	0.086
Model 8	Year of capture	3	546.9	1.79	0.078

Table S3: Model selection procedure (dredge function from the MuMin package, automatic selection procedure). Only models with a ΔAICc < 2 are presented. The selected best model is highlighted in bold. k : number of parameters in the model. AICc : corrected Akaike Information Criterion. ΔAICc : difference between the AICc of the candidate model and the AICc of the selected model. AICcw: AICc weight (measures the relative likelihood that a given model is the best among all the fitted models).

Global model: Glycated albumin ~ (Population + Sex + Body mass + Glycemia)^2					
	Independent variables	**k**	**AICc**	**ΔAICc**	**AICc weights**
Model 1	**Mass + Population + Year of capture + Population x Year of capture**	**6**	**174.2**	**0.00**	**0.654**
Model 2	Population + Year of capture + Population x Year of capture	5	175.5	1.28	0.346

Table S4: **Model selection procedure (dredge function from the MuMin package, automatic selection procedure) with ΔAICc < 2 for the factors explaining glycated albumin**. The selected best model is highlighted in bold. k : number of parameters in the model. AICc : corrected Akaike Information Criterion. ΔAICc : difference between the AICc of the candidate model and the AICc of the selected model. AICcw: AICc weight (measures the relative likelyhood that a given model is the best among all the fitted models).

Predictor	Estimate	SE	t	p-value
Intercept	22.45723	1.16884	19.213	<2e-16 ***
Body Mass	0.10936	0.05735	1.907	0.06322 ·
Population [Chizé]	-1.88307	0.59881	-3.145	0.00301 **
Year of capture [2022]	1.39180	0.55625	2.502	0.01623 *
Population [Chizé] x Year of capture [2022]	2.47930	0.78530	3.157	0.00291 **
Random effect	**Variance**	**SD**		
ID (N=46)	0.00	0.00		
Residuals	1.84	1.36		

Table S5. Parameters of the selected model explaining glycated albumin levels in wild roe deers, with ID as random factor (n=44 samples; Marginal R^2/ Conditionnal R^2 = 0.283 / NA) (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). Adding ID as random factor did not change the model's output.

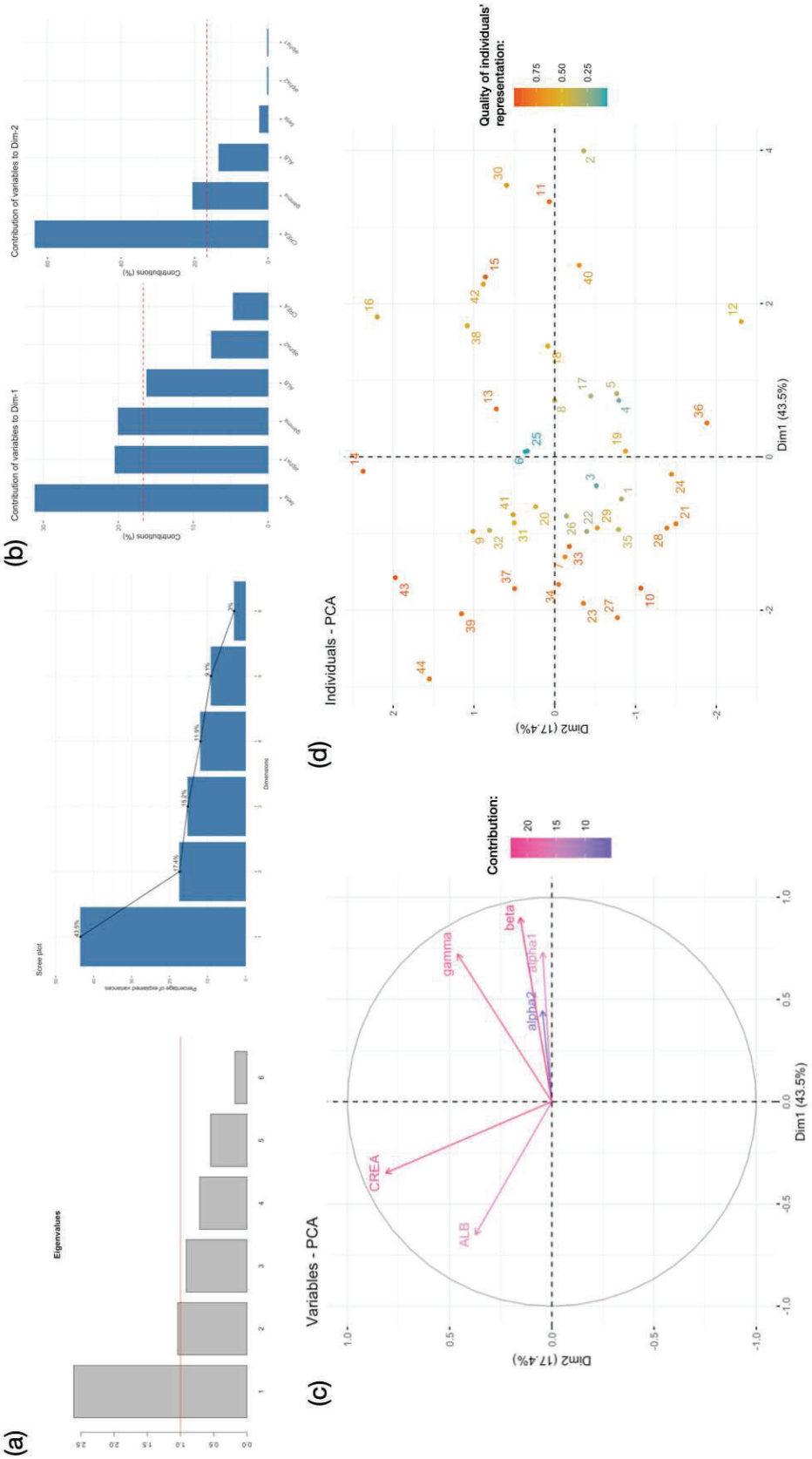

Figure S1: PCA of the haematological variables known to experience senescence in wild roe deers. (a) Principle components' (PCs) eigenvalues plot. (b) Decomposition of variance among PCs. (c) Correlation circle showing the projection of each variable on PCs. (d) Individuals' map showing individuals' position along the PCs.

Global model: Glycated albumin ~ PC1 * Population + PC2*Population					
	Independent variables	**k**	**AICc**	**ΔAICc**	**AICc weights**
Model 1	**PC1 + Population + PC1:Population**	**5**	**163.2**	**0.00**	**0.69**
Model 2	PC1	3	164.8	1.6	0.31

Table S6: **Model selection table (dredge function from the MuMin package, automatic selection procedure) with ΔAICc < 2 for the haematological factors explaining glycated albumin**. The selected best model is highlighted in bold. k : number of parameters in the model. AICc : corrected Akaike Information Criterion. ΔAICc : difference between the AICc of the candidate model and the AICc of the selected model. AICcw: AICc weight (measures the relative likelyhood that a given model is the best among all the fitted models).

Dependent variable: Glycated albumin levels (GA)				
Fixed factor	**Estimate**	**SE**	**t**	**p**
Intercept	26.15	0.30	86.56	<2e-16 ***
PC1	0.06	0.20	0.32	0.7516
Population [Chizé]	-0.61	0.44	-1.39	0.1732
PC1:Population [Chizé]	-0.58	0.27	-2.14	0.0381 *
Random effect	**Variance**	**SD**		
ID (N=46)	0.00	0.00		
Residuals	2.02	1.42		

Table S7. Parameters of the selected model explaining glycated albumin levels in relation to haematological parameters, with ID as random factor (n=44 samples; Marginal R^2/ Conditionnal R^2 = 0.208 / NA) (***** $p < 0.05$; ******* $p < 0.001$). Adding ID as random factor did not change the model's output.

Appendix n°4: Review Published in Biology Letters
(corresponding to the general introduction of this manuscript)

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Review

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Glycation resistance and life-history traits: lessons from non-conventional animal models

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Glycation reactions play a key role in the senescence process and are involved in numerous age-related pathologies, such as diabetes complications or Alzheimer's disease. As a result, past studies on glycation have mostly focused on human and laboratory animal models for medical purposes. Very little is known about glycation and its link to senescence in wild animal species. Yet, despite feeding on high-sugar diets, several bat and bird species are long-lived and seem to escape the toxic effects of high glycaemia. The study of these models could open new avenues both for understanding the mechanisms that coevolved with glycation resistance and for treating the damaging effects of glycations in humans. Our understanding of glycaemia's correlation to proxies of animals' pace of life is emerging in few wild species; however, virtually nothing is known about their resistance to glycation, nor on the relationship between glycation, species' life-history traits and individual fitness. Our review summarizes the scarce current knowledge on the links between glycation and life-history traits in non-conventional animal models, highlighting the predominance of avian research. We also investigate some key molecular and physiological parameters involved in glycation regulation, which hold promise for future research on fitness and senescence of individuals.

1. Introduction

Since the observation of glycation reactions *in vivo* in living organisms [1], we have learned that glycation damage, notably through the formation of so-called advanced glycation end-products (AGEs), heavily contributes to senescence and ageing-related diseases [1,2]. As their name suggests, AGEs are produced late in the chain of reactions occurring during the glycation process, their particularity being that they are stable and, therefore, hardly degradable by the body. Consequently, they tend to accumulate in cells and tissues and can lead to altered protein conformation and functionality via the formation of protein aggregates. By binding to specific receptors, AGEs also promote oxidative stress and inflammatory reactions, thus playing a major role in the senescence process [3]. To date, most research has been conducted in human medicine, with the main aim of combating glycation's deleterious health consequences.

Interestingly, some wild and/or non-conventional animal models do not seem to suffer from any of the known consequences of glycation on health. Despite their long lifespans, birds indeed usually express blood glucose levels similar to those observed in hyperglycaemic humans but without the expected deleterious effects [4]. A similar paradox can be found in nectarivorous and frugivorous bats, which are among the longest-lived mammals and do not seem to show any apparent signs of age-related diseases, despite relying on a high-sugar diet [5]. If glycaemia *per se* has been previously

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suggested to be correlated with proxies of species' pace-of-life [6,7] or individual fitness [8] mainly based on energetic explanations, we hypothesize here that such a relationship also implies that evolution might have acted through the selection of glycation regulatory and/or resistance mechanisms. Resisting glucotoxicity via resistance to glycation, while glucose remains the main energy source in the environment, may well fall into a novel view of senescence evolution, not only based on trade-offs but also on the conjunction of structural, cellular, physiological or behavioural traits pushing further the limits imposed by physiological constraints, and so extending physiological homeostasis in life-threatening conditions [9].

Our primary objective here is to focus on non-conventional animal models and see what they can teach us in terms of (i) the links between glycation and life-history traits, at the intra- and interspecific levels, and (ii) the mechanisms that evolved to escape the deleterious effects of glycation (thereafter glycation stress). Because this knowledge remains in its infancy, we outline gaps and promote promising avenues of research, as they should enable us to better understand how glucose and glycation may support life-history traits' evolution.

2. A few words on the glycation reaction and its known effects on health and senescence

The glycation reaction consists of the non-enzymatic binding of a reducing sugar to a free amino group of a protein. The unstable newly formed compound then undergoes a series of reactions, resulting in the formation of so-called Amadori compounds within a few days. The latter includes essential well-studied biomarkers such as glycated haemoglobin (HbA1c) or glycated albumin (GA). Those compounds can then undergo further irreversible reactions over a few weeks or months to finally form stable AGEs [10]. Because of their spontaneous character, rates of glycation reactions are expected to vary with blood glucose levels, but this idea is based on human medicine.

In biomedicine, senescence is characterized by the progressive deterioration of the structural integrity and function of cells and tissues over time, which leads to an increased risk of death. Lopez-Òtin *et al*. defined several major hallmarks of ageing [11] and, later, of health [12,13], among which glycation is likely to play a key role [3]. AGEs and Amadori compounds interfere in different physiological and cell mechanisms well known to alter senescence-related mechanisms, such as oxidative stress [14], chronic inflammation [15] or alteration of proteins' structures and functionalities, which ultimately leads to ageing-related diseases [2]. More information on glycation's contribution to the senescence process is given in table 1.

In evolutionary biology, senescence (i.e. the decreasing fertility and increasing mortality rates with age [25]) stands as a key variable, as it has been shown to vary considerably between species [26] and individuals [27]. In this context, any senescence-related mechanism may have been the target of selection. How glycation stress covaries among and within species with contrasted pace-of-life or fitness, and under which genetic and environmental influences, remains an open question.

3. Exploring the links between glycation and life-history traits in non-conventional animal models

Evolutionary biologists consider that a trait is susceptible to natural selection if it meets the following conditions: (i) variability in its expression within a population, (ii) heritability and transmissibility between generations, and (iii) impact on fitness [28]. It has been demonstrated in humans that some glycation products (HbA1c, GA and carboxymethyl-lysine (CML)) meet the first two conditions [29]. Moreover, given that glycation levels are higher in patients suffering from ageing-related diseases (the latter increasing the risk of mortality), this suggests that glycation most likely has an impact on at least fitness-related traits. If such data concerning glycation are not yet available in animals other than humans, we, however, know that glycaemia is a trait that varies with life-history stage and environmental conditions [30]. Montoya *et al.* also showed that glucose regulation is a repeatable trait that is affected by handling in zebra finches (*Taeniopygia guttata*) [31]. This regulation capacity can be impaired by stress events, and it has been suggested that the coping ability of individuals to glucose-related physiological challenges could impact their fitness [32]. It, therefore, appears that glucose regulation may be a key physiological trait under selection [32–34]. As glycation rates are usually expected to be positively correlated with glycaemia [29,35], we might expect glycation levels and glycation regulation to also be of prime importance in defining individual fitness, and moreover in shaping species' life-history. However, it is important to mention that the relationship between glycation and glycaemia is not systematic [36]. Indeed, high glycaemia levels may be beneficial for fitness (by relaxing energy-based trade-offs), while low glycation levels may be concomitantly selected as an anti-senescence adaptation. This may account for the lack of (e.g. interspecific) correlation between the two traits, while both are related to individual fitness. Hence, glycaemia and glycation seem to be proxies of individual fitness and not just labile variables that follow the nutritional status of organisms. This is probably owing to their central energetic role and, at the same time, to their chemical characteristics that, hypothetically, make them harmful molecules for fitness. What evidence do we have to suggest such an evolutionary role?

(a) Glycation in relation to reproduction and survival

Fitness is optimized differently in different species, and local selection pressure has shaped specific trade-offs on life-history traits such as those related to reproduction and survival, thereby defining the pace-of-life of species [37]. Several studies have therefore tested the relationship between HbA1c levels and individuals' reproduction in birds. In collared flycatchers (*Ficedula albicollis*), higher HbA1c levels correlated with increased adult reproductive success, namely bigger clutch size for females and higher number of fledged young for both males and females, after correction for laying date [38]. A positive correlation between HbA1c levels and growth rates of nestling has also been reported in American kestrels (*Falco sparverius*) [39]. Although the

Table 1. Examples of glycation's contribution to each hallmark of ageing (as defined by Lopez-Otìn *et al*. [3,11,15–24]).

hallmark of ageing (as defined by [11])	examples of glycations' contribution
genome instability	• glycation of nucleic acids and altered three-dimensional structure of DNA • altered DNA repair mechanisms (because of impaired function of proteins and enzymatic pathways)
telomere attrition	• accelerated shortening of telomeres (owing to DNA damage, increased ROS and pro-inflammatory metabolites production)
epigenetic alterations	• incomplete degradation of CML in tumours
loss of proteostasis	• glycation of amino acids and altered protein conformation • impaired functionality of proteins • deregulated protein clearance
disabled macroautophagy	• altered protein three-dimensional structure and functionality
deregulated nutrient sensing	• altered enzymatic pathways
mitochondrial dysfunction	• altered respiratory function • altered permeability of the membrane • increased ROS production
cellular senescence	• alteration of cellular membranes integrity (owing to glycated proteins and lipids) • increased ROS production
stem cell exhaustion	• abnormal activation or inhibition of Wnt/β-catenin signaling • AGE–RAGE interaction • abnormal differentiation of primary stem cells
altered intercellular communication	• alteration of cellular membranes integrity (owing to glycated proteins and lipids) • upregulation of RAGE pathways
chronic inflammation	• upregulation of RAGE pathways • increased ROS production • increased production of pro-inflammatory metabolites
dysbiosis	• change in gut microbiota's composition (richness, diversity…) because of dietary AGEs

CML, N∈-(carboxymethyl)lysine; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species.

links reported are not proven to be causal, those two studies still suggest that high HbA1c levels reflect a higher investment in reproduction effort and growth. However, it should be noted that Récapet *et al*. did not find any correlations between HbA1c and other markers of reproductive success (i.e. probability to successfully fledge at least one offspring and number of total chicks) in collared flycatchers [40]. Still, they found a negative correlation between HbA1c levels and a proxy of survival (number of recaptures after a capture–marking–recapture protocol). According to the authors, these different results suggest that reproduction and growth, which both induce high metabolic demands, are life-history traits likely to trigger elevated glycation levels, ultimately leading to increased mortality rates. Thus, HbA1c, more than a simple variable that changes according to nutritional status, should be considered as a physiological variable that modulates individuals' life-history trade-offs. However, those previous measurements of HbA1c levels in birds suffer from various methodological drawbacks (see Box 1), as discussed in Suo *et al*. [41] and in Brun *et al*., who showed no detectable HbA1c in zebra finches using a highly specific method [36]. It remains that little is known about the relationship between glycation levels and individual fitness, and that the few available studies solely focused on birds. Intra-population variability and heritability of glycation levels remain undefined in most animal species. Such data would enable us to assess whether glycation and the proteins (receptors, enzymes) involved in related regulatory pathways can be targeted through natural selection.

(b) Correlations between glycation and body mass

Among the different traits that determine the life-history of a species, body mass is one of the most important [42], because it allows interspecific allometric relationships among traits to be assessed as well as intraspecific variability in relation to individual quality. However, to our knowledge, only one correlational study in zebra finches addressed the links between glycation and body mass at an intraspecific level, and no correlation was found between plasma protein glycation levels and body mass [36]. On the other hand, some interspecific studies have been conducted on this subject. A recent work focusing on plasma AGE concentrations in seven birds and 16 captive mammal species of different body sizes showed that birds displayed higher AGE levels than mammals, but no significant correlation with body mass was detected in either class, even after phylogenetic correction [43]. Similarly, another

Box 1. Methodological issues related to protein glycation analysis in most studies involving non-conventional animal models

To date, most studies measuring HbA1c in non-conventional animal models have been using chromatography methods [38] or commercial kits based on either boronate affinity [39] or cation exchange chromatography coupled to UV detection. Capillary electrophoresis has also been used by Oikonomidis *et al*. to quantify HbA1c in dogs. Concerning glycated plasma proteins measurements, competitive ELISA kits are the most used. AGEs, on the other hand, are often detected using fluorescence methods. However, all these methods lack specificity and sensitivity (see [41] for more detail). It should also be noted that commercial kits are targeted specifically at humans or laboratory mice and rats, which may induce an additional bias. Moreover, some studies performed HbA1c measurements on whole blood [39] instead of isolated red blood cells, with the result that other blood proteins most likely interfered with the final values measured. The gold standard method to measure glycation levels is mass spectrometry, as used by Brun *et al*. [36]. This methodology ensures specific glycation sites in proteins are targeted with high sensitivity.

study in Canidae failed to correlate plasma AGE levels with average body mass in each species [44]. An identical result was obtained when restricting the analysis to domestic dog strains, although dog breeds vary widely in body size [44]. This suggests that glycation is a variable likely reflecting a biological feature that stands above a simple body condition index, as it has been recently suggested for glycaemia [45,46], a hypothesis that needs a broader exploration at the inter- and intraspecific levels.

(c) Glycation in relation to longevity

In humans, it is widely admitted that the levels of glycation-derived molecules (and more specifically of AGEs, the ultimate products) increase with age, thus taking part in the biological modifications that accompany the senescence process (e.g. loss of skin elasticity, crystalline opacification, etc.) [22]. Similarly, because glycation is associated with ageing-related diseases, it contributes to an increased probability of death. This is in accordance with the pro-senescent role of glycation and AGEs in humans, owing to the lack of selection pressure to set up any anti-glycation protection systems.

In non-conventional animal models, the picture is more mixed. The dynamic of glycation with age has been studied at an intraspecific level in a few species from different taxa. In mammals, while no correlation between age and HbA1c levels was found in the domestic dog (*Canis familiaris*) [47], a positive correlation between skin pentosidine levels (a specific cross-linking AGEs) and age was highlighted in seven mammal species (respectively least shrew, rat, domestic dog, cow, pig, squirrel monkey and rhesus monkey), with tighter relationships in shorter-lived species [48]. This is in line with the human observations. In insects, it was shown that older flies *Drosophila melanogaster* displayed 44% less AGEs than younger ones [49]. This may reflect a selective disappearance over time of individuals with higher AGEs levels, reinforcing the idea that the rate at which glycated products are accumulated may impact individual fitness.

When looking at the interspecific level, a negative correlation was underlined between the rate of formation of pentosidine in skin collagen and maximum lifespan in mammals [48]. This highlights that shorter-lived species accumulate pentosidine more quickly than longer-lived ones, which might be coherent with the fact that glycation accelerates actuarial senescence (e.g. the increase in mortality with age), and therefore might contribute partly to a shorter lifespan, both between and within species. Interestingly, in their extensive study of plasma AGEs levels in mammals and birds, Baker *et al.* found no correlation between age and AGEs levels in birds [43]. When focusing on 16 mammal species, after correction for phylogeny, they also did not highlight any correlation between age and plasma AGEs [43]. The absence of correlation between the plasma levels of AGEs or HbA1c and age was also observed at a smaller scale in wild canid species and domestic dogs of different breeds [44,47].

Taken together, these conflicting results suggest that there is not necessarily a direct link between AGEs accumulation and age. The dynamic of glycation with age is likely to be a species-specific trait, which might be largely dependent on (i) the metabolic adaptation to high sugar diet (i.e. which absolutely needs to be buffered for), (ii) the flying ability (see §4.1), or (iii) the methodological issues related to the type of tissue considered and on the nature of the glycated compound used as a marker of senescence. Indeed, we might expect to observe a more marked accumulation of glycation products in tissues with slow protein turnover rates [50]. Furthermore, there is a higher probability of observing an age-dependent accumulation of AGEs rather than Amadori compounds, as AGEs are stable over the long term owing to their (supposed so far) non-degradable status and difficult clearance. Conversely, Amadori compounds have short lifespans, as they are among the first products formed during the glycation reaction chain, and they are prone to undergo further reactions owing to their relative instability. They can also be degraded by specific mechanisms (see §4). It, therefore, seems more likely to observe Amadori compounds correlating with glycaemia in the short term (as observed in diabetic patients) rather than with age. However, a slight increase in their levels with age in the context of a longitudinal follow-up cannot be ruled out, given that glucose tolerance (i.e. the ability of an individual to efficiently regulate their blood glucose level and to avoid potentially harmful effects of high glycaemia through specific mechanisms) is known to decline with age [51].

Other important differences, such as the sex-dependent differences in glycation (see [47,52–54]) and their implication in sex–ageing patterns also need further examination. Longevity differences between males and females can be observed in many species, and one could expect the longest-lived sex to exhibit lower levels owing to their ability to prevent their early formation. We indeed already know that AGEs levels can differ between genders in patients with ageing-related diseases [52] or in pathological laboratory rats [54].

Table 2. Known correlations between glycation products and age, glycaemia and/or life-history traits in non-conventional animal models at (*a*) intraspecific and (*b*) interspecific levels. + and - indicate positive and negative correlations, respectively, NS indicates no significant correlation.

glycation product	factor	correlation	species	comments	reference
(a) intraspecific studies					
HbA1c	age	NS	*Ficedula albicollis*	wild, adults	[38]
		NS	*Canis familiaris*	domestic, all ages, ANOVA on 3 age classes	[47]
	sex	NS	*Ficedula albicollis*	wild, adults	[38]
		NS	*Falco sparverius*	wild, nestlings	[39]
		NS	*Canis familiaris*	domestic, all ages	[47]
	body mass	NS	*Ficedula albicollis*	wild, adults	[38]
	clutch size	+	*Ficedula albicollis*	wild, adults	[38]
	number of offspring fledged	+	*Ficedula albicollis*	wild, adults	[38]
		NS	*Ficedula albicollis*	wild, adults	[40]
	arrival date on reproduction site	–	*Ficedula albicollis*	wild, adults	[38]
	return rate	–	*Ficedula albicollis*	wild, adults	[40]
	nestling growth rate	+	*Falco sparverius*	wild, nestlings	[39]
	number of brood mates	NS	*Falco sparverius*	wild, nestlings	[39]
GA	age	NS	*Taeniopygia guttata*	captive, adults	[36]
	glycaemia	NS	*Taeniopygia guttata*	captive, adults	[36]
TPGP	age	NS	*Taeniopygia guttata*	captive, adults	[36]
	sex	NS	*Taeniopygia guttata*	captive, adults	[36]
	body mass	NS	*Taeniopygia guttata*	captive, adults	[36]
	glycaemia	NS	*Taeniopygia guttata*	captive, adults	[36]
glycated serrotransferrin	age	NS	*Taeniopygia guttata*	captive, adults	[36]
	glycaemia	+	*Taeniopygia guttata*	captive, adults	[36]
glycated carbonic anhydrase	age	+	*Taeniopygia guttata*	captive, adults	[36]
	glycaemia	NS	*Taeniopygia guttata*	captive, adults	[36]
pentosidine (skin)	Age	+	*Cryptotis parva*	captive, 0–3 y.o.	[48]
		+	*Rattus norvegicus*	captive, 5–25 y.o.	[48]
		+	*Canis familiaris*	domestic, 2–14 y.o., beagle breed	[48]
		+	*Bos taurus*	captive, 0–14 y.o.	[48]
		+	*Sus scrofa*	captive, 0–15 y.o.	[48]
		+	*Saimiri sciureus*	captive, 3–21 y.o.	[48]
		+	*Macaca mulatta*	captive, 5–27 y.o.	[48]
(b) interspecific studies					
plasma AGEs	age	NS	mammals	captive (zoos)	[43]
		NS	birds	captive (zoos)	[43]
		NS	wild canids & domestic dog breeds	captive (zoos) and domestic	[44]
	age/MLSP	NS	mammals	captive (zoos)	[43]
		NS	birds	captive (zoos)	[43]
		NS	wild canids and domestic dog breeds	captive (zoos)	[44]
	body mass	NS	mammals	captive (zoos)	[43]
		NS	birds	captive (zoos)	[43]
		NS	wild canids and domestic dog breeds	captive (zoos) and domestic	[44]

(*Continued.*)

Table 2. (*Continued.*)

glycation product	factor	correlation	species	comments	reference
pentosidine (skin)	MLSP	–	mammals	captive	[48]

GA, glycated albumin; MLSP, maximum lifespan; TPGP, total plasma glycated proteins (GA, glycated serotransferrin and glycated carbonic anhydrase).

In conclusion, it appears that the present knowledge on how glycation might be associated with life-history traits and age in non-model species remains highly heterogeneous, and even contradictory in some specific cases, whatever the scale (inter- or intraspecific) or the parameter considered (see table 2). One possible explanation lies in the methodology of the studies carried out to date. They are all population-based and therefore cross-sectional, and it cannot be ruled out that the selective disappearance of the weakest individuals or those with the highest glycation levels does play a role in the heterogenous results obtained. Long-term studies with follow-up of individuals appear necessary to assess the possible links between glycation and age. It is also interesting to note that most studies looked at each life-history trait separately, although all these traits are intrinsically linked and influence each other. A global approach integrating the whole panel of traits defining the species' pace-of-life and several markers of glycation at once is still lacking. Only such an approach might assess how glycation and life-history traits may have been shaped in relation to environmental challenges by evolution. Finally, we need more precise descriptions of the metabolic and cellular pathways through which glycation resistance may be acquired, and how such mechanisms may be related to higher individual fitness. Because (hyper)glycaemia levels are different among clades (i.e. mammals and birds), it is likely that the resistance mechanisms that emerged and their importance as modulators of fitness will differ among them.

4. Mechanisms of glycation resistance that need to be explored in a broad range of animals

The apparent resistance of bats and birds to high blood glucose levels may be explained by specific physiological and molecular mechanisms aimed at mitigating glycation stress. Interestingly, HbA1c levels measured in some bird and bat species showed lower levels than those of diabetic humans (e.g. 3.9% in the *Glossophaga soricina* bat [55], between 0.73 and 3.72% in collared flycatchers [40], no detection in zebra finches [36]), which supports the aforementioned hypothesis. Still, Baker *et al*. [43] found that AGEs levels are higher in birds than in mammals. This completes the resistance hypothesis by suggesting that resistance to high glycaemia can be reached at different levels (i.e. through early or late-glycation buffering). Thus, a more detailed exploration of the potential regulatory and/or resistance mechanisms in apparently high glycaemia tolerant animal species could lead to a better understanding of the glycation–life-history traits nexus.

(a) Fast and effective glucose utilization: the case of flying vertebrates

Active glucose metabolism enables rapid glucose utilization and/or effective storage in cells immediately after food digestion, which can reduce the amount of blood glucose available for glycation reactions. Birds and bats both undertake active flight, which is one of the most energy-demanding activities that has evolved in multiple clades [56–59]. It has been shown in nectar- and fruit-feeding bats that flight is one of the key factors in post-prandial blood glucose regulation [55,60]. Studies on bats have shown that post-prandial blood glucose levels reach peaks that would be harmful in other mammals, but they drop quickly and reach fasting levels within 60–70 min after the bats spent 70–75% of their time flying [55,60], a behavioural-based mechanism that might almost replace insulin-mediated pathways in the *Glossophaga soricina* bat [55].

In addition to the above-mentioned behavioural adaptations (flight), physiological mechanisms are also primarily involved in glucose metabolism. Floral nectar is roughly composed of a 35 : 35 : 30% ratio of glucose, fructose and sucrose [61]. *Phyllostomoid* bats seem to rely on a well-developed arsenal of intestinal sucrases, which are responsible for the efficient hydrolysis of sucrose [62], and therefore, favour the rapid absorption of sugar molecules. Birds also seem to be adapted to high-sugar regimes, as the maximal enzymatic activity of sucrases was found to be up to two times higher in hummingbirds than in nectarivorous bats [62].

In bats, ingested sugars are usually directly consumed instead of being converted into fat and stored in adipose tissue or as glycogen in liver and muscles. This is notably the case for nectarivorous bats, both at rest and while flying and foraging [63–65]. To explain such a fast utilization of absorbed sugars, muscles would be the ideal compartment to study. To date, we only have data from some studies that highlighted that adult bats' erythrocytes have a higher permeability to glucose than most mammals and birds, thanks to a rapid GLUT-1 mediated glucose transport [66]. This is even more remarkable because high permeability to glucose in erythrocytes is a feature usually only observed during fetal and neonatal life in mammals [67,68], except for humans, higher primates, small odontocete whales and bats, where it continues into adulthood [66]. Still, this metabolic particularity cannot be generalized to other sugars, as bats' red blood cells permeability to fructose seems really low [66], which conversely might reduce exposure of erythrocyte proteins to fructose toxicity. The case of avian erythrocytes is very different from that in bats: studies on domestic geese showed very low permeability to glucose [69] and facilitated hexose transport seems to be almost absent in pigeons [70]. It should be noted that, unlike mammals, including bats, avian red blood cells are nucleated and possess functional mitochondria [71,72], thus making these more prone to be fuelled by fatty acids instead of glucose, a possible indirect mechanism of protection against glycation.

While the insulin-dependent cascade is conserved across most vertebrates [73], it differs substantially in birds [74]. Indeed, mammals heavily rely on GLUT-4 for insulin-induced glucose uptake into cells, but some studies mention the absence of the

GLUT-4 transporter in birds [75,76], while others claim to have detected it [77,78]. One hypothesis to explain these conflicting results might be because avian genomes remain incompletely annotated owing to complexity. The absence of GLUT-4 could also be compensated by other transporters, such as GLUT-12, which appear to play a predominant role in few avian species [74,79,80]. Recent studies have also shown that the Slc2a4 gene encoding GLUT-4 had undergone changes under selective pressure in bat species relying on high-sugar diets in comparison to their insectivorous counterparts [81]. Moreover, it is estimated that bats heavily depend on intestine passive paracellular absorption, which constitutes over 70% of their total glucose absorption [82–84]. While this far exceeds what is seen in non-flying mammals [83,85–87], it is worth noticing that similar observations have been made in small birds [86–89]. Some authors suggested that this heightened reliance on passive paracellular transport might stem from the necessity to compensate for a proportionally smaller intestinal tissue mass compared to their overall body mass [86]. In the same vein but among non-flying vertebrates, marmosets, primarily gum-feeders with a smaller intestine relative to mammals of comparable size, also demonstrate a significant 30% paracellular glucose transport [90].

(b) Protection of biomolecules

(i) Protein turnover

Protein turnover contributes to the maintenance of protein homeostasis, which can be defined as the preservation of the stability and functionality of the proteome. Several intracellular mechanisms mediated by the proteasome and the lysosome are involved in degrading structurally altered proteins to avoid their possible toxicity. Various studies in different animal models have demonstrated that the rate of accumulation of AGEs in proteins is positively correlated with their half-life, and therefore, negatively with their turnover rate [48,50,91,92]. One study suggested that enzymes and intracellular proteins with a rapid turnover may, therefore, be protected from high rates of glycation [93]. However, it is now widely admitted that senescence is accompanied by a loss of proteostasis, namely a decline in the synthesis of chaperones and a decline in the activity of the proteolytic systems [94], which could then partly explain the accumulation of AGEs in proteins and cells over life [22,95]. In addition, proteostasis itself does not escape glycation stress [96].

(ii) Specific amino acid composition and three-dimensional conformation of proteins

Protein glycation mainly targets lysine and arginine residues [97–100]. However, several studies demonstrated that not all lysines or arginines in the same protein are subject to the same glycation rates. For example, of the 44 amino acids in the alpha and beta chains of human HbA1c, only five appear to be potential glycation sites, namely three lysines and two valines [101]. This site specificity obviously depends on the amino acid composition of a given protein, but also and above all, on its multidimensional conformation, with theoretically glycatable amino acids being either exposed at the surface or, on the contrary, inaccessible because hidden. Thus, the lower number of lysines in chicken albumin and the fact that most of these residues are located in the inner part of the protein (only one lysine on the surface) seem to explain why it is glycated at a lower rate than bovine or human albumin (three lysines on the surface) when exposed to the same increasing concentrations of glucose [102,103]. Moreover, the presence of certain amino acids (such as aspartic acid or specific binding sites for phosphate or phosphorylated molecules) in the neighbourhood of the preferential glycation sites also seems to be of importance [104]. It is also interesting to notice that birds from the Psittacidae family, which includes parrots and parakeets, have more lysine residues than other birds, yet cases of diabetes have been reported in parrots [105].

(c) Buffering glycated proteins and advanced glycation end-products

(i) Deglycating enzymes

Several defence mechanisms occur *in vivo* to regulate the accumulation of glycation products at different stages of the Maillard reaction. Although research in this area is still in its infancy, researchers have already identified several enzymes capable of detaching the sugar from the amine function of the fructosamine, thus reversing the formation of Amadori products and stopping the glycation reaction at its early steps [106]. To date, three major classes of deglycating enzymes have been described, which differ in their physiological role and their catalytic mechanisms: fructosamine oxidases (in fungi and bacteria) [107], fructosamine-3-kinase (in bacteria, mammals [107] and birds [108]) and fructoselysine-6-phosphate deglycase (in bacteria) [106].

(ii) Receptor for advanced glycation end-products

Originally known for binding AGEs [109], the receptor for advanced glycation end-products (RAGE) is now recognized as a multi-ligand receptor of the immunoglobulin superfamily [110]. It is expressed in various tissues [111] and is involved in inflammatory and immunological pathways [112]. RAGE is found under two main forms: transmembrane, the binding of which activates different signaling cascades, and as a free circulating form that can bind ligands without activating any pathway [113].

Among the AGEs capable of binding RAGE, preferential candidates include CML and hydroimidazolone [112]. The consequences of AGE–RAGE interactions at the membrane include a significant increase in reactive oxygen species (ROS) production, the activation of the NFκB transcription factor, an upregulation of inflammatory pathways, increased cell adhesion to the extracellular matrix, as well as facilitated AGEs accumulation [112,114–116]. Studies in diabetic patients have shown

higher RAGE levels in the kidneys, particularly in the context of nephropathies, as well as in atheroma plaques of patients suffering from atherosclerosis [117,118]. A positive correlation between RAGE and HbA1c levels has also been highlighted [118]. In addition, genetically modified mice that do not express RAGE appear to resist ageing-related diseases associated with hyperglycaemia [119–122]. Furthermore, it is worth noting that soluble RAGE is suspected to mitigate the negative impact of AGEs by sequestrating them [123].

For a long time, RAGEs were believed to be unique to mammals, with birds lacking them, which appeared to be a potential explanation for birds' resistance to glycation [124]. An evolutionary study points out that RAGEs emerged with mammals while being lost in birds [125]. However, recent research indicates that some bird species may possess genomic sequences related to RAGE, thus leading to an emerging debate [126,127].

5. Concluding remarks and future prospects

Our review clearly outlines the critical lack of research on glycation in species other than humans or laboratory mice and rats. However, as we saw before, some taxa such as bats and birds seem to have evolved tolerance and/or resistance to high glycaemia and/or glycation, while having an extended lifespan relative to non-flying animals. Studying the mechanisms of tolerance to high levels of circulating glucose in bats and birds, notably in wild conditions (as captive conditions might not accurately reproduce and reflect what happens in nature), will likely put us on the trail to new treatments for diseases such as diabetes in humans. A better understanding of the nature of the mechanisms involved in glycation resistance will also help us to determine how evolution has led to develop tolerances to the adverse effects of high glycaemia and glycation, and how glycation patterns and life-history traits are intertwined between and within species. Indeed, we already know that glycation interferes with mechanisms involved in life-history trade-offs such as oxidative stress and telomere shortening [20,128]. Thus, studying glycation could provide key data for explaining senescence rate variability among vertebrates. In depth studies on the relationship between glycation and fitness are necessary, which will require long-term records of individuals' life trajectories with repeated sampling over life. Furthermore, defining the rates of glycation accumulation over life should allow us to assess if glycation may be part of the process triggering organisms' entry into senescence, as suggested by previous results [129]. Long-term studies of glycation dynamics at the individual level will enable us to gather key information on three major points in the evolution of glycation resistance. That is, (i) the inter-individual variability of glycaemia and glycation levels, (ii) the heritability of glycation levels (could there be a genetic transmission of this resistance), and (iii) the effect of glycation in determining individual adult phenotype, physiological performances and ultimately inter-individual variations in fitness through the evaluation of reproductive and actuarial senescence and lifelong reproductive success.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. This article has no additional data.

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Authors' contributions. C.D.: writing—original draft, writing—review and editing; F.C.: writing—review and editing; F.B.: writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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Glycation: an additional biomarker of aging in mammals? Insights from non-conventional species

Résumé

Les réactions de glycation sont reconnues pour leur contribution au vieillissement et aux pathologies chroniques associées chez l'homme. Cependant, peu d'études se sont penchées sur cette réaction chez des espèces autres que l'homme ou les modèles animaux de laboratoire classiques, alors que certaines espèces sauvages semblent présenter une résistance aux effets délétères des glycations sur la santé. Dans cette thèse, nous proposons donc d'explorer les liens entre glycation des protéines, sénescence et traits d'histoire de vie chez différentes espèces de mammifères non conventionnelles. Nos travaux montrent que les taux de protéines glyquées augmentent de manière significative chez les espèces à rythme de vie plus lent, suggérant l'existence d'une tolérance aux effets néfastes des protéines glyquées chez les espèces plus longévives. D'autre part, nous montrons que les chauves-souris semblent manifester une résistance et/ou une tolérance accrue aux glycations par rapport aux autres mammifères. Il semble que la glycation des protéines ne soit pas un marqueur de sénescence chez tous les mammifères. Chez certaines espèces, comme les chauves-souris, elle pourrait plutôt refléter une qualité individuelle, influencée par des facteurs tels que la capacité à réguler le stress oxydatif. En outre, chez le chevreuil, les variations dans les niveaux de glycation semblent dépendre de la qualité de l'environnement et de l'état de santé des individus, suggérant un lien complexe entre l'état physiologique et les processus de glycation. Ces résultats soulignent l'importance d'étudier la glycation dans des modèles animaux non conventionnels afin de mieux comprendre l'évolution des mécanismes de résistance à cette réaction. Cette approche pourrait ouvrir de nouvelles perspectives pour traiter les effets pathologiques des glycations chez l'homme.

Mots-clés : glycation, sénescence, traits d'histoire de vie, espèces non-conventionnelles, mammifères

Résumé en anglais

Glycation reactions are recognized for their contribution to aging and associated chronic pathologies in humans. However, few studies have focused on this reaction in species other than humans or traditional laboratory animal models, even though some wild species appear to exhibit resistance to the detrimental effects of glycation on health. In this thesis, we aim to explore the links between protein glycation, senescence, and life history traits in various unconventional mammalian species. Our findings demonstrate that the levels of glycated proteins significantly increase in species with slower life histories, suggesting the existence of a tolerance to the harmful effects of glycated proteins in longer-lived species. Furthermore, we show that bats appear to exhibit increased resistance and/or tolerance to glycation compared to other mammals. It seems that protein glycation is not a universal marker of senescence across all mammals. In some species, such as bats, it may instead reflect individual quality, influenced by factors such as the ability to regulate oxidative stress. Additionally, in roe deer, variations in glycation levels appear to depend on environmental quality and the health status of individuals, suggesting a complex relationship between physiological state and glycation processes. These results underscore the importance of studying glycation in unconventional animal models to gain a better understanding of the evolution of resistance mechanisms to this reaction. Such an approach may open new avenues for addressing the pathological effects of glycation in humans.

Key words: glycation, senescence, life-history traits, non-conventional models, mammals