nature portfolio

Peer Review File

Constitutively Active Glucagon Receptor Drives High Blood Glucose in Birds

Corresponding Author: Professor Cheng Deng

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version. Parts of this Peer Review File have been redacted as indicated to remove third-party material. Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The study investigates the high blood glucose levels observed in birds compared to other vertebrates, hypothesizing that the glucagon receptor (GCGR) plays a central role. Researchers found that avian GCGR exhibits constitutive activity, which is unusually high compared to other vertebrates. The paper explores this phenomenon through various experiments, demonstrating that this constitutive activity of GCGR in birds leads to sustained high blood glucose levels and is critical for their unique metabolic adaptations.

This study is novel and explores a very interesting research question and the experimental evidence the authors provide is abundant and solid. This is an impressive and thorough work. However, the way the manuscript is written now makes it very difficult to follow the author's logic and thus to properly assess the quality of the research.

Therefore, my major comment is that the structure of the manuscript needs to be significantly revised to make the findings more accessible to the reader. I have some specific examples of where the structure could be improved:

1) The first two paragraphs of the results don’t provide any background or reasoning on why it is important to talk about it.

2) The comparative analysis of the constitutive activity and expression of GCGR across different vertebrate groups, highlighting the unique characteristics of avian GCGR (showing both constitutive activity and high expression), would serve as a strong starting point. This sets the context for why the research was done. Although the authors seem to follow this logic by including this in Figure 1a, the necessity of the preceding analysis is not well-explained.

3) Positive selection signal in GCGR seems like a minor point, as well as a detailed analysis of the effect of amino acid substitutions on the structural properties as it doesn't inform the following analysis and now seems to be out of place. I would recommend discussing this closer to the end of the manuscript unless the author could explain the reason for this analysis being placed here better.

4) A more general comment: for each new section of the results, the authors should provide a clear motivation for the following steps. This will help the reader understand the progression of the research and its logical flow.

Comments regarding the analyses:

1) The authors performed PAML positive selection analysis using the placental mammals' ancestral branch as the foreground and birds and non-placental mammals as the background. This approach is surprising given that the main hypothesis suggests that the constitutive activity of GCGR was likely subject to natural selection in birds, providing a selective advantage during the transition to flight and supplying birds’ increased metabolic demands. If the evolutionary hypothesis focuses on the loss of constitutive activity in placental mammals, then positive selection may not be the appropriate type of selection to look for, unless there was a selective advantage for placental mammals in losing the

constitutive activity. I suggest that the authors communicate the evolutionary hypothesis more clearly and then perform the appropriate tests to match the hypothesis.

2) Figure 1A and Figure 2A both represent comparative analysis across vertebrate groups, but they have a very different set of species analyzed. Was there a good reason to not keep the set of analyzed species consistent?

3) Figure 5 sets up a comparison of null, hsGCGR (human receptor), and scGCGR (canary receptor). However, hsGCGR data is missing from panels aa and ab, as well as Supplementary Fig. 21a,b. The reason for excluding these data should be provided.

4) The choice of organisms for in vivo tests is confusing. The analysis of the effects of avian GCGR downregulation (Fig. 3) is conducted in Gallus gallus and Lonchura striata, while single-cell RNAseq is done in Melopsittacus undulatus. The authors should explain the reasoning behind the selection of these organisms.

Minor comments regarding wording and data presentation:

1) There are several points in the introduction that seem poorly explained:
Line 65-66: “Constitutive activity has been documented in many GPCRs and other receptors, resulting in the production of a second messenger even in the absence of ligand.” - This is the definition of constitutive activity, so it is more accurate to say “which means” instead of “resulting”.

2) Line 119: the authors use “phylogenetic analysis” to refer to PAML analysis when it is not phylogenetic analysis, but a positive selection test.

3) In many figures, the authors are truncating Y-axis and it is misleading. Specifically in: Fig. 3b,j,n,r,v; Fig. 4g, Fig. 5aa, and Fig. 6b,d,e.

4) Figure 5c presents a timeline for the enzymatic activity of Gpa and Pepck as barplots, when in previous panels the same type of data was presented with lineplots. I would suggest being consistent and using lineplots throughout as barplots look more crowded and confusing.

5) This paper uses “safflower” chickens and “safflower” chicken GCGRs for a number of results. It’s unclear what safflower chickens are, as they are not present in a list of chicken breeds.

6) Fig. 7a: repetition of “high expression” in the mammalian panel. I assume the left one needs to be corrected to “low expression”.

Referee #2

(Remarks to the Author)
The authors conducted a very thorough, comprehensive study to understand mechanisms underpinning the high blood glucose concentrations found in birds compared to other vertebrates, especially mammals. They show in a large number of diverse experiments that the avian glucagon receptor is constitutively active and that high hepatic expression of avian glucagon receptors, even when expressed in non-avian species, result in high blood glucose, high lipid mobilization and high metabolic rate. Their study suggests that the constitutive activity of the glucagon receptor is a phylogenetically basal trait that has been lost in placental mammals. They also elucidated the molecular nature of constituent activity in the avian glucagon receptor.

Main comments:
1. My biggest comment on this manuscript is a disconnect between the Introduction, the remarkable experiments that were conducted, and the Discussion. The Introduction focuses on high blood glucose in birds as compared to most other vertebrates and the mechanisms that may underlie this pattern. It sets up the main study question as a proximate issue – to find out the molecular mechanisms that lead to high blood glucose levels in birds. This is the question that the study was designed to address, and it did so in a very comprehensive way.

However, in the Discussion (especially from L 559 onwards) the authors turn to discussing the functional relevance of high glucose levels, and associated traits like metabolic rates and body mass. While their hypothesis and the detailed pathways illustrated in Fig 7 are interesting, and data on metabolic rates and mass fit with this hypothesis, in principle this study was not designed to address functional questions. It may well be that the constitutively active avian GCGR played a permissive role in the evolution of flight physiology. But why do some reptile taxa like Agamidae have blood glucose levels and metabolic rates as high as in birds? These reptiles do not fly. The authors do not address this issue. Also, bats, i.e. flying mammals, were not included. I suspect this is because of a lack of data for bats, but at least they should be included in the framework. I think the best the authors could argue is that the avian GCGR may have permitted/facilitated the evolution of flight - and I would urge the authors to tone down statements like ‘adaptations to flight’. In fact, one wonders whether the question should be turned around and ask why placental mammals have lost this trait.

In Fig 1, the authors compiled data on a nice number of different bird species, some of which are migratory (and migrate in various ways like soaring or flapping and for various distances), some are predators that rely on fast flight to catch prey (like

peregrine falcons), some have a cheaper way for obtaining food (like granivores that often hop to gather food instead of flying) than others, some show amazing acrobatic courtship displays that look rather costly like manakins etc. For addressing functional questions, data from these different groups of birds could have been compared.

Also, what about other modes of locomotion like swimming? The authors did include fishes in their study, but did not interpret their data in terms of their locomotory mode. What about the hopping of amphibians? This is the kind of study design that would allow the authors to comment on functional aspects of glucagon receptor functioning.

I suggest that the authors rewrite their Discussion and tailor it to their main aim(s) as outlined in the Introduction and fitting the experiments they conducted.

2. I am surprised that bats are not considered at all in this paper – after all they are flying mammals and because of their primary mode of locomotion should be closer in physiological adaptations to birds than mammals. The authors may want to check for data on bats and include those if available. If not available, the issue of bats should at least be touched on in the Discussion. They could play a really interesting role in hypothesis testing.

3. The manuscript is very impressive in its completeness. However, at times it is also overwhelming because it contains so much data. Some of it may not be necessary and even detract the reader from the more crucial aspects of the work. The authors may want to go over their Results and Figure section of the main manuscript and focus it on addressing the main question.

4. As a non-specialist reader, I was quite often confused by the large number of abbreviations used and had to go back and forth to find what these referred to. Sometimes I found definitions in the text of the manuscript and other times, I had to search the legends of the Figures or the Supplementary Materials. That was a bit of a hassle. I suggest to limit abbreviations to the most frequently used terms and write out the rest.

5. The Figure legends often are insufficient in explaining what was done to obtain the data. Also, I could not find legends for any of the Tables.

6. I was puzzled by the statistics. Some statistics seem to test the data in multiple ways, a technique that is not considered correct in other fields. For example, when there were 3 groups, then one group was tested against the two other groups in two different tests, which is repeated testing of the same data set that requires an adjustment in the p-value. I know that for data sets with n=3 complex statistics are not an option. However, for some experiments you had larger sample sizes and more than two groups. For these experiments I suggest you use analysis of variance (ANOVA) or general linear model (GLM) statistics where you can compare all three groups simultaneously in one model instead of comparing two groups at a time.

7. Again regarding statistics: it was often unclear whether animals were measured repeatedly, which could have been a valuable method because it reduces individual variation in the data. However, repeated-measures data need to be analyzed with statistics that account for it, for example paired t-test, repeated measures ANOVA or GLM with animal ID included as a variable.

8. Not sure the experiments/section described in L 473-500 are needed?

9. As far as I know, glucagon receptors are not only expressed in the avian liver but also in adipocytes, the brain and perhaps other tissues. Can the authors touch on that issue, at least in the discussion, to outline how these fit with the maintenance of high blood glucose envisioned in the Discussion?

Specific comments:
L 94-95: "… glycogen content in avian liver remained relatively stable…" This statement does not match Suppl Fig 2b, where you indicate a significant difference at $p < 0.01$ in glycogen content between fed and fasted states in both chicken and budgerigar. Please correct your statement.
L 101: "levels of THE tuatara reptiles…": since you only examined one species from that family, I suggest to reword the 'THE tuatara" into "A tuatara"
L 102: "decreased with age" – I could not find the stats supporting this statement. Please add.
L 109 – 112. That sentence is a bit hard to read, perhaps reword it.
L 115: what is an "activity-high slope"? See also one sentence below. Also, I could not find stats to back up your statement.
L 127 ff. The work explained here is interesting. Of course, chicken have undergone intensive artificial selection for egg or meat production. If the authors really wanted to make statements about the evolution of the glucagon receptor in birds, they should include wild species into their analyses. I realize that this may technically not be possible. However, this limits the generality of the findings presented here.
L 202: stats on protein expression levels?
L 213: the legend of Fig 2 states that you used unpaired student t-tests. Which compare two groups. It is unclear to me which statistics you used here. Comparing certain groups multiple times against other groups would clearly be a repeated testing of the same data set and would require correction of the p-value – or the use of different statistics.
L 216: could you please explain what kind of inhibitor this YMxxx is?
L 227: this section hinges on understanding what an 'AAV delivery system' is. Please explain – not sure I found any details on this.

L 230: it should be “FIG 3 E&F” since Fig 3e does not show concentrations. Also, please explain or redo the stats – these must be repeated measures data? I only found reference to unpaired t-tests.
L 232: please delete “and 3h”
In general, Fig 3 is loaded with graphs and I wonder whether the numbers of panels in that Fig could be reduced.
L 233: not sure what a Gpa enzyme is and did not find an explanation easily.
L 276: on Fig 3 there is no panel labelled ‘ab’
L 315: please explain PPAR
Likewise, Fig 4 is loaded with graphs and I wonder whether the numbers of panels in that Fig could be reduced. For example the axis labeling on many graphs is already so small that it is hard to read (e.g. Fig 4L).
L 336: glucose intolerance and insulin resistance are not explained.
L 356: Figs 5 h & i look like repeated measures data, and if the case, should be analyzed in that way
L 378: what are db/db mice, and their phenotypes?
L 419: Fig 5aa only shows data for the scGCGR and null groups, please correct your statement here (hsGCGR is not shown and I am not sure whether data exist for that group). Same for statement in L 422!
L 450: In Suppl Fig 23 the two groups are not labeled – one has to go back to Fig 6 and assume the groups are coded in the same way. In this line Suppl Fig 23 H should be referred to, not ‘L”
L 459ff: if the data in this section all refer to F3 individuals, please indicate it here again. It is quite confusing because in the F2 generation there was only 1 C/C male, while in the F3 generation, there were apparently n=3 C/C individuals. That makes quite a difference. Please also clarify on Fig 6 and Suppl Fig 23.
L 522: Fig. 3a shows the schematic of the three types of injection, but data on the GCG antagonist are not presented in this Fig!
Fig 7 is an interesting depiction of the main conclusions by the authors that the constitutively active GCGR plays a role in adaptations to flight. While that is a reasonable hypothesis, in L 541 the authors also admit that Agamidae have high blood glucose and high basal metabolic rates. Agamidae don’t fly… (as well as the other families mentioned in L 553) and the authors do not explain how other taxa of ‘lower vertebrates’ that also have constitutively active GCGR (e.g., Fig 1a) fit in this picture.
L 547ff: “… birds eat non-stop…”. This is an oversimplification. Sure, this may be so in chicken, maybe also in granivorous bird species, frugi/nectarivores and many insectivores. But there are also birds of prey that eat intermittently… Also, small mammals with high metabolic rate may likewise eat ‘non-stop’, simply because like birds, they cannot carry much energy reserves because of their small size. I am not sure this statement is needed or helpful here. Also, the Figs you refer to (Fig 5h and Suppl Fig 17o) are for mice…
L 604: This study has many strengths. But there are also several more limitations that I commented on above that should be touched on here.
L 611: I suggest to tone down that statement, especially the parts relating to “that independently

Referee #3

(Remarks to the Author)
In this manuscript, the authors report on the well-known phenomenon that glucose production and utilization is increased in birds relative to other vertebrates. Consistent with the higher glucose demand and production, which is considered a physiological adaptation to flying, the authors show that the avian glucagon receptor (GCGR) shows higher constitutive activity relative to GCGR in other vertebrates (including humans), and also in comparison to the avian receptors for GLP-1, GLP-2, and GIPR. Using viral-mediated knockdown of GCGR, the authors convincingly demonstrate that the higher constitutive activity of GCGR, along with increased hepatic expression of GCGR, accounts for the higher levels of blood glucose in birds. Consolidating these data, the authors performed snRNAseq studies across multiple species, showing that hepatic expression of GCGR is increased in birds relative to other vertebrates and that expression of avian GCGR in mice results in higher levels of blood glucose and decreased body weight.

The manuscript is excellently written and easy to follow. It deals with an interesting and important question, the role of the avian GCGR in regulating glucose homeostasis in birds. The proposed mechanism of enhanced GCGR action is elegantly demonstrated using state-of-the art technologies, and the proposed studies are sufficiently clear and reliable to support the authors hypotheses. The proposed mechanism of higher constitutive GCGR activity makes sense, given that glucagon is a very strong promoter of de novo glucose production. The manuscript gained much from the demonstration that viral-mediated GCGR knock-down decreases blood glucose in birds, whereas the induced expression of avian GCGR in mice increases levels of blood glucose. Overall, this is a beautiful study that unmistakably demonstrates the importance of avian GCGR in glucose homeostasis in birds.

The data presented are elegant and logical, and it is really difficult to find some criticism here, which I highly appreciate. I have only a few comments and suggestions that the authors may want to consider to further strengthen the manuscript. I wonder whether non-flying birds have lower GCGR activity and blood glucose levels relative to flying birds? On page 1, the authors correctly state that birds have higher levels of blood glucose relative to other vertebrates, almost twice as high as mammals and 7-fold higher than amphibians. While correct, we have to keep in mind here that that birds also have a higher metabolic rate, and hence higher glucose demand, relative to other vertebrates, particularly mammals. Accordingly, the manuscript would gain from addressing the question whether birds produce glucose at a higher rate than what is expected based on their higher metabolic rate? When comparing birds with a vertebrate of equally high metabolic rate, do birds still have higher blood glucose, GCGR activity and hepatic GCGR expression? Just comparing blood glucose levels without taking into account species-related differences in metabolic rate is a bit misleading, and undermines the beauty of these studies. In case birds do show higher glucose production than expected by metabolic rate (requires regression analysis with ANCOVA to demonstrate this), then this is a very important message. On another note, the authors may also want to mention

that utilization of glucose is faster relative to utilization of fat, which further aligns with the physiological adaptation to flying. This said, fat is a very suboptimal energy substrate for initiation of flying, while fat is the preferred substance for migratory birds that have to fly over a very long time. I wonder whether higher constitutive GCGR action got lost in non-flying birds (like Ostrich)? Nonetheless, non-flying birds are very fast runner, which also requires high glucose utilization. On another point, I wonder where in evolution the higher constitutive GCGR activity occurred? Birds separated quite early from other vertebrates, so is the higher constitutive GCGR activity also observed in very early birds? The demonstration that constitutive activity of GCGR is also observed in the Urochordata, and lost in placental-vertebrates, would support this.

The observation that avian GCGR maintains high expression in the liver is actually expected, since hepatic GCGR expression is highest across all tissues in all other animals as well, consistent with the liver as the primary organ for de novo glucose production. The same also accounts for the demonstration that avian GCGR signaling is primarily Gas-coupled, which is the case also for GCGR signaling in all other species. Is the rate of hepatic GCGR expression also a function of metabolic rate, or in other words, do animals with higher MR also have a higher expression of GCGR? Is the higher constitutive GCGR activity also relevant for thermogenesis in the birds? Did the authors measure thermogenesis after the viral-mediated gene KD in the birds? Do other animals living in the cold have higher hepatic GCGR expression as well?

I highly discourage the authors from normalizing the energy expenditure data shown in Fig. 5aa-ac, and Fig 5ad-af by deviation to body weight, since such methodology doesn't discriminate between mass-dependent and -independent effects on metabolic rate (PMID: 22205519, PMID: 23520145, PMID: 34489606). The more appropriate way of doing this is by using a regression analysis and ANCOVA with e.g. body weight as co-variate (PMID: 22205519, PMID: 23520145, PMID: 34489606). Notably, such methodology also allows to calculate a bar graph based on the ANCOVA corrected data (see mentioned publications for an example)

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)
I appreciate that authors significantly restructured the manuscript, improving the manuscript's clarity, structure, and readability. I believe that the authors sufficiently addressed my comments and I would be pleased to see this work published.

Referee #2

(Remarks to the Author)
The authors are to be congratulated for their extensive revision of the manuscript, which improved its already considerable quality in a major way. I am impressed by their even adding more data (e.g. on bats) and re-analyzing many of the existing ones. They also extensively restructured and reworded their manuscript.

Main comments:
The Intro, Results and Discussion parts are now better connected, and the authors have nuanced their interpretation that the constitutively active GCGR contributed to the evolution of flight in birds.
I have a few more suggestions.
Specific comments:
L 1: please write out 'GCGR' in the title
L10: Abbreviation 'Gs'is not explained.
L 114: briefly state findings for budgies here? You do mention 'birds', i.e. both chicken and budgie results a few sentences down.
Suppl Fig 1: the sequence of the panels is obscure, especially for the panels following panel i. Please reorder so that the reader can more easily find the relevant panel. Also, three panels (those below j & l & q) now have no letter assigned to them. Please revise.
L 143: should you refer to Suppl Fig 4'A'?
L 147: please delete the crossed-out reference to Fig 5
L156: is 'human' missing at the end of this line?
L 172: add 'and budgerigar' here?
L197: Fig 1 & 2 do not show age-related trends in hepatic expression of GCGR genes. Please delete the reference to those Figs.
Suppl Fig 11: I find the x-axis legend referring to 'hatch (days)' confusing. The Fig legend doesn't explain it further. These are pre-hatch or incubation days, correct? If so, I'd rather label them 'incubation days' and explain in the legend that day 0 or 1 was the first day of incubation (or however else you defined it).
L 206: I cannot find the stats for Fig 2a and suppl Fig 12 that would support your statement. In the legend to Fig S12 you write that stats are provided above the western blots, but I didn't find anything. There are numbers above the plots, but not sure what these refer to.
L 213ff: likewise, I am missing the stats for Fig S 13. Please state the type of stats you used and p-levels (same for Fig S12)
L 558: "massive energy expenditure": is this backed up by data (no citation is provided)? When food is scarce, animals can also resort to decreasing metabolic rate and increasing rest periods, which can help them to reduce depleting endogenous energy stores at a fast pace. Lowering of body temperature can also be a strategy to save energy. Therefore I am not entirely convinced by this argument.

L 562: please insert ‘can/could’ or something along that line before ‘utilize’. You have not tested whether constitutively active GCGR are involved in flying or migration. Also, again, bats can also fly and migrate.
L 636: please add ‘may’ here.
L 882ff: Please add a statement to the legend for Suppl Fig 1 to make the reader aware that that the scale of the y-axes differ for panels a-i.
Legends for Supplementary Tables: Thank you for now providing titles for the supplementary tables. However, more information is required. For example, for Suppl. Table1 the statistical measure of the variance is not stated. Are the data provided as means ± std errors, or standard deviation or confidence intervals…? Please add such information to all Tables (where it applies).

Referee #3

(Remarks to the Author)
I have no further comments. The manuscript improved significantly during the revision and I think that it is an important manuscript

Referee expertise:

Referee #1: avian molecular evolution

Referee #2: avian evolutionary physiology

Referee #3: glucagon signaling/physiology

Referees' comments:

Referee #1 (Remarks to the Author):

The study investigates the high blood glucose levels observed in birds compared to other vertebrates, hypothesizing that the glucagon receptor (GCGR) plays a central role. Researchers found that avian GCGR exhibits constitutive activity, which is unusually high compared to other vertebrates. The paper explores this phenomenon through various experiments, demonstrating that this constitutive activity of GCGR in birds leads to sustained high blood glucose levels and is critical for their unique metabolic adaptations.

This study is novel and explores a very interesting research question and the experimental evidence the authors provide is abundant and solid. This is an impressive and thorough work. However, the way the manuscript is written now makes it very difficult to follow the author's logic and thus to properly assess the quality of the research. Therefore, my major comment is that the structure of the manuscript needs to be significantly revised to make the findings more accessible to the reader. I have some specific examples of where the structure could be improved:

R: Many thanks for your encouraging expertise comments. Throughout this point-by-point response, we typed in red to present our answers to the questions and comments of reviewers while we used gray highlights to present our revisions in the manuscript. Parts of the manuscript that only were moved to a different location are marked in a lighter gray. We also provide manuscript and figure files with highlights of changes for the convenience of reviewers/editors. New and altered figures were highlighted in yellow. Figures or parts that were moved (e.g., to Supplemental Figures) were highlighted in green. Figures that are included in the replies to referees are denoted with the letter R.

1. The first two paragraphs of the results don't provide any background or reasoning on why it is important to talk about it.

R: Thanks for your important points, we have made the following changes to the Introduction part of the manuscript.

The former first paragraph (about the flight of birds) was moved into the Discussion part, on lines 593-598, was revised as:

As one of the most diverse extant vertebrate groups, birds have unique physiological adaptations and exaptations for flight, e.g. low body weight in relation to size combined with strong muscles and high metabolic rate[97]. As the main fuel for short-term flight of birds[11], glucose probably facilitates rapid carbohydrate energy supply during the takeoff phase[31,98–100], and the high blood glucose supported by constitutive activity GCGR **(Fig. 3** and **4)** provides a large energy pool for birds.

In the manuscript, on lines 26-31, was revised as:

As one of the most successful and diverse vertebrate lineages, birds possess numerous physiological characteristics distinct from other vertebrates[1,2], among which high blood glucose stands out as one of the most prominent and crucial characteristics. Glucose, a vital metabolic compound intimately tied to numerous physiological aspects of organisms, renders the study of blood glucose regulation mechanisms in birds instrumental in unraveling their origins and evolution.

In the manuscript, on lines 51-58, was revised as:

Previous studies have shown differences in the tissue expression profiles of the glucagon receptor family between mammals and birds[17–22]. E.g., in chickens, GCGR is expressed at high levels in various tissues, with the highest in liver and adipose tissue, followed by breast muscle, brain, proventriculus, and kidney[22]. In mammals, such as rats and mice, GCGR expression is highest in the liver, followed by the kidney, and lesser amounts are found in other tissues[17–21]. GCGR primarily regulates glucose metabolism processes in the liver, including glycogenolysis, gluconeogenesis, and glycolysis[23,24].

In the manuscript, on lines 61-68, was revised as:

In birds, studies regarding their high blood glucose primarily focused on the role of ligands involved in blood glucose regulation[27]. A previous study has shown that an antagonist of GCG does not affect blood glucose levels in birds[28], suggesting that the maintenance of high blood glucose in birds may not rely on ligand-mediated regulation. The rise in blood glucose levels is primarily regulated by hepatic glycogenolysis and gluconeogenesis[29,30]. In vertebrates, gluconeogenesis and glycogenolysis are conserved and occur mainly in the liver, producing approximately 85% of

endogenous glucose[31].

In the manuscript, on lines 77-82, was revised as:

The GCG-Gs axis also upregulated downstream genes involved in lipid metabolism, e.g., the peroxisome proliferator-activated receptor alpha (*Pparα*)[41]. Thus, the GCG–GCGR axis plays a central role in regulating blood glucose and glucose homeostasis[4,42–45], while the upstream molecular regulatory mechanisms appear to differ between birds and mammals.

In the manuscript, on lines 84-94, was revised as:

Constitutive activity has been documented in many GPCRs and other receptors, which means that a second messenger is produced without a ligand; this form of GPCR constitutive activity is associated with various potential physiological functions[46–48]. Constitutive activity can still be further activated by its ligand. Some constitutively active GPCRs, like the human adenosine A2B receptor (A2BR), human histamine H4 receptor (hH4R), and human H3receptor (hH3R), have been demonstrated that their cognate ligands can further activate the corresponding receptors[49–51]. In this study, we have discovered that the avian GCGR exhibits highly constitutive activity and maintains high expression levels in the liver. We postulate that the evolution of GCGR associated with glucose regulation contributed to the physiological characteristic of high blood glucose levels in birds.

2. The comparative analysis of the constitutive activity and expression of GCGR across different vertebrate groups, highlighting the unique characteristics of avian GCGR (showing both constitutive activity and high expression), would serve as a strong starting point. This sets the context for why the research was done. Although the authors seem to follow this logic by including this in Figure 1a, the necessity of the preceding analysis is not well-explained.

R: We have added motivation to analyze constitutive activity and expression of GCGR receptors of different animals in the resubmitted version:

In the Introduction part of the manuscript, on lines 61-65, we added literature support for the motivation to analyze constitutive activity, was revised as:

In birds, studies regarding their high blood glucose primarily focused on the role of ligands involved in blood glucose regulation[27]. A previous study has shown that an antagonist of GCG does not affect blood glucose levels in birds[28], suggesting that the maintenance of high blood glucose in birds may not rely on ligand-mediated regulation.

We added motivation to analyze constitutive activity and expression of GCGR in the Result part of the manuscript, on lines 98-101, was revised as:

The glucagon receptor family functions mainly by activating the signaling pathways through ligand stimulation[52]. Because the glucagon receptor family plays such a critical role in blood glucose metabolism, we investigated the activation of several receptors in HEK293 cells.

On lines 117-124, was revised as:

Compared with mice, blood glucose levels, hepatic glycogen content, and GPa enzymes were less affected by feeding and fasting in birds (**Supplementary Fig. 2a**,**b** and **c**). Hepatic GCGR expression was significantly upregulated after fasting in mice and birds (**Supplementary Fig. 2d**). Based on the above results, we hypothesized that constitutively active GCGR, which continuously activates the downstream signaling pathway independent of ligands, may be a regulator of glucose homeostasis and an essential contributor to the phenotype of high blood glucose levels in birds.

On lines 165-169, was revised as:

The above experimental results indicate that the constitutive activity of GCGR is not unique to birds. Although GCGR exhibits high constitutive activity in reptiles, amphibians, and fish, their average blood glucose levels are lower than in birds. This suggests that constitutive activity may not be the sole determinant of elevated blood glucose levels. Therefore, we next examined expression levels.

On lines 187-191, was revised as:

The birds and one branch of the reptiles (*Agamidae*), exhibiting both high constitutive activity and high hepatic expression of GCGR, showed minimal concentrations of glycogen stores in their livers compared to other vertebrates, and showed relatively high blood glucose levels (**Fig. 2a** and **Supplementary Fig. 10a**).

3. Positive selection signal in GCGR seems like a minor point, as well as a detailed analysis of the effect of amino acid substitutions on the structural properties as it doesn't inform the following analysis and now seems to be out of place. I would recommend discussing this closer to the end of the manuscript unless the author could explain the reason for this analysis being placed here better.

R: We agree and deleted everything concerning the positive selection analysis and retained structure function-analysis based on previous findings that the activation of hsGCGR depended on the cooperation of multiple domains (PMID: 32193322, PMID: 23863937, PMID: 28514451) and selected residues in these domains that were conserved in all birds aligned but were different in placental mammals.

Consequently, we deleted **Fig 1b** and **Fig 1d,e,** as well as **Fig 1f,g** to the supplementary figures as **Supplementary Fig. 6 and 8**. We also deleted the former **Supplementary Fig. 6b** and

Supplementary Table 4 and **5**).

In the results section, we deleted the entire part related to "positive selection signal in GCGR" and replaced it in the manuscript as follows, lines 144-160:

Furthermore, testing for constitutive activity of GCGRs after domain swapping showed that the constitutive activity of non-placental vertebrate GCGR depended on the cooperation of multiple domains (the stalk, extracellular cell loop ECL1 and intracellular cell loop ICL2, and possibly the C-terminus) (**Supplementary Fig. ~~5~~-8)**. Consistently, previous studies have shown that these domains may function in the activation of hsGCGR (the ICL2 of hsGCGR interacts with Gs as determined in the crystal structure[56], and structural differences in the stalk, ECL1, and the C-terminus were detected in the activated and inactive hsGCGR states[57,58]). Hence, we modified several amino acid residues in these domains, based on their high conservation in birds while different in placental mammals. Perhaps, the putative adaptive evolution of GCGR (loss of constitutive activity) may have benefitted placental mammals, e.g., allowing them to retrieve stored energy, when food is scarce. Avian GCG concentrations in plasma are five-fold higher than those in mammals[59]. As expected, GCGR, devoid of constitutive activity, was more sensitive to GCG (**Fig. 1c** and **Supplementary Fig. 9**). When the body needs energy, GCGR can quickly and sensitively activate downstream pathways in response to GCG stimuli, facilitating placental mammals to adapt to different external environments.

In the Discussion of the manuscript, lines 548-553, was revised as:

Here, we show that the avian glucagon receptor (GCGR) evolved differently from that of placental mammals and maintained constitutive activity, dependent on the interaction of the stalk, ECL1, and ICL2 (**Supplementary Fig. 5-8**). By screening the human SNP data from NCBI, we also have uncovered naturally occurring point mutations (hsGCGR^{H339R}) in the ICL3 of human GCGR with a weak constitutive activity (**Supplementary Fig. 24**).

4. A more general comment: for each new section of the results, the authors should provide a clear motivation for the following steps. This will help the reader understand the progression of the research and its logical flow.

R: We added motivation for several sections of the results. (Please see reply to questions 2), and we have also made the following changes：

In the manuscript, on lines 291-298, was revised as:

The results in **Fig. 3** and **Supplementary Fig. 18** show that the changes in downstream pathways were similar after *GCGR* gene expression was knocked down in the liver. Moreover, *Melopsittacus undulatus* (budgerigars) has high hepatic *GCGR* gene expression (**Fig. 2b**), as well as high constitutive activity (**Fig. 1a**). To investigate the regulatory mechanisms responsible for the high glucose levels in birds, endogenous *GCGR* gene expression in liver of adult male *Melopsittacus*

undulatus (mu) was downregulated by RNA interference employing intravenous AAV-sh(muGCGR) injection.

In the manuscript, on lines 351-354, was revised as:

However, the expression of GCGRs was maintained only for a short time after ADV injection (**Supplementary Fig. 20b**). Therefore, AAV injection was chosen to achieve a longer duration of constitutively active GCGR expression in mouse liver.

5. The authors performed PAML positive selection analysis using the placental mammals' ancestral branch as the foreground and birds and non-placental mammals as the background. This approach is surprising given that the main hypothesis suggests that the constitutive activity of GCGR was likely subject to natural selection in birds, providing a selective advantage during the transition to flight and supplying birds' increased metabolic demands. If the evolutionary hypothesis focuses on the loss of constitutive activity in placental mammals, then positive selection may not be the appropriate type of selection to look for, unless there was a selective advantage for placental mammals in losing the constitutive activity. I suggest that the authors communicate the evolutionary hypothesis more clearly and then perform the appropriate tests to match the hypothesis.

R: The reviewer is absolutely right that positive selection is not the appropriate type of selection to look for (see also reply to your question number 3).

Hence, we deleted everything pertaining to positive selection and retained structure function-analysis based on previous findings that the activation of hsGCGR depended on the cooperation of multiple domains (PMID: 32193322, PMID: 23863937, PMID: 28514451) and selected residues in these domains that were conserved in all birds aligned but were different in placental mammals. In birds, high blood glucose is attributed to both high constitutive activity and high hepatic expression of GCGR. However, other species, such as fishes, frogs, and most lizard lineages, which retain GCGR's constitutive activity, do not exhibit the phenotype of high blood glucose due to their low high hepatic expression of GCGR. Therefore, the avian high blood glucose is not driven by the adaptive evolution of GCGR activity but rather by its high hepatic expression. Regarding the loss of constitutive activity in placental mammals, we have added explanations to this evolutionary hypothesis in the manuscript.

In the manuscript, the corresponding part, on lines 144-160, was revised as：

Furthermore, testing for constitutive activity of GCGRs after domain swapping showed that the constitutive activity of non-placental vertebrate GCGR depended on the cooperation of multiple domains (the stalk, extracellular cell loop ECL1 and intracellular cell loop ICL2, and possibly the C-terminus) (**Supplementary Fig. ~~5~~ 8)**. Consistently, previous studies have shown that these domains may function in the activation of hsGCGR (the ICL2 of hsGCGR interacts with Gs as determined in the crystal structure[56], and structural differences in the stalk, ECL1, and the C-terminus were detected in the activated and inactive hsGCGR states[57,58]). Hence, we modified several amino acid residues in these domains, based on their high conservation in birds while different in placental mammals. Perhaps, the putative adaptive evolution of GCGR (loss of constitutive activity) may have benefitted placental mammals, e.g., allowing them to retrieve stored energy, when food is scarce. Avian GCG concentrations in plasma are five-fold higher than those in mammals[59]. As expected, GCGR, devoid of constitutive activity, was more sensitive to GCG (**Fig. 1c** and **Supplementary Fig. 9**). When the body needs energy, GCGR can quickly and sensitively activate downstream pathways in response to GCG stimuli, facilitating placental mammals to adapt to different external environments.

In the manuscript, the corresponding part, on lines 626-632, was revised as：

To adapt to new environments or challenges, organisms who evolved one such putative adaptation, such as the constitutive activity of GCGR, use additional "adjusting screws" or "setscrews" to further enhance or counteract the effects, such as enhanced or reduced *GCGR* gene expression levels and many other (e.g., downstream) mechanisms, for example, when birds occupied a different temporary or permanent environment or behavior, such as the ground (flight-less or poor) or even the water (swimming, diving).

On lines 634-637, was revised as：

In conclusion, constitutively active GCGR with high hepatic expression underlies sustained high blood glucose in birds and is the consequence of a “switchless gene” that contributed, among many other adaptations and exaptations, to the evolution of flight in birds and their ancestors.

6. Figure 1A and Figure 2A both represent comparative analysis across vertebrate groups, but they have a very different set of species analyzed. Was there a good reason to not keep the set of analyzed species consistent?

R: The reasons for not keeping the consistency of the species analyzed are as follows:

The species in **Fig. 1a** are used to research GCGR constitutive activity in cells (species for which genomic data are available). The species in **Fig. 2a** were used for the expression of hepatic GCGR and blood glucose test based on the live animals with unprotected status and readily available to obtain (*Ictalurus punctatus, Danio rerio, Pelophylax nigromaculatus, Xenopus laevis, Orthriophis, taeniurus, Pogona vitticeps, Phrynocephalus theobaldi, Laudakia sacra, Eublepharis macularius,*

Chrysemys picta picta, *Crocodylus siamensis*, *Taeniopygia guttata*, *Lonchura striata*, *Serinus canaria*, *Carpodacus vinaceus*, *Passer domesticus*, *Parus monticolus*, *Muscicapa ferruginea*, *Phylloscopus magnirostris*, *Melopsittacus undulatus*, *Anas platyrhynchos*, *Gallus gallus*, , *Petaurus breviceps*, *Capra hircus*, *Mus musculus*, *Macaca mulatta*). It is, therefore, difficult to achieve consistency.

In the manuscript, the corresponding part, on lines 176-187, was revised as：

In vertebrates, the GCG–GCGR axis regulates the rate of hepatic glucose production by promoting glycogenolysis and gluconeogenesis[43]. We quantified the glycogen content, blood glucose, and GCGR expression in the liver of animals with unprotected status and availability, including the following birds (*Gallus gallus*, *Anas platyrhynchos*, *Melopsittacus undulatus*, *Phylloscopus magnirostris*, *Muscicapa ferruginea*, *Parus monticolus*, *Passer domesticus*, *Carpodacus vinaceus*, *Serinus canaria*, *Lonchura striata* and *Taeniopygia guttata*), mammals (*Macaca mulatta*, *Mus musculus*, *Capra hircus*, and *Petaurus breviceps*), reptiles (*Crocodylus siamensis*, *Chrysemys picta picta Eublepharis macularius*, *Laudakia sacra*, *Phrynocephalus theobaldi*, *Pogona vitticeps* and *Orthriophis taeniurus*), amphibians (*Xenopus tropicalis* and *Pelophylax nigromaculatus*), and fish (*Danio rerio* and *Wallago attu*).

7. Figure 5 sets up a comparison of null, hsGCGR (human receptor), and scGCGR (canary receptor). However, hsGCGR data is missing from panels aa and ab, as well as Supplementary Fig. 21a,b. The reason for excluding these data should be provided.

R: We repeated the experiments and set up a comparison of null, hsGCGR (human receptor), and scGCGR (canary receptor) about oxygen consumption (VO_2), carbon dioxide generation (VCO_2), and energy consumption, and then we revised the graphs (**Fig. 5v** and **Supplementary Fig. 27a-c**).

8. The choice of organisms for in vivo tests is confusing. The analysis of the effects of avian GCGR downregulation (Fig. 3) is conducted in Gallus gallus and Lonchura striata, while single-cell RNAseq is done in Melopsittacus undulatus. The authors should explain the reasoning behind the selection of these organisms.

R: We performed hepatic GCGR knockdown experiments in *Gallus gallus* (chicken), *Lonchura striata* (white-rumped munia), and *Melopsittacus undulatus* (budgerigars) and examined downstream gene expression using the real-time qPCR assay (**Fig. 3k,o, 4f,** and **Supplementary Fig. 18b,e, 19e-g**). And we selected *Melopsittacus undulatus* (budgerigar) for our in vivo

experiments and single-cell RNA sequencing for the following reasons:

Firstly, the results in **Fig. 3** and **Supplementary Fig. 18** show that the changes in downstream pathways were similar after GCGR expression was knocked down in the liver of birds.

Secondly, our study focuses on evolution, so we examined hepatic GCGR expression in various bird species available for purchase. The results indicated that hepatic GCGR expression was highest in budgerigars (**Fig. 2b**).

Moreover, Budgerigars are not a protected species in China and are more readily obtainable in large quantities, which is advantageous for our experiments. They exhibit minimal individual variability and consistent quality, making them ideal for our study.

Finally, our experimental results also demonstrated that the knock-down effect in budgerigars was superior (Expression of GCGR decreased approximately from 25% to about 10%) (**Fig. 4d**) compared to that observed in *Gallus gallus* (chicken) (approximately from 9% to about 5%) (**Fig. 3k**) and *Lonchura striata* (white-rumped munia) (**Fig. 3o**) (approximately from 20% to about 13%) So we conducted single-cell RNA seq in budgerigars.

In the manuscript, the corresponding part, on lines 291-298, we explain the reasoning behind the selection of *Melopsittacus undulatus*, was revised as:

The results in **Fig. 3** and **Supplementary Fig. 18** show that the changes in downstream pathways were similar after *GCGR* gene expression was knocked down in the liver. Moreover, *Melopsittacus undulatus* (budgerigars) has high hepatic *GCGR* gene expression (**Fig. 2b**), as well as high constitutive activity (**Fig. 1a**). To investigate the regulatory mechanisms responsible for the high glucose levels in birds, endogenous *GCGR* gene expression in liver of adult male *Melopsittacus undulatus* (mu) was downregulated by RNA interference employing intravenous AAV-sh(muGCGR) injection.

There are several points in the introduction that seem poorly explained:

9. Line 65-66: “Constitutive activity has been documented in many GPCRs and other receptors, resulting in the production of a second messenger even in the absence of ligand.” - This is the definition of constitutive activity, so it is more accurate to say “which means” instead of “resulting”.

R: On lines 84-86, was revised as:

Constitutive activity has been documented in many GPCRs and other receptors, which means that a second messenger is produced without a ligand; this form of GPCR constitutive activity is associated with various potential physiological functions[46–48].

10. Line 119: the authors use “phylogenetic analysis” to refer to PAML analysis when it is not phylogenetic analysis, but a positive selection test.

R: Once more, please refer to answers to question 3 and/or 5

11. In many figures, the authors are truncating Y-axis and it is misleading. Specifically in: Fig. 3b,j,n,r,v; Fig. 4g, Fig. 5aa, and Fig. 6b,d,e.

R: In the current manuscript, we standardized the Y-axis scale across the graphs, or at least aligned the starting values of the Y-axis. However, in certain cases, such as the significant difference in blood glucose levels between birds and lizards, unifying the full range of the Y-axis was not feasible (**Fig. 3b,j,n,r,v, Fig. 4b,c, Fig. 5b,c,l,r,v, Fig. 6c,d, and Supplementary Fig. 20c, 24b, 25b, 29, 30, 31**). For mice, the Y-axis values of blood glucose data started from 5 (mmol/L), and the Y-axis values of body weight data ranged from 80 to 120 (%initial). For chicken breeding, the Y-axis values of blood glucose starting from 8 (mmol/L) and body weight data starting from 0 (Kg).

12. Figure 5c presents a timeline for the enzymatic activity of Gpa and Pepck as barplots, when in previous panels the same type of data was presented with lineplots. I would suggest being consistent and using lineplots throughout as barplots look more crowded and confusing.

R: In the current manuscript, we changed the graphs to line plots (**Supplementary Fig 20d,e**).

13. This paper uses “safflower” chickens and “safflower” chicken GCGRs for a number of results. It’s unclear what safflower chickens are, as they are not present in a list of chicken breeds.

R: In the current manuscript, we have added breed information.

Lines 478-481, was revised as:

In order to obtain chickens with greater differences in blood glucose and expectedly more eSNPs, a common Chinese farm chicken breed, Chinese yellow-feathered broiler (outbred), was selected for high or low blood glucose (**Supplementary Fig. 29a**).

In the Materials and Methods part of manuscript, on lines 1777-1778, was revised as:

The chicken breed was the Chinese yellow-feathered broiler, provided by Guangdong Wens Nanfang Poultry Breeding Co. Ltd. (Guangzhou, China).

14. Fig. 7a: repetition of “high expression” in the mammalian panel. I assume the left one needs to be corrected to “low expression”.

R: In the current manuscript (**Fig. 7a**), we added information and modified the representation in the figure to make it more straightforward：

As shown in Fig. R1 below, GCGR is highly endogenously expressed in the liver but lacks constitutive activity (green receptor). It can only activate downstream the metabolic pathway upon ligand stimulation (left side of the diagram). When constitutively active GCGR is exogenously overexpressed in placental mammals, downstream signaling is activated even without ligand stimulation (right side of the diagram).

FIGURE REDACTED

Fig R1. A schematic of the activation state of downstream signals GCGRs of different species in the presence and absence of ligand agonist stimulation.

In the current manuscript, we added an explanation for **Fig. 7a** in the figure legend, on lines 853-869, was revised as:

a, Model compares the classic ligand-induced activation of GPCRs versus the constitutive activity of receptors under the control of hepatic glucose output. Non-placental mammalian GCGRs have constitutive activity, promote glycogenolysis, gluconeogenesis, lipolysis through high expression without relying on GCG agonist, and play a role in raising blood glucose and NEFAs output in liver. High expression of constitutively active GCGRs also enhances energy metabolism. Upper left: In placental mammals, GCGR with no constitutive activity is highly expressed in the liver, activating the Gs signaling pathway and upregulating downstream the glycolipid metabolic pathway only upon ligand stimulation. Upper right: When constitutively active GCGR is heterologously overexpressed in the liver of mice, the downstream Gs signaling pathway is activated even without ligand stimulation, leading to the upregulation of the glycolipid metabolic pathway. Lower left: GCGR with constitutive activity is lowly expressed in the liver, activating the Gs signaling pathway and upregulating downstream the glycolipid metabolic pathway only upon ligand stimulation. Lower right: In birds or *Agamidae*, constitutively active GCGR is highly expressed in the liver, the downstream Gs signaling pathway is activated even without ligand stimulation, enhancing glycolipid metabolism.

Referee #2 (Remarks to the Author):

The authors conducted a very thorough, comprehensive study to understand mechanisms underpinning the high blood glucose concentrations found in birds compared to other vertebrates, especially mammals. They show in a large number of diverse experiments that the avian glucagon receptor is constitutively active and that high hepatic expression of avian glucagon receptors, even when expressed in non-avian species, result in high blood glucose, high lipid mobilization and high metabolic rate. Their study suggests that the constitutive activity of the glucagon receptor is a phylogenetically basal trait that has been lost in placental mammals. They also elucidated the molecular nature of constituent activity in the avian glucagon receptor.

R: Many thanks for your encouraging expertise comments. Throughout this point-by-point response, we typed in red to present our answers to the questions and comments of reviewers while we used gray highlights to present our revisions in the manuscript. Parts of the manuscript that only were moved to a different location are marked in a lighter gray. We also provide manuscript and figure files with highlights of changes for the convenience of reviewers/editors. New and altered figures were highlighted in yellow. Figures or parts that were moved (e.g., to Supplemental Figures) were highlighted in green. Figures that are included in the replies to referees are denoted with the letter R.

Main comments:

1. My biggest comment on this manuscript is a disconnect between the Introduction, the remarkable experiments that were conducted, and the Discussion. The Introduction focuses on high blood glucose in birds as compared to most other vertebrates and the mechanisms that may underlie this pattern. It sets up the main study question as a proximate issue – to find out the molecular mechanisms that lead to high blood glucose levels in birds. This is the question that the study was designed to address, and it did so in a very comprehensive way. However, in the Discussion (especially from L 559 onwards) the authors turn to discussing the functional relevance of high glucose levels, and associated traits like metabolic rates and body mass. While their hypothesis and the detailed pathways illustrated in Fig 7 are interesting, and data on metabolic rates and mass fit

with this hypothesis, in principle this study was not designed to address functional questions. It may well be that the constitutively active avian GCGR played a permissive role in the evolution of flight physiology.

R: Good point, we revised the Introduction and rewrote the Discussion.

For the Introduction part:

The former first paragraph (about the flight of birds) was moved into the Discussion part, on lines 593-598, was revised as:

As one of the most diverse extant vertebrate groups, birds have unique physiological adaptations and exaptations for flight, e.g. low body weight in relation to size combined with strong muscles and high metabolic rate[97]. As the main fuel for short-term flight of birds[11], glucose probably facilitates rapid carbohydrate energy supply during the takeoff phase[31,98–100], and the high blood glucose supported by constitutive activity GCGR **(Fig. 3** and **4)** provides a large energy pool for birds.

In the manuscript, on lines 26-31, was revised as:

As one of the most successful and diverse vertebrate lineages, birds possess numerous physiological characteristics distinct from other vertebrates[1,2], among which high blood glucose stands out as one of the most prominent and crucial characteristics. Glucose, a vital metabolic compound intimately tied to numerous physiological aspects of organisms, renders the study of blood glucose regulation mechanisms in birds instrumental in unraveling their origins and evolution.

In the manuscript, on lines 51-58, was revised as:

Previous studies have shown differences in the tissue expression profiles of the glucagon receptor family between mammals and birds[17–22]. E.g., in chickens, GCGR is expressed at high levels in various tissues, with the highest in liver and adipose tissue, followed by breast muscle, brain, proventriculus, and kidney[22]. In mammals, such as rats and mice, GCGR expression is highest in the liver, followed by the kidney, and lesser amounts are found in other tissues[17–21]. GCGR primarily regulates glucose metabolism processes in the liver, including glycogenolysis, gluconeogenesis, and glycolysis[23,24].

In the manuscript, on lines 61-68, was revised as:

In birds, studies regarding their high blood glucose primarily focused on the role of ligands involved in blood glucose regulation[27]. A previous study has shown that an antagonist of GCG does not affect blood glucose levels in birds[28], suggesting that the maintenance of high blood glucose in birds may not rely on ligand-mediated regulation. The rise in blood glucose levels is primarily regulated by hepatic glycogenolysis and gluconeogenesis[29,30]. In vertebrates, gluconeogenesis and glycogenolysis are conserved and occur mainly in the liver, producing approximately 85% of

endogenous glucose[31].

In the manuscript, on lines 77-82, was revised as:

The GCG-Gs axis also upregulated downstream genes involved in lipid metabolism, e.g., the peroxisome proliferator-activated receptor alpha (*Pparα*)[41]. Thus, the GCG–GCGR axis plays a central role in regulating blood glucose and glucose homeostasis[4,42–45], while the upstream molecular regulatory mechanisms appear to differ between birds and mammals.

In the manuscript, on lines 84-94, was revised as:

Constitutive activity has been documented in many GPCRs and other receptors, which means that a second messenger is produced without a ligand; this form of GPCR constitutive activity is associated with various potential physiological functions[46–48]. Constitutive activity can still be further activated by its ligand. Some constitutively active GPCRs, like the human adenosine A2B receptor (A2BR), human histamine H4 receptor (hH4R), and human H3receptor (hH3R), have been demonstrated that their cognate ligands can further activate the corresponding receptors[49–51]. In this study, we have discovered that the avian GCGR exhibits highly constitutive activity and maintains high expression levels in the liver. We postulate that the evolution of GCGR associated with glucose regulation contributed to the physiological characteristic of high blood glucose levels in birds.

For the Discussion, on lines 513-564 was revised as:

Several explanations have been proposed to elucidate why birds have relatively high blood glucose levels, the most important of which are mechanisms involving insulin and glucagon[5,80–82]. However, since these explanations were first proposed by Minick in 1986, other studies have found no correlation between insulin and avian blood glucose[83,84]. Due to the absence of insulin, pancreatic resection in mammals leads to hyperglycemia and diabetes[85]. In avian species, the excision of the pancreatic gland usually gives only rise to a transient impact on blood glucose, with glycemic levels returning to normal shortly thereafter[9,10,86,87]. In birds, such as chickens, GCGR expression was highest in liver and adipose tissue, followed by muscle, brain, proventriculus, and kidney[22]. Gluconeogenesis occurs mainly in the liver and kidney[31], with the liver producing about 70% of glucose in the fasted state, and the high expression of constitutively active GCGR in the liver supports the physiological characteristics of high blood glucose in birds. The high expression of avian GCGR may contribute to glycolysis processes in other tissues, forming a stable state of blood glucose regulation throughout the entire organism. In vertebrates, the conserved GCG–GCGR axis is central to regulating blood glucose and glucose homeostasis[43,44,88]. Exogenous GCG has previously been shown to markedly elevate circulating glucose concentrations in chickens, but it was unclear whether they are also controlled by endogenous GCG[89]. Additionally, circulating glucose concentrations were unaffected by the administration of a GCG antagonist in fed chicks[90]. Our results also showed that a GCGR antagonist, desHis^{1}Pro^{4}-glucagon amide, did not affect blood glucose (**Fig. 3a**)[24,43,91] suggesting that the GCG–GCGR axis may not play a major role in the high blood glucose phenotype of birds. Except for non-placental mammals, which have a weak constitutively active GCGR, the placental mammals lack a constitutively active GCGR, but maintain high hepatic *GCGR* gene expression **(Fig. 2a)**. In contrast, most non-mammalian vertebrates (fish, amphibians, and most reptiles) have low hepatic *GCGR* gene expression but strong

constitutive GCGR activity **(Fig. 2a)**. Only birds and *Agamidae* (possibly including *Lacertidae*, *Varanidae*, *Chamaeleonidae*, *Iguanidae*, *Phrynosomatidae*, and *Dactyloidae*) have a high constitutively active GCGR and also maintain high hepatic expression **(Fig. 2a** and **Supplementary Fig.15)**. The knockdown of *GCGR* gene expression or inhibition of GCGR constitutive activity both downregulated the carbohydrate metabolism pathway in birds (**Fig. 3**,**4**). Thus, both the constitutive activity and the hepatic expression levels of *GCGR* gene are crucial factors in maintaining high blood glucose levels in birds and *Agamidae*.

Here, we show that the avian glucagon receptor (GCGR) evolved differently from that of placental mammals and maintained constitutive activity, dependent on the interaction of the stalk, ECL1, and ICL2 (**Supplementary Fig. 5-8**). By screening the human SNP data from NCBI, we also have uncovered naturally occurring point mutations (hsGCGR^{H339R}) in the ICL3 of human GCGR with a weak constitutive activity (**Supplementary Fig. 24**). Mice with highly expressed hsGCGR^{H339R} expression in liver exhibit avian-like metabolism including high blood glucose and light weight (**Supplementary Fig. 24**). We presume that the loss of constitutive activity of GCGR in placental mammals may have led to avoidance of disadvantages caused by the excessive activation of GCGR signaling, such as the inability to cope with the massive energy expenditure of the body when food is scarce. The function of non-constitutive active GCGRs is strictly regulated by ligand binding, and the response is more sensitive (**Supplementary Fig. 9**). Therefore, precise signal transduction and regulation can be achieved, allowing eutherian species to adapt to various external environments. In contrast, birds utilize the energy support of constitutively active GCGR for flight and evolved other strategies (including migration) to cope with environmental changes[92].

on lines 593-604 was revised as:

As one of the most diverse extant vertebrate groups, birds have unique physiological adaptations and exaptations for flight, e.g. low body weight in relation to size combined with strong muscles and high metabolic rate[97]. As the main fuel for short-term flight of birds[11], glucose probably facilitates rapid carbohydrate energy supply during the takeoff phase[31,98–100], and the high blood glucose supported by constitutive activity GCGR **(Fig. 3** and **4)** provides a large energy pool for birds. First, high blood glucose levels supported by GCGR help to meet the intensive utilization of glucose needs in birds, especially bursts of flight[31,100]. Previous studies have found higher circulating glucose concentrations in *Neognathae* compared to the flightless ratites (*Palaeognathae*)[31]. The constitutive activity and hepatic expression levels of GCGR in *Palaeognathae* are relatively low compared to those in flight-capable birds (**Fig. 2b** and **Supplementary Fig. 1r**).

on lines 615-637 was revised as:

Finally, avian GCGR expression levels are associated with high basal metabolic rates related to the evolution of flight. Flight is the locomotion with the highest metabolic rate[103]. A previous study showed that flightless birds have a relatively low basal metabolic rate[104]. Our results are consistent with the high energy consumption rate required for sustained flapping flight[105], and an important physiological shift towards a higher basal metabolic rate that occurred in birds compared to non-

avian dinosaurs and reptiles as part of the adaptations and exaptations for flight (**Fig. 5v-aa**, **Supplementary Fig. 27**, and **28**)[106].

Thus, we suggest that the avian GCGR as a highly expressed “switchless gene” contributed to the adaptive flight physiology of birds to some extent (high blood glucose, fat utilization, and high basal metabolic rates) (**Fig. 7**). To adapt to new environments or challenges, organisms who evolved one such putative adaptation, such as the constitutive activity of GCGR, use additional "adjusting screws" or "setscrews" to further enhance or counteract the effects, such as enhanced or reduced *GCGR* gene expression levels and many other (e.g., downstream) mechanisms, for example, when birds occupied a different temporary or permanent environment or behavior, such as the ground (flight-less or poor) or even the water (swimming, diving).

In conclusion, constitutively active GCGR with high hepatic expression underlies sustained high blood glucose in birds and is the consequence of a “switchless gene” that contributed, among many other adaptations and exaptations, to the evolution of flight in birds and their ancestors.

2. But why do some reptile taxa like Agamidae have blood glucose levels and metabolic rates as high as in birds? These reptiles do not fly. The authors do not address this issue. Also, i.e. flying mammals, were not included. I suspect this is because of a lack of data for bats, but at least they should be included in the framework.

R: Changes in energy metabolism were just one of many prerequisites for the evolution of flight in birds. Likely, the initial increase in GCGR constitutive activity has not yet been an adaptation for flight (see *Agamidae*). By alteration of many additional factors (light bones, exaptation of feathers for flight, birds eventually could evolve the ability to fly. They could further modulate their energy metabolisms and not by reversing the mutations that lead to constitutive activity of GCGR, but by potentially numerous other means, depending on the ecological niche they occupy or their “lifestyle”, e.g., migration, etc. This is in line with the constant trial-and-error and random “tinkering” that evolution has to rely on, as it does not have the capability of planning and designing (PMID: 860134). The question on the aptations (adaptations and/or exaptations) (doi:10.1017/S0094837300004310) for flight in bats is interesting (just as it would be for insects). One would not expect that re-gaining of constitutive GCGR activity in bats (we have shown that it did not) alone could lead to the evolution of flight in these mammals. Additionally, directing our efforts on the interesting question on further adaptations and exaptations leading to flightless mammals would go beyond the scope of this already extensive study. Comparing flight in bats and

birds would probably unveil common aptations, but also many different ones. Previous studies have shown that birds often use lipids as flight fuel, while bats mostly use glucose (PMID: 20639431).

[redacted]

[redacted] The results show that the GCGR of bats does not have constitutive activity (as shown Fig. R1 below), and likely there other genes involved in supporting bat flight. Indeed, future experiments would be very important to answer these questions.

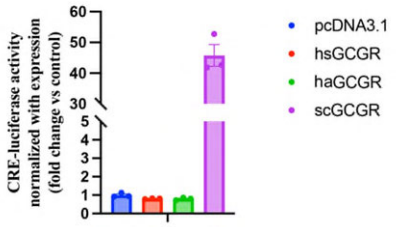

Fig. R1: GCGR of bat (*Hipposideros armiger*) has no constitutive activity. *Homo sapiens* (hs)GCGR, *Hipposideros armiger* (ha)GCGR, *Serinus canaria* (sc)GCGR.

3. I think the best the authors could argue is that the avian GCGR may have permitted/facilitated the evolution of flight - and I would urge the authors to tone down statements like 'adaptations to flight'.

R: We agree. In the current manuscript, we changed "adaptations to flight" to: permitted/facilitated the evolution of flight or we stressed that constitutive activity of GCGR might be one of many aptations to flight, or we used putative adaptation.

In the abstract part of the manuscript, the corresponding part, on lines 14-17, was revised to:

By identifying molecular changes after breeding selection in chickens and testing one natural human GCGR mutation (hsGCGR^{H339R}), our study demonstrated that constitutively active GCGR establishes the high blood glucose and may have contributed to the evolution of flight in birds.

In the Discussion part of the manuscript, the corresponding part, on lines 624-637, was revised to:

Thus, we suggest that the avian GCGR as a highly expressed "switchless gene" contributed to the adaptive flight physiology of birds to some extent (high blood glucose, fat utilization, and high basal metabolic rates) (**Fig. 7**). To adapt to new environments or challenges, organisms who evolved one such putative adaptation, such as the constitutive activity of GCGR, use additional "adjusting screws" or "setscrews" to further enhance or counteract the effects, such as enhanced or reduced *GCGR* gene expression levels and many other (e.g., downstream) mechanisms, for example, when

birds occupied a different temporary or permanent environment or behavior, such as the ground (flight-less or poor) or even the water (swimming, diving).

In conclusion, constitutively active GCGR with high hepatic expression underlies sustained high blood glucose in birds and is the consequence of a "switchless gene" that contributed, among many other adaptations and exaptations, to the evolution of flight in birds and their ancestors.

4. In fact, one wonders whether the question should be turned around and ask why placental mammals have lost this trait.

R:： The reviewer is absolutely right that positive selection is not the appropriate type of selection to look for. Hence, we deleted everything pertaining to positive selection and retained structure function-analysis based on previous findings that the activation of hsGCGR depended on the cooperation of multiple domains (PMID: 32193322, PMID: 23863937, PMID: 28514451) and selected residues in these domains that were conserved in all birds aligned but were different in placental mammals. Regarding the loss of constitutive activity in placental mammals, we have added explanations to this evolutionary hypothesis in the manuscript.

In the manuscript, the corresponding part, on lines 141-160, was revised to：

The constitutive activity was also found in the ancestral GCGR in Urochordata[55], indicating that the constitutive activity of GCGR had been lost in placental mammals (**Supplementary Fig. 4**). Furthermore, testing for constitutive activity of GCGRs after domain swapping showed that the constitutive activity of non-placental vertebrate GCGR depended on the cooperation of multiple domains (the stalk, extracellular cell loop ECL1 and intracellular cell loop ICL2, and possibly the C-terminus) (**Supplementary Fig. ~~5~~ 8)**. Consistently, previous studies have shown that these domains may function in the activation of hsGCGR (the ICL2 of hsGCGR interacts with Gs as determined in the crystal structure[56], and structural differences in the stalk, ECL1, and the C-terminus were detected in the activated and inactive hsGCGR states[57,58]). Hence, we modified several amino acid residues in these domains, based on their high conservation in birds while different in placental mammals. Perhaps, the putative adaptive evolution of GCGR (loss of constitutive activity) may have benefitted placental mammals, e.g., allowing them to retrieve stored energy, when food is scarce. Avian GCG concentrations in plasma are five-fold higher than those in mammals[59]. As expected, GCGR, devoid of constitutive activity, was more sensitive to GCG (**Fig. 1c** and **Supplementary Fig. 9**). When the body needs energy, GCGR can quickly and sensitively activate downstream pathways in response to GCG stimuli, facilitating placental mammals to adapt to different external environments

5. In Fig 1, the authors compiled data on a nice number of different bird species, some of which are migratory (and migrate in various ways like soaring or flapping and for various distances), some

are predators that rely on fast flight to catch prey (like peregrine falcons), some have a cheaper way for obtaining food (like granivores that often hop to gather food instead of flying) than others, some show amazing acrobatic courtship displays that look rather costly like manakins etc. For addressing functional questions, data from these different groups of birds could have been compared.

R: We tried to analyze the data of the birds in **Fig. 1** as suggested above (as shown in Fig. R1 below). However, we encountered many difficulties, such as comparing migration distance, because the migration route and migration strategy of different populations of the same species in different regions are different. The data analysis of these birds needs to be combined with their constitutive activity and GCGR expression data, Still, the species in **Fig. 1a** are used for the research of GCGR constitutive activity in cells (species for which genomic data are available), The species in **Fig. 2a** were used for the expression of hepatic GCGR and blood glucose test based on the live animals with unprotected status and readily available to obtain). It isn't easy to achieve consistency. Moreover, the choice of flight form of birds depends on the specific situation they face; it is difficult to classify, and different distances of flight use different fuel (birds use glucose as fuel for short-burst flight and fatty acid for long-distance flight)). Furthermore, there are limitations to our data, such as the fact that when birds are grouped according to characteristics such as flight ability or species, data from only one or two birds per group do not allow convincing conclusions to be drawn (as shown in Fig. R1 below).

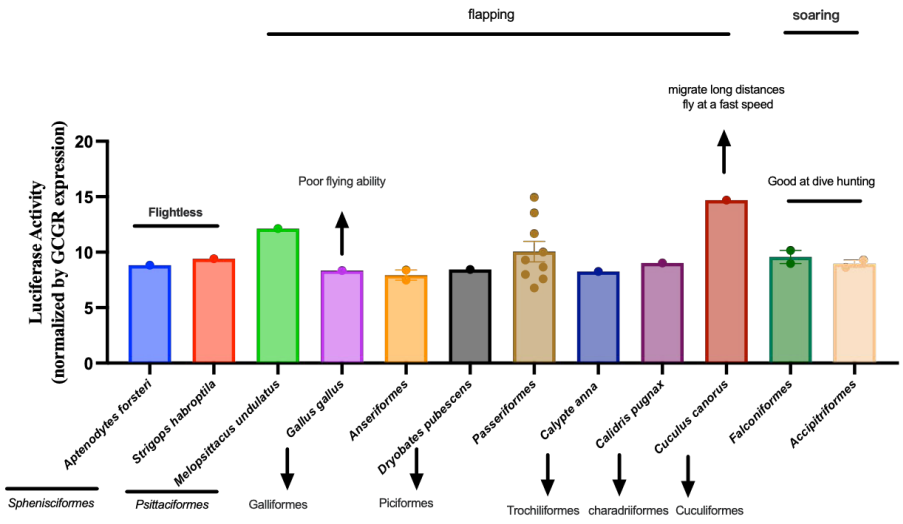

Fig. R1: Analysis of taxonomic and constitutive activity intensities of birds. Cuculus canorus is a migratory bird that and it migrates long distances (PMID: 26549318).

6. Also, what about other modes of locomotion like swimming? The authors did include fishes in their study, but did not interpret their data in terms of their locomotory mode. What about the hopping of amphibians? This is the kind of study design that would allow the authors to comment on functional aspects of glucagon receptor functioning.

R: The species in **Fig. 1a** are used for the research of GCGR constitutive activity in cells (species for which genomic data are available), and the species in **Fig. 2a** were used for the expression of hepatic GCGR and blood glucose test based on the live animals with unprotected status and readily available to obtain). It is, therefore, difficult to achieve consistency. Furthermore, even assuming that all animals use glucose as fuel for these different modes of locomotion. It's also difficult to analyze because the energy consumption of locomotion also needs to consider the animal's weight difference, the resistance of motion at different speeds to air or water, and the efficiency of the muscles.

7. I suggest that the authors rewrite their Discussion and tailor it to their main aim(s) as outlined in the Introduction and fitting the experiments they conducted.

R: We rewrote the Discussion. Please see reply to question 1.

8. I am surprised that bats are not considered at all in this paper – after all they are flying mammals and because of their primary mode of locomotion should be closer in physiological adaptations to birds than mammals. The authors may want to check for data on bats and include those if available. If not available, the issue of bats should at least be touched on in the Discussion. They could play a really interesting role in hypothesis testing.

R: Please see the reply to question 2.

9. The manuscript is very impressive in its completeness. However, at times it is also overwhelming because it contains so much data. Some of it may not be necessary and even detract

the reader from the more crucial aspects of the work. The authors may want to go over their Results and Figure section of the main manuscript and focus it on addressing the main question.

R: In the current manuscript, we moved the fatty acid metabolism-related data to the supplementary figures and simplified the description of this part of the results.

The changes are as follows:

We moved fatty acid metabolism-related data from former **Fig. 3ac-ag** to **Supplementary Fig. 18a-i**, data from former **Fig. 4a,c,l,m,o-q** to **Supplementary Fig. 19a-g**, data from former **Fig. 5a-d** to **Supplementary Fig. 20**, and data from former **Fig. 5g, h, j** to **Supplementary Fig. 21f,g,k**. and **deleted Fig. 4k** (because the data information of **Fig. 4k** is similar to **Fig. 4a**).

In the manuscript, on lines 274-280, was revised as:

The GCG-Gs axis also upregulated genes involved in lipid metabolism, and we detected the lipid metabolism-related genes of these species (**Supplementary Fig. 18**). Knockdown of constitutively active GCGR in the liver of birds and *Pogona vitticeps* downregulated the lipid metabolism pathway (**Supplementary Fig. 18a-i**), while overexpression of constitutively active GCGR in the liver of *Eublepharis macularius* and *Danio rerio* upregulated the lipid metabolism pathway (**Supplementary Fig. 18j-n**).

10. As a non-specialist reader, I was quite often confused by the large number of abbreviations used and had to go back and forth to find what these referred to. Sometimes I found definitions in the text of the manuscript and other times, I had to search the legends of the Figures or the Supplementary Materials. That was a bit of a hassle. I suggest to limit abbreviations to the most frequently used terms and write out the rest.

R: We have left out some abbreviations accordingly. However, for the reader's convenience, we also provide all abbreviations in the **Supplementary Table 13**.

11. The Figure legends often are insufficient in explaining what was done to obtain the data. Also, I could not find legends for any of the Tables.

R: In our resubmitted manuscript, Figure legends have been reworked to explain how the data was obtained in as much detail as possible, and legends for the tables have been added in the first sheet

of Supplementary Tables.

12. I was puzzled by the statistics. Some statistics seem to test the data in multiple ways, a technique that is not considered correct in other fields. For example, when there were 3 groups, then one group was tested against the two other groups in two different tests, which is repeated testing of the same data set that requires an adjustment in the p-value. I know that for data sets with n=3 complex statistics are not an option. However, for some experiments you had larger sample sizes and more than two groups. For these experiments I suggest you use analysis of variance (ANOVA) or general linear model (GLM) statistics where you can compare all three groups simultaneously in one model instead of comparing two groups at a time.

R: As suggested by you, we used analysis of variance (ANOVA) for data analysis for more than two groups and updated and legends accordingly. Overall, the significant differences calculated using the t-test and ANOVA are statistically significant (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

13. Again regarding statistics: it was often unclear whether animals were measured repeatedly, which could have been a valuable method because it reduces individual variation in the data. However, repeated-measures data need to be analyzed with statistics that account for it, for example, paired t-test, repeated measures ANOVA, or GLM with animal ID included as a variable.

R: Please see reply to question 12 above.

14. Not sure the experiments/section described in L 473-500 are needed?

R: This experimental result (hsGCGR^{H339R}) further proves that the high expression of constitutively active human GCGR leads to the phenotype of high blood glucose and upregulated the glucose metabolism pathway. In the resubmitted version, we moved figures about the section to **Supplementary Fig. 24,** and combined the description of the result with the mouse experiment (**Fig. 5**) to make the main line of the manuscript clearer.

The part was moved to lines 409-429:

SNPs can help explain susceptibility differences in various diseases across a population. Among these mutations, we selected and screened 68 missense mutations in the protein-coding region of GCGR, which could impact the constitutive activity of GCGR (see accompanying paper). As established by experiments in HEK293 cells, the hsGCGR^{H339R} exhibited weak constitutive activity (**Supplementary Fig. 24a**), similar to pbGCGR (**Supplementary Fig. 24a** and accompanying paper). Based on the hsGCGR^{H339R} with weak Gs constitutive activity, we synthesized an AAV for delivery in mouse liver (**Supplementary Fig. 24**). Blood glucose levels increased in the hsGCGR$^{H339R}{}_{H}$ (H: high dose) AAV injection group, and also resulted in weight loss compared to the control groups (hsGCGR and null) (**Supplementary Fig. 24b**,**c**). *Pck1* and *G6pc1* expression were significantly increased (**Supplementary Fig. 24d**). Moreover, GPa enzyme activity increased, and hepatic glycogen content decreased in the hsGCGR$^{H339R}{}_{H}$ AAV injection group (**Supplementary Fig. 24e**,**f**). Lipolysis transcript genes *Lpl* increased, but no changes were observed in lipogenesis genes (*Acc*, Acyl-CoA thioesterase 7- *Acot7*, *Fasn*, *Pparγ*, CCAAT/enhancer-binding protein-alpha- *Cebpα*) after hsGCGR$^{H339R}{}_{H}$ AAV injection (**Supplementary Fig. 24g**,**h**) and also no changes in serum and hepatic TGs, NEFAs (**Supplementary Fig. 24i**,**j**). Mice injected with hsGCGR$^{H339R}{}_{H}$ exhibited a phenotype (blood glucose levels increased) similar to pbGCGR four days after injection (**Supplementary Fig. 24k**). However, scGCGR-injected mice showed a higher phenotype significance than both of them (**Fig. 5b**,**d**,**l**,**r**).

15. As far as I know, glucagon receptors are not only expressed in the avian liver but also in adipocytes, the brain and perhaps other tissues. Can the authors touch on that issue, at least in the discussion, to outline how these fit with the maintenance of high blood glucose envisioned in the Discussion?

R: You are correct. chicken GCGR expression was highest in liver and adipose tissue, followed by muscle, kidney, and brain (as shown in Fig. R1a (data reference from PMID: 18299131) and Fig. R1b (our result below). In birds, gluconeogenesis occurs mainly in the liver and kidney, and the high expression of constitutive GCGR in both tissues supports the physiological characteristics of high blood glucose in birds. Blood glucose is transported to muscles, kidneys, brain, and other tissues for utilization, and the high expression of GCGR may contribute to glycolysis processes in these tissues, forming a stable state of blood glucose regulation throughout the bird's body.

On lines 520-527, was revised as:

In birds, such as chickens, GCGR expression was highest in liver and adipose tissue, followed by muscle, brain, proventriculus, and kidney[22]. Gluconeogenesis occurs mainly in the liver and kidney[31], with the liver producing about 70% of glucose in the fasted state, and the high expression

of constitutively active GCGR in the liver supports the physiological characteristics of high blood glucose in birds. The high expression of avian GCGR may contribute to glycolysis processes in other tissues, forming a stable state of blood glucose regulation throughout the entire organism.

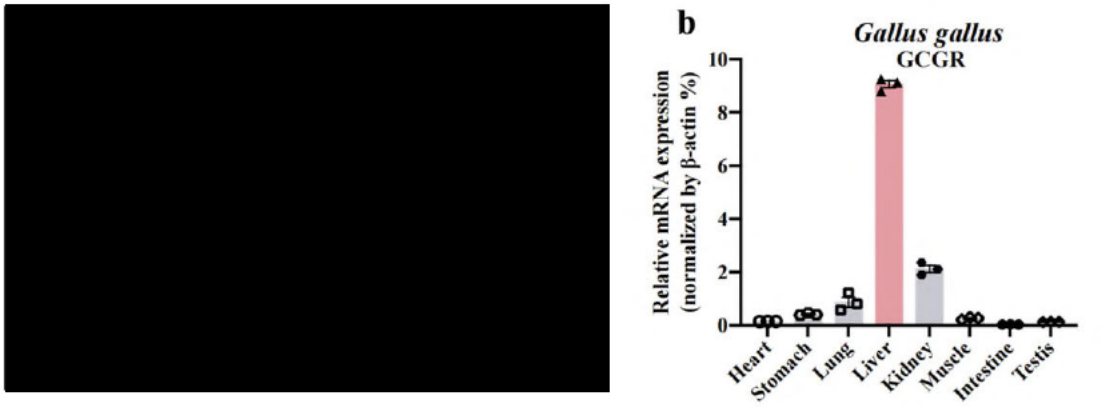

Fig. R1: GCGR expression profile of individual chicken tissues [redacted] (b) Figure from **Supplementary Fig. 12e** in our manuscript,

16. L 94-95: "… glycogen content in avian liver remained relatively stable…" This statement does not match Suppl Fig 2b, where you indicate a significant difference at $p < 0.01$ in glycogen content between fed and fasted states in both chicken and budgerigar. Please correct your statement.

R: On lines 115-117, was revised as:

As reported previously[53,54], mice showed a more drastic glycogen depletion in response to fasting compared to the glycogen content in the avian liver (**Supplementary Fig. 2b**).

17. L 101: "levels of THE tuatara reptiles…": since you only examined one species from that family, I suggest to reword the 'THE tuatara" into "A tuatara"

R: On lines 200-202, was revised as:

In a tuatara reptile (*Pogona vitticeps*), blood glucose and hepatic GCGR expression levels decreased somewhat with age, but remained elevated (**Supplementary Fig. 11b**,**d**).

18. L 102: "decreased with age" – I could not find the stats supporting this statement. Please add.

R: On lines 200-202, was revised as:

In a tuatara reptile (*Pogona vitticeps*), blood glucose and hepatic GCGR expression levels decreased somewhat with age, but remained elevated (**Supplementary Fig. 11b**,**d**).

19. L 109 – 112. That sentence is a bit hard to read, perhaps reword it.

R: On lines 130-135, was revised as:

Ex vivo expression of the different flag-tagged non-placental vertebrate GCGR constructs exhibited a broad range of GCGR measured as protein. Therefore, in **Fig.1a**, we normalized GCGR expression to the same levels and detected significantly stronger constitutive activity of Gs signaling in non-mammalian vertebrates and weaker constitutive activity in non-placental mammals, but no constitutive activity in placental mammals.

20. L 115: what is an "activity-high slope"? See also one sentence below. Also, I could not find stats to back up your statement.

R: The "activity-high slope" means the CRE-luciferase activity induced by GCGR rise as a function of receptor expression was significantly steeper. We modified the sentence to make it easier to understand and added an explanation of the slope in the legend of such graphs.

On lines 137-141, was revised as:

Compared with placental mammals, non-mammalian vertebrates showed the strongest constitutive activity (high slope factor, CRE-luciferase activity induced by the rise of GCGR as a function of receptor expression was significantly steeper), In contrast, non-placental mammals only showed weak constitutive activity (low slope factor) (**Fig. 1b**).

On lines 662-664, was revised as:

b, Constitutive activities of GCGRs in different species (green: placenta mammals, blue: non-placenta mammals, and red: birds). A higher value of slope indicates a higher level of Gs constitutive activity.

On lines 926-927, was revised as:

pmGCGR/GLP1R-like and ciGCGR/GLP1R-like have constitutive activity. A higher value of slope indicates a higher level of Gs constitutive activity.

21. L 127 ff. The work explained here is interesting. Of course, chicken have undergone intensive artificial selection for egg or meat production. If the authors really wanted to make statements about the evolution of the glucagon receptor in birds, they should include wild species into their analyses. I realize that this may technically not be possible. However, this limits the generality of the findings presented here.

R: It is important to note that upon breeding, the chicken GCGR gene undergoes additional

mutations, which were used as the basis in the experiments mentioned. Since the genome of the wild form (red jungle fowl) is available (GRCg6a/galGal6), we compared the GCGR gene to the chicken genome used (GRCg7b/galGal1, a kind of domestic chicken) and found only three mismatches at the nucleotide level, all of them synonymous substitutions. We have added relevant explanations in the figure legend of **Supplementary Fig. 6a** of the resubmitted manuscript.

In the Figure legend, on lines 939-947, was revised as:

a, Functional tests of those ggGCGR mutants well expressed in HEK293 cells. Expressed at a similar level, the ICL2 mutant showed reduced constitutive activity compared with wild-type ggGCGR. The CDS sequences of the chicken *GCGR* gene that we used were derived from the GRCg7b/galGal1 genome assembly. Compared to the sequence of the wild form (red jungle fowl) (available as GRCg6a/galGal6), we only found three mismatches at the nucleotide level, all synonymous substitutions.

22. L 202: stats on protein expression levels?

R: We have done stats on protein expression levels, and the statistical results were also added to **Supplementary Fig. 12n**.

23. L 213: the legend of Fig 2 states that you used unpaired student t-tests. Which compare two groups. It is unclear to me which statistics you used here. Comparing certain groups multiple times against other groups would clearly be a repeated testing of the same data set and would require correction of the p-value – or the use of different statistics.

R: As suggested by you, we used analysis of variance (ANOVA) for data analysis for more than two groups and updated and legends accordingly. Overall, the significant differences calculated using the t-test and ANOVA are statistically significant (****$p < 0.0001$; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

24. L 216: could you please explain what kind of inhibitor this YMxxx is?

R: YM-254890 is a selective Gq/11 protein inhibitor isolated from *Chromobacterium* sp. (PMID: 30055466)

On lines 220-223, was revised as:

Moreover, the stimulation of *Pck1* and *G6pc1* transcription could largely be inhibited by protein kinase A inhibitor H89 and the allosteric inhibitor adomeglivant (Ad), which inhibit constitutively active GCGR, but not by Gq/11 inhibitor YM254890[62] (**Supplementary Fig. 14a-d**).

25. L 227: this section hinges on understanding what an 'AAV delivery system' is. Please explain – not sure I found any details on this.

R: On lines 231-234, was revised as:

To further investigate the role of GCGR in regulating blood glucose and glycogen content after decreased expression, we interfered with the hepatic expression of GCGR using the recombinant adeno-associated virus (AAV) delivery system in *Aves* and *Agamidae* (**Fig. 3**)

26. L 230: it should be "FIG 3 E&F" since Fig 3e does not show concentrations. Also, please explain or redo the stats – these must be repeated measures data? I only found reference to unpaired t-tests.

R: As you suggested, we redo the stats (ANOVA) and update the graph accordingly.

27. L 232: please delete "and 3h".

In general, Fig 3 is loaded with graphs and I wonder whether the numbers of panels in that Fig could be reduced.

R: As you suggested, we moved **Fig 3ac-ag** (related to lipid metabolism) to **Supplementary Fig. 18a-i** and increased the font size.

On lines 237-238, we deleted "and 3h", was revised as:

Notably, one month after injection, hepatic *GCGR* gene expression and its corresponding blood glucose had recovered to normal levels (**Fig. 3f**, **g**).

28. L 233: not sure what a Gpa enzyme is and did not find an explanation easily.

R: We mentioned the full name of GPa in the Introduction in the former manuscript, to make this sentence easier to understand, we added full name of GPa.

On lines 238-240, was revised as:

The decrease in receptor expression led to an increase in glycogen content and a decrease of glycogen phosphorylase a (GPa) enzyme activity (**Fig. 3h**).

29. L 276: on Fig 3 there is no panel labelled ‘ab’

R: We added it to our resubmitted manuscript.

30. L 315: please explain PPAR

R: On lines 309-314, was revised as:

sNuc-RNAseq results indicated that the downregulation of *GCGR* gene expression in the liver leads to a decreased expression of fatty acid metabolism, carbon metabolism (which comprises carbohydrate metabolism, energy metabolism, and amino acid metabolism) and peroxisome proliferator-activated receptor (PPAR, a group of transcription factors) pathway signaling (**Fig. 4j**).

31. Likewise, Fig 4 is loaded with graphs and I wonder whether the numbers of panels in that Fig could be reduced. For example the axis labeling on many graphs is already so small that it is hard to read (e.g. Fig 4L).

R: As you suggested, we moved **Fig. 4a,c,k,o-p,** and the figure on the right of **Fig.4l** to **Supplementary Fig. 19** and **deleted k** (because the data information of **Fig. 4k** is similar to **Fig. 4a**). We also increased the font size. We also moved fatty acid metabolism-related data from **Fig. 3** and **5** to Supplementary Figure. Please see reply to question 9 above.

32. L 336: glucose intolerance and insulin resistance are not explained.

R: We added the explanation, on lines 345-349, was revised as:

Furthermore, with ggGCGR overexpression, the mice developed glucose intolerance and insulin resistance on day 4 (**Supplementary Fig. 20g**). The rapid increase in hepatic glucose production in mice may disrupt normal glucose homeostasis and induce glucose intolerance and insulin resistance[69,70].

33. L 356: Figs 5 h & i look like repeated measures data, and if the case, should be analyzed in that way

R: As you suggested, we redo the stats (ANOVA) and updated the graph accordingly.

34. L 378: what are db/db mice, and their phenotypes?

R: db/db mice are an animal model for diabetes; we included this information in the "Materials and Methods section":

On lines 1448-1451, was revised as:

C57BL/6J (wide type and DIO) and *db*/*db* (a diabetes model with a mutation in the leptin receptor gene *Lepr*) were purchased from GemPharmatech Co., Ltd., China. Mice were housed under conventional conditions in filter-top protected cages and cared for in accordance with the Experimental Animal Laboratory Center of West China Hospital.

35. L 419: Fig 5aa only shows data for the scGCGR and null groups, please correct your statement here (hsGCGR is not shown and I am not sure whether data exist for that group). Same for statement in L 422!

R: We repeated the experiments and set up a comparison of null, hsGCGR (human receptor), and scGCGR (canary receptor) about oxygen consumption (VO_2), carbon dioxide generation (VCO_2), and (ab) Energy consumption, and then we revised the graphs (**Fig. 5v** and **Supplementary Fig. 27a-c**).

36. L 450: In Suppl Fig 23 the two groups are not labeled – one has to go back to Fig 6 and assume the groups are coded in the same way. In this line Suppl Fig 23 H should be referred to, not 'L"

R: We added and corrected labeling in our resubmitted manuscript. For the clarity of the figures, we divided the **Supplementary Fig. 22** into three Supplementary figures **(Supplementary Fig. 29-31**)

37. L 459ff: if the data in this section all refer to F3 individuals, please indicate it here again. It is quite confusing because in the F2 generation there was only 1 C/C male, while in the F3 generation, there were apparently n=3 C/C individuals. That makes quite a difference. Please also clarify on Fig 6 and Suppl Fig 23.

R: We added the information about generation in **Fig. 6** and **Supplementary Fig. 29-31**.

38. L 522: Fig. 3a shows the schematic of the three types of injection, but data on the GCG antagonist are not presented in this Fig!

R: We optimized the graph's presentation to make it look clearer in the resubmitted version (Fig. 3b-d). As shown in Fig. R1 below, the data for the GCG antagonist (Des) are represented using regular triangles. We made the figure easier to understand by using different background colors to represent the different groups (Fig. R1b below).

FIGURE REDACTED

Fig. R1: a-d, Schematic of drug treatment via *i.v.* injection in chicks (a). The effect of a GCGR antagonist (desHis^{1}Pro^{4}-glucagon amide) and allosteric inhibitor (adomeglivant- Ad) on blood glucose levels and glycogen content (b), hepatic GCGR and *G6pc2* mRNA expression (c), and GPa enzyme activity (d) in chicks after 4 hours treatment.

39. Fig 7 is an interesting depiction of the main conclusions by the authors that the constitutively active GCGR plays a role in adaptations to flight. While that is a reasonable hypothesis, in L 541 the authors also admit that Agamidae have high blood glucose and high basal metabolic rates. Agamidae don't fly… (as well as the other families mentioned in L 553) and the authors do not explain how other taxa of 'lower vertebrates' that also have constitutively active GCGR (e.g., Fig 1a) fit in this picture.

R: This is an important point. In **Fig. 7a** (as shown in Fig. R1 below), we have categorized four scenarios based on the presence or absence of constitutive activity and expression levels of GCGR, summarizing each as follows, 'lower vertebrates' (Fish, Amphibians, and most reptiles) fit in the "Lower left" category:

Upper left：In placental mammals, GCGR with no constitutive activity is highly expressed in the liver, activating the Gs signaling pathway and upregulating downstream the glycolipid metabolic pathway only upon ligand stimulation.

Upper right: When constitutively active GCGR is heterologously overexpressed in the liver of mice, the downstream Gs signaling pathway is activated even without ligand stimulation, leading to the

upregulation of the glycolipid metabolic pathway.

Lower left: In 'lower vertebrates' (Fish, Amphibians, and most reptiles), GCGR with constitutive activity is lowly expressed in the liver, activating the Gs signaling pathway and upregulating downstream the glycolipid metabolic pathway only upon ligand stimulation.

Lower right: In birds or Agamidae, constitutively active GCGR is highly expressed in the liver, the downstream Gs signaling pathway is activated even without ligand stimulation, enhancing glycolipid metabolism. and **Fig. 7b** primarily depicts the birds; the high expression of constitutively active GCGR in the liver promotes downstream the glycolipid metabolic pathway and respiratory metabolism. This one of the potential factors that facilitated the flight capabilities of birds. However, this figure has limitations as it does not address a group of reptiles exhibiting high hepatic constitutively active GCGR expression. Glucose homeostasis in reptiles remains unexplored mainly (PMID: 21871969). Glucose seemingly plays a secondary role in metabolism (PMID: 11352549). Instead, more research focuses on glucose's role in response to external stimuli (stress, high temperatures, etc.) (PMID: 12765656, PMID: 11352549). Although these reptiles exhibit similar phenotypic characteristics in blood glucose levels to birds, their high blood glucose may support distinct physiological functions (even though their metabolic rates are higher than those of other reptiles, they are still significantly lower compared to birds (**Supplementary Fig. 28**). Furthermore, bird flight necessitates the support of numerous other systems, such as the respiratory and cardiovascular systems. While the primary focus of our manuscript revolves around the mechanisms of high blood glucose in birds, it is indeed essential for us to include a discussion on the exceptions of these reptiles (in fact, our unpublished work explores that constitutively active GCGR drives the physiological regulation of body temperature in lizards). In our resubmitted version, we have included a discussion on these reptiles.

FIGURE REDACTED

Fig R1. A schematic of the activation state of downstream signals GCGRs of different species in the presence and absence of ligand agonist stimulation.

In the current manuscript, we added explanation for **Fig. 7** in the figure legend, on line 853-879, was revised as:

a, Model compares the classic ligand-induced activation of GPCRs versus the constitutive activity of receptors under the control of hepatic glucose output. Non-placental mammalian GCGRs have constitutive activity, promote glycogenolysis, gluconeogenesis, lipolysis through high expression without relying on GCG agonist, and play a role in raising blood glucose and NEFAs output in liver. High expression of constitutively active GCGRs also enhances energy metabolism. Upper left: In placental mammals, GCGR with no constitutive activity is highly expressed in the liver, activating the Gs signaling pathway and upregulating downstream the glycolipid metabolic pathway only upon ligand stimulation. Upper right: When constitutively active GCGR is heterologously overexpressed in the liver of mice, the downstream Gs signaling pathway is activated even without ligand stimulation, leading to the upregulation of the glycolipid metabolic pathway. Lower left: GCGR with constitutive activity is lowly expressed in the liver, activating the Gs signaling pathway and upregulating downstream the glycolipid metabolic pathway only upon ligand stimulation. Lower right: In birds or *Agamidae*, constitutively active GCGR is highly expressed in the liver, the downstream Gs signaling pathway is activated even without ligand stimulation, enhancing glycolipid metabolism.
b, Schematic of hypothesis of the putative role of GCGR for flight adaptation in birds. The high expression of constitutively active GCGR in the liver promotes the downstream glycolipid metabolic pathway and respiratory metabolism. This is one of the potential factors that facilitated the flight capabilities of birds. Green GCGR symbol: not constitutively active GCGR; red GCGR: Constitutively active GCGR. Blue shape: glycogen. Green shape: G6PC protein. Polygonal line: transient activity (grey) and constitutive activity (red). Black dots: GCG. Black dashed arrow: transient nutrition and transient activation. Black and red solid arrow: continuous nutrition and constitutive activity. Black triangle: cAMP accumulated by transient activation. Red triangle: cAMP accumulated by constitutive activity.

40. L 547ff: "… birds eat non-stop…". This is an oversimplification. Sure, this may be so in chicken, maybe also in granivorous bird species, frugi/nectarivores and many insectivores. But there are also birds of prey that eat intermittently… Also, small mammals with high metabolic rate may likewise eat 'non-stop', simply because like birds, they cannot carry much energy reserves because of their small size. I am not sure this statement is needed or helpful here. Also, the Figs you refer to (Fig 5h and Suppl Fig 17o) are for mice…

R: Good point. Like all small birds, e.g., *Passerine*, many birds are very susceptible to starvation because their metabolism is extremely quick. They also keep slim when food is abundant (PMID: 30241777). Here, we can only propose that many birds appear to eat non-stop due to the permanently activated and switchless GCGR. In contrast, other animals, e.g., many small mammals, with high

metabolic rates, may eat 'non-stop' and rely on other high metabolic mechanisms but not GCGR. We also deleted this statement about "eat non-stop".

Lines 586-591, was revised as:

Because the permanent and "switchless GCGR" requires external energy, we propose that this is provided by increased food intake in our chow-food diet mouse model ~~and it provides an explanation why birds usually eat non-stop~~ (**Supplementary Fig. 21 k**,**o**). Meanwhile, non-placental mammals with weak constitutively active GCGR and placental mammals lacking constitutive activity of GCGR maintain relatively high hepatic GCGR expression levels (**Fig. 1a** and **2a**,**b**).

41. L 604: This study has many strengths. But there are also several more limitations that I commented on above that should be touched on here.

R: Thank you for your kind suggestion. We added the limitation to our resubmitted manuscript and have already solved the other limitations you commented on above. Please see the replies to questions 2, 6, 8, 15, and 21.

R: Lines 644-650, was revised as:

Thirdly, under the protective regulations by the National Forestry and Grassland Administration of China (https://www.forestry.gov.cn/c/www/gkgjlyjgb/509743.jhtml), the tissue samples from different species we could obtain were limited, which imposed certain constraints on our data. Finally, due to the limited number of bird species for which both GCGR sequences and tissue samples are available, the limited data allow us only to suggest a contribution of constitutively active GCGR to the flying ability of birds.

42. L 611: I suggest to tone down that statement, especially the parts relating to "that independently

R: Lines 634-637, was revised as:

In conclusion, constitutively active GCGR with high hepatic expression underlies sustained high blood glucose in birds and is the consequence of a "switchless gene" that contributed, among many other adaptations and exaptations, to the evolution of flight in birds and their ancestors.

Referee #3 (Remarks to the Author):

In this manuscript, the authors report on the well-known phenomenon that glucose production and utilization is increased in birds relative to other vertebrates. Consistent with the higher glucose demand and production, which is considered a physiological adaptation to flying, the authors show that the avian glucagon receptor (GCGR) shows higher constitutive activity relative to GCGR in other vertebrates (including humans), and also in comparison to the avian receptors for GLP-1, GLP-2, and GIPR. Using viral-mediated knockdown of GCGR, the authors convincingly demonstrate that the higher constitutive activity of GCGR, along with increased hepatic expression of GCGR, accounts for the higher levels of blood glucose in birds. Consolidating these data, the authors performed snRNAseq studies across multiple species, showing that hepatic expression of GCGR is increased in birds relative to other vertebrates and that expression of avian GCGR in mice results in higher levels of blood glucose and decreased body weight.

The manuscript is excellently written and easy to follow. It deals with an interesting and important question, the role of the avian GCGR in regulating glucose homeostasis in birds. The proposed mechanism of enhanced GCGR action is elegantly demonstrated using state-of-the art technologies, and the proposed studies are sufficiently clear and reliable to support the authors hypotheses. The proposed mechanism of higher constitutive GCGR activity makes sense, given that glucagon is a very strong promoter of de novo glucose production. The manuscript gained much from the demonstration that viral-mediated GCGR knock-down decreases blood glucose in birds, whereas the induced expression of avian GCGR in mice increases levels of blood glucose. Overall, this is a beautiful study that unmistakably demonstrates the importance of avian GCGR in glucose homeostasis in birds.

The data presented are elegant and logical, and it is really difficult to find some criticism here, which I highly appreciate. I have only a few comments and suggestions that the authors may want to consider to further strengthen the manuscript.

R: Many thanks for your encouraging expertise comments. Throughout this point-by-point response,

we typed in red to present our answers to the questions and comments of reviewers while we used gray highlights to present our revisions in the manuscript. Parts of the manuscript that only were moved to a different location are marked in a lighter gray. We also provide manuscript and figure files with highlights of changes for the convenience of reviewers/editors. New and altered figures were highlighted in yellow. Figures or parts that were moved (e.g., to Supplemental Figures) were highlighted in green. Figures that are included in the replies to referees are denoted with the letter R.

1. I wonder whether non-flying birds have lower GCGR activity and blood glucose levels relative to flying birds?

R: This is an important point. Previous studies have found higher circulating glucose concentrations in *Neognathae* than in the flightless ratites (*Palaeognathae*) (as shown in Fig. R1a below). (DOI:10.1016/B978-0-12-819770-7.00005-0).

And we added the experiments and set up a comparison of constitutive activity and hepatic expression of GCGR between *Neognathae* and *Palaeognathae* (as shown in Fig. R1b, c below), from our limited results, we observed that the constitutive activity of GCGR in *Palaeognathae* is relatively lower than that in flight-capable birds with lower expression level. This suggests that the constitutive activity and expression of GCGR may support flight. However, our data is limited, and a single gene cannot determine avian flight ability. For instance, amino acid changes in ATGL^{Ser321Gly} and ACOT7^{Ala197Val} have been associated with the loss of flight in birds (PMID: 31227702).

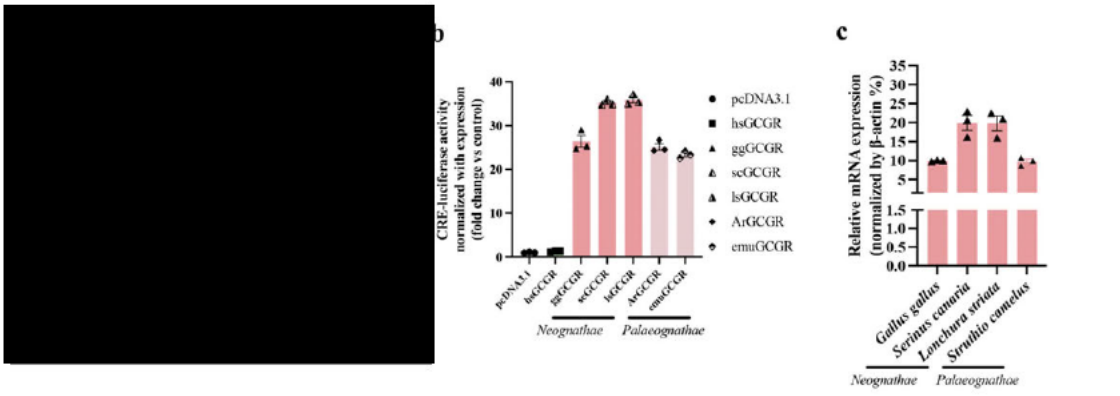

Fig. R1: Constitutive activity of GCGRs from flightless and flying birds.

[redacted] Comparison for constitutive activity of GCGR between *Neognathae* and *Palaeognathae*. (c) Hepatic expression of GCGRs in *Neognathae* and *Palaeognathae*.

In our resubmitted manuscript, on lines 600-604, was revised as:

Previous studies have found higher circulating glucose concentrations in *Neognathae* compared to the flightless ratites (*Palaeognathae*)[31]. The constitutive activity and hepatic expression levels of GCGR in *Palaeognathae* are relatively low compared to those in flight-capable birds (**Fig. 2b** and **Supplementary Fig. 1r**).

2. On page 1, the authors correctly state that birds have higher levels of blood glucose relative to other vertebrates, almost twice as high as mammals and 7-fold higher than amphibians. While correct, we have to keep in mind here that that birds also have a higher metabolic rate, and hence higher glucose demand, relative to other vertebrates, particularly mammals. Accordingly, the manuscript would gain from addressing the question whether birds produce glucose at a higher rate than what is expected based on their higher metabolic rate? When comparing birds with a vertebrate of equally high metabolic rate, do birds still have higher blood glucose, GCGR activity and hepatic GCGR expression? Just comparing blood glucose levels without taking into account species-related differences in metabolic rate is a bit misleading, and undermines the beauty of these studies.
In case birds do show higher glucose production than expected by metabolic rate (requires regression analysis with ANCOVA to demonstrate this), then this is a very important message.

R：This is an important point. Our results showed that constitutively active GCGR upregulated the downstream metabolic rate and the glucose metabolic pathway. As far as the analysis of our results is concerned, it cannot be concluded that "birds produce glucose at a higher rate than expected based on their higher metabolic rate." We analyzed species in our collected data with both glucose and metabolic rate data (as shown in Fig. R1a, b below), and no correlation was found between them.

			BMR (ml O2/g*hour)	Blod glucose (mmol/L)	Referance
Mammal	*Tolypeutes matacus*	Cingulata	0.181034483	4.8	doi:10.4236/fns.2016.74026
	Dasypus novemcinctus	Cingulata	0.243012048	3.4	doi:10.4236/fns.2016.74026
	Canis lupus	Carnivora	0.27	5.6	doi:10.4236/fns.2016.74026
	Petaurus breviceps	Diprotodontia	0.72	4.2	test in our study
	Desmodus rotundus	Chiroptera	1.180272109	4.9	doi:10.4236/fns.2016.74026
	Babyrousa babyrussa	Rodentia	1.440104167	6	doi:10.4236/fns.2016.74026
	Molossus molossus	Chiroptera	1.442307692	5.8	doi:10.4236/fns.2016.74026
	Glossophaga soricina	Chiroptera	2.602941176	3	doi:10.4236/fns.2016.74026
Birds	*Buteo buteo*	Accipitriformes	0.638636364	20.4	doi:10.1016/s1095-6433(98)10039-9
	Columba livia	Columbiformes	0.530192719	17.1	test in our study
	Phoeniculus purpureu	Coraciiformes	1.576395623	19.8	doi:10.1016/j.cbpa.2013.09.017
	Guira guira	Cuculiformes	1.327857143	17.8	doi:10.1016/j.cbpa.2013.09.017
	Falco sparverius	Falconiformes	0.981034483	18.1	doi:10.1016/j.zool.2014.04.002
	Corvus corax	Passeriformes	0.786783042	19.9	doi:10.1016/j.cbpa.2013.09.017
	Zosterops virens	Passeriformes	2.347457627	18.5	doi:10.1016/j.cbpa.2013.09.017
	Fulmarus glacialis	Procellariiformes	0.956067588	9.2	doi:10.1016/j.cbpa.2013.09.017
	Nestor notabilis	Psittaciformes	0.91277333	16.4	doi:10.1016/j.cbpa.2013.09.017
	Bubo virginianus	Strigiformes	0.7467	20	doi:10.1007/s11515-012-1206-2
	Calypte anna	Trochiliformes	2.666666667	16.7	doi:10.1016/s1095-6433(98)10039-9

Fig. R1: Correlation analysis of blood glucose and metabolic rate in different species. [redacted] **(c) Corresponding metabolic rate data, blood glucose, references data for different species.**

3. On another note, the authors may also want to mention that utilization of glucose is faster relative to utilization of fat, which further aligns with the physiological adaptation to flying. This said, fat is a very suboptimal energy substrate for initiation of flying, while fat is the preferred substance for migratory birds that have to fly over a very long time.

R: This is a good point; we mentioned it in the discussion part of our resubmitted manuscript.

On lines 598-604, was revised as:

First, high blood glucose levels supported by GCGR help to meet the intensive utilization of glucose needs in birds, especially bursts of flight[31,100]. Previous studies have found higher circulating glucose concentrations in *Neognathae* compared to the flightless ratites (*Palaeognathae*)[31]. The

constitutive activity and hepatic expression levels of GCGR in *Palaeognathae* are relatively low compared to those in flight-capable birds (**Fig. 2b** and **Supplementary Fig. 1r**).

4. I wonder whether higher constitutive GCGR action got lost in non-flying birds (like Ostrich)? Nonetheless, non-flying birds are very fast runner, which also requires high glucose utilization.

R: Please see the reply to question 1.

5. On another point, I wonder where in evolution the higher constitutive GCGR activity occurred? Birds separated quite early from other vertebrates, so is the higher constitutive GCGR activity also observed in very early birds?

R: GCGRs of *Palaeognathae* (arGCGR and dnGCGR) also have constitutive activity (as shown in Fig. R1 below) (**Supplementary Fig.1r** in manuscript). The demonstration that constitutive activity of GCGR is also observed in the constitutive activity of GCGR is also observed in Urochordata (ancestral gene of GCGR) (as shown in Fig. R2 below) (**Supplementary Fig.4b** in manuscript) and lost in placental mammals. Regarding this, we have added explanations to this evolutionary hypothesis in the manuscript.

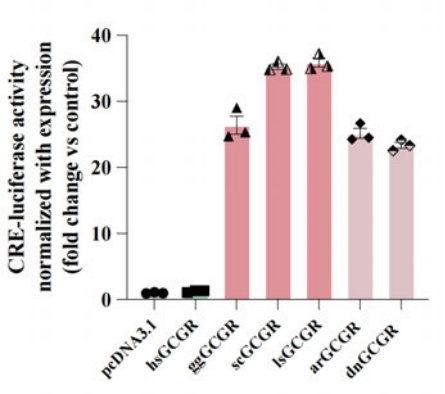

Fig. R1: Constitutive activities of GCGRs in different species, normalized by the expression of the plasmids. hs: *Homo sapiens*; gg: *Gallus gallus*; sc: *Serinus canaria*; ls: *Lonchura striata*; ar: *Apteryx rowi* (*Palaeognathae*); dn: *Dromaius novaehollandiae* (*Palaeognathae*).

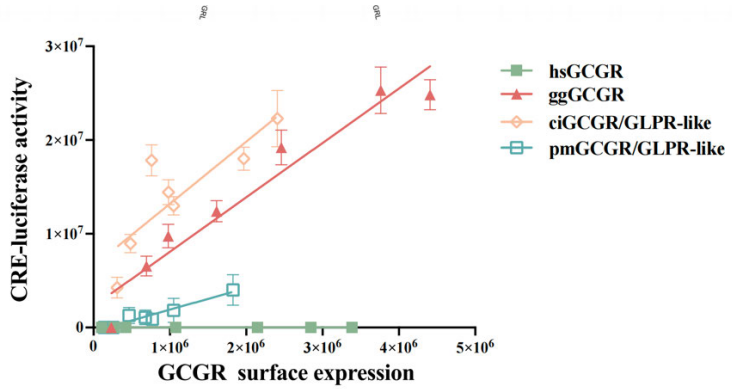

Fig. R2: Putative ancestral GCGR genes (*Phallusia mammillata* pmGCGR/GLP1R-like and *Ciona intestinalis*-ciGCGR/GLP1R-like) have constitutive activity.

In the manuscript, on lines 141-160, was revised as：

The constitutive activity was also found in the ancestral GCGR in Urochordata[55], indicating that the constitutive activity of GCGR had been lost in placental mammals (**Supplementary Fig. 4**). Furthermore, testing for constitutive activity of GCGRs after domain swapping showed that the constitutive activity of non-placental vertebrate GCGR depended on the cooperation of multiple domains (the stalk, extracellular cell loop ECL1 and intracellular cell loop ICL2, and possibly the C-terminus) (**Supplementary Fig. ~~5~~ 8)**. Consistently, previous studies have shown that these domains may function in the activation of hsGCGR (the ICL2 of hsGCGR interacts with Gs as determined in the crystal structure[56], and structural differences in the stalk, ECL1, and the C-terminus were detected in the activated and inactive hsGCGR states[57,58]). Hence, we modified several amino acid residues in these domains, based on their high conservation in birds while different in placental mammals. Perhaps, the putative adaptive evolution of GCGR (loss of constitutive activity) may have benefitted placental mammals, e.g., allowing them to retrieve stored energy, when food is scarce. Avian GCG concentrations in plasma are five-fold higher than those in mammals[59]. As expected, GCGR, devoid of constitutive activity, was more sensitive to GCG (**Fig. 1c** and **Supplementary Fig. 9**). When the body needs energy, GCGR can quickly and sensitively activate downstream pathways in response to GCG stimuli, facilitating placental mammals to adapt to different external environments.

6. The observation that avian GCGR maintains high expression in the liver is actually expected, since hepatic GCGR expression is highest across all tissues in all other animals as well, consistent with the liver as the primary organ for de novo glucose production. The same also accounts for the demonstration that avian GCGR signaling is primarily Gas-coupled, which is the case also for GCGR signaling in all other species. Is the rate of hepatic GCGR expression also a function of metabolic rate, or in other words, do animals with higher MR also have a higher expression of GCGR? Is the higher constitutive GCGR activity also relevant for thermogenesis in the birds? Did the authors measure thermogenesis after the viral-mediated gene KD in the birds?

R: We added the experiments and knocked down the hepatic GCGR expression of birds, measuring metabolic rate and thermogenesis, as well as viral-mediated gene expression.

As shown in Fig. R1 below, the results show that the knockdown of hepatic GCGR in birds leads to the downregulation of thermogenic genes, indicating that the expression of constitutively active GCGR is associated with thermogenesis.

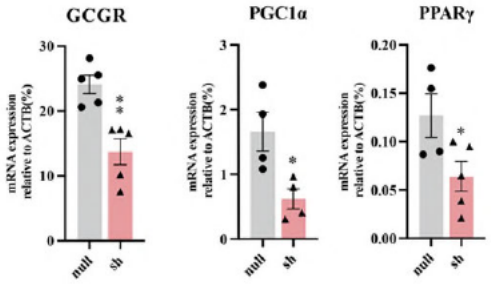

Fig. R1: Endogenous expression of GCGR after knockdown of hepatic GCGR in birds, as well as expression of downstream thermogenic-related genes *Pgc1α*, and *Pparγ*.

Although our results indicate that the expression of constitutively active GCGR is associated with thermogenesis, not all high-metabolism species exhibit the same pattern as birds with high expression of constitutively active GCGR. For example, bats have a relatively high metabolic rate, but their GCGR lacks constitutive activity and is not as highly expressed as in birds (as shown in Fig. R2 below). Besides GCGR, other genes can also regulate metabolic rates in animals.

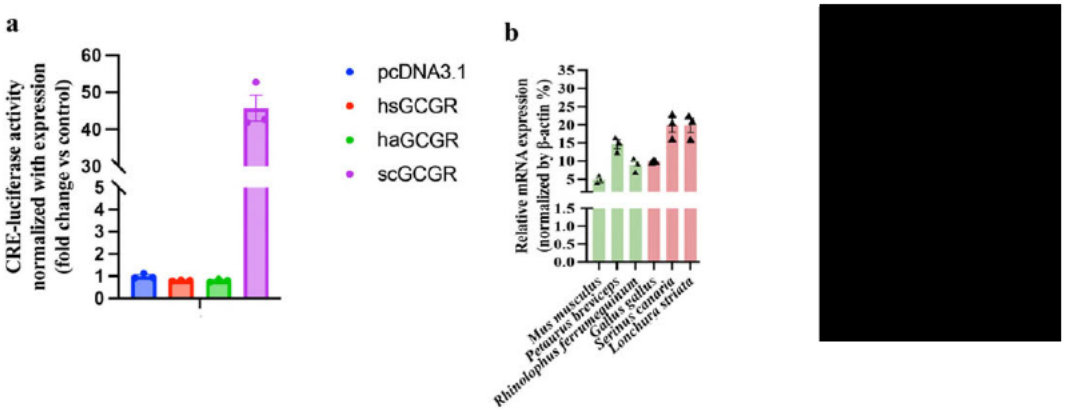

Fig. R2: (a) GCGR of bat (*Hipposideros armiger*) has no constitutive activity. *Homo species* (hs)GCGR, *Hipposideros armiger* (ha)GCGR, *Serinus canaria* (sc)GCGR. (b) mRNA expression levels of GCGR in the liver of different species. "*Rhinolophus ferrumequinum*" is a wild bat (we captured from the Shennongjia area in China). (c)

7. Do other animals living in the cold have higher hepatic GCGR expression as well?

R: That's a good question, but we do not know, and it would not be trivial to obtain livers from Arctic animals, for example, to answer this question in the present study.

As shown in Fig. R1, [redacted] and the results showed that hepatic GCGR mRNA level of Antarctic fish was lower than fish living in water with normal temperatures (*Danio rerio, Oreochromis mossambicus*) (as shown Fig. R1a below). Correspondingly, [redacted] We think the animals living in the cold may not have higher hepatic GCGR expression. However, this question needs to be answered by more data support.

Fig. R1

8. I highly discourage the authors from normalizing the energy expenditure data shown in Fig. 5aa-ac, and Fig 5ad-af by deviation to body weight, since such methodology doesn't discriminate between mass-dependent and -independent effects on metabolic rate (PMID: 22205519, PMID: 23520145, PMID: 34489606). The more appropriate way of doing this is by using a regression analysis and ANCOVA with e.g. body weight as co-variate (PMID: 22205519, PMID: 23520145, PMID: 34489606). Notably, such methodology also allows to calculate a bar graph based on the ANCOVA corrected data (see mentioned publications for an example)

R: That's a good point. We conducted the stats using a regression analysis and ANCOVA, then updated the graph accordingly (**Supplementary Fig. 27e-j**).

Referee #1 (Remarks to the Author):

I appreciate that authors significantly restructured the manuscript, improving the manuscript's clarity, structure, and readability. I believe that the authors sufficiently addressed my comments and I would be pleased to see this work published.

R: Many thanks for your encouraging expertise comments.

Referee #2 (Remarks to the Author):

The authors are to be congratulated for their extensive revision of the manuscript, which improved its already considerable quality in a major way. I am impressed by their even adding more data (e.g. on bats) and re-analyzing many of the existing ones. They also extensively restructured and reworded their manuscript.

R: Thanks for your important points on our comparison and especially for your suggestion of extrapolation.
Throughout this point-by-point response, we typed in red to present our answers to the questions and comments of reviewers while we used gray highlights to present our revisions in the manuscript. We also provide manuscript and figure files with highlights of changes for the convenience of reviewers/editors

Main comments:
The Intro, Results and Discussion parts are now better connected, and the authors have nuanced their interpretation that the constitutively active GCGR contributed to the evolution of flight in birds. I have a few more suggestions.
Specific comments:
1. L 1: please write out ‘GCGR’ in the title
R: Many thanks for your encouraging expertise comments.
On line 1, we revised as:

Constitutively Active Glucagon Receptor Drives High Blood Glucose in Birds

2. L10: Abbreviation ‘Gs’is not explained.
R: Thanks for your comments.
G protein can refer to two distinct families of proteins. Heterotrimeric G proteins, sometimes referred to as the "large" G proteins, are activated by G protein-coupled receptors and are made up of alpha (α), beta (β), and gamma (γ) subunits. "Small" G proteins (20-25kDa) belong to the Ras superfamily of small GTPases. These proteins are homologous to the alpha (α) subunit found in heterotrimers, but are in fact monomeric, consisting of only a single unit. However, like their larger relatives, they also bind GTP and GDP and are involved in signal transduction. Receptor-activated G proteins are bound to the inner surface of the cell membrane. They consist of the Gα and the tightly associated Gβγ subunits. There are four main families of Gα subunits:

Gαs (G stimulatory, Gs), Gαi (G inhibitory, Gi), Gαq/11, and Gα12/13. They behave differently in the recognition of the effector molecule, but share a similar mechanism of activation. Gs as one of G proteins, is important signal transducing molecules in cells. Gs activates adenylate cyclase, increasing cAMP levels and stimulating downstream signaling pathways such as protein kinase A (PKA) (PMID 27515397, PMID 27515397, ISBN 978-0-8153-4244-1, www.ebi.ac.uk). So, we maintained the "Gs" in the abstract and added "G protein Gs" in the main text.
On line 413, we revised as:

Avian GCGR constitutively activated downstream G protein Gs signaling and persistently consumed its substrate (glycogen), thus stimulating gluconeogenesis and lipolysis to alter physiological functions in the form of high blood glucose, low body weight and high basal metabolic rate (**Fig. 4**).

3. L 114: briefly state findings for budgies here? You do mention 'birds', i.e. both chicken and budgie results a few sentences down.
R: Thanks for your comments.
On line 83, we revised as:

Accordingly, chicken and budgerigar (*Melopsittacus undulatus*, *mu*) blood glucose levels and glycogen phosphorylase a (GPa) enzyme activity were significantly higher compared to mice under fed and fasted conditions (**Extended Data Fig. 1s,u**).

4. Suppl Fig 1: the sequence of the panels is obscure, especially for the panels following panel i. Please reorder so that the reader can more easily find the relevant panel. Also, three panels (those below j & l & q) now have no letter assigned to them. Please revise.
R: Thanks for pointing out the mismatch. We have reformatted **Supplementary Fig. 1, show as Extended data 1**.

5. L 143: should you refer to Suppl Fig 4'A'?
R: Thanks for your comments.
On line 102, we revised as:

Constitutive GCGR activity was also found in Urochordata[41], indicating that the constitutive activity of GCGR had been lost in placental mammals (**Supplementary Information Fig. 1b,c**).

6. L 147: please delete the crossed-out reference to Fig 5
R: Thanks for your careful reading and finding the mistake.
On line 107, we changed as:
(the stalk, extracellular cell loop ECL1 and intracellular cell loop ICL2, and possibly the C-terminus) (**Extended Data Fig. 2** and **Supplementary Information Fig. 2**).

7. L156: is 'human' missing at the end of this line?
R: Thanks for your comments.
On line 114, we revised as:

As expected, GCG stimulated its corresponding receptor with an 50% maximal efficacy (EC50) of 37nM for ggGCGR, and 1.04nM for hsGCGR **(Fig. 1c)**. All GCGRs with high constitutive activity could be further activated by both hsGCG and ggGCG with similar potency in a dose-dependent manner, but with lower maximal efficacy (Emax) **(Extended Data Fig. 1w-z)**.

8. L 172: add 'and budgerigar' here?

R: Thanks for your comments.

On line 83, we revised as:

Accordingly, chicken and budgerigar (*Melopsittacus undulatus*, *mu*) blood glucose levels and glycogen phosphorylase a (GPa) enzyme activity were significantly higher compared to mice under fed and fasted conditions (**Extended Data Fig. 1s,u**).

9. L197: Fig 1 & 2 do not show age-related trends in hepatic expression of GCGR genes. Please delete the reference to those Figs.

R: Thanks for your suggestion.

on line 133, we revised as:

Among all non-placental vertebrates, both *Aves* and *Agamidae* (lizards) maintained high constitutive activity and hepatic expression of *GCGR* genes throughout their entire lives (**Extended Data Fig. 3d**,**f**).

10. Suppl Fig 11: I find the x-axis legend referring to 'hatch (days)' confusing. The Fig legend doesn't explain it further. These are pre-hatch or incubation days, correct? If so, I'd rather label them 'incubation days' and explain in the legend that day 0 or 1 was the first day of incubation (or however else you defined it).

R: Thanks for pointing out the misunderstandings. We have reformatted **Supplementary Fig. 11, show as Extended data 6c-f**.

On **Extended data 6c-f** in figure legend, we revised as:

c-f, Blood glucose levels and GCGR mRNA expression during the whole life in *Gallus gallus* (day 1 is defined as the first day of egg incubation)

11. L 206: I cannot find the stats for Fig 2a and suppl Fig 12 that would support your statement. In the legend to Fig S12 you write that stats are provided above the western blots, but I didn't find anything. There are numbers above the plots, but not sure what these refer to.

R: Thanks for reminding us of the important point. We reanalyzed all our data using analysis of variance (ANOVA) and updated the figures and legends accordingly. Overall, the significant differences calculated using the t-test and ANOVA are statistically significant. Detailed information included in the accordingly figure legends.

12. L 213ff: likewise, I am missing the stats for Fig S 13. Please state the type of stats you used and p-levels (same for Fig S12)

R: Yes，please see the reply to question 5 about stats.

13. L 558: “massive energy expenditure”: is this backed up by data (no citation is provided)? When food is scarce, animals can also resort to decreasing metabolic rate and increasing rest periods, which can help them to reduce depleting endogenous energy stores at a fast pace. Lowering of body temperature can also be a strategy to save energy. Therefore I am not entirely convinced by this argument.

R: Yes, writing “massive” is inaccurate and we replaced the term.

On line 396, we revised as:

We presume that the loss of constitutive activity of GCGR in placental mammals may have countered disadvantages caused by excessive activation of GCGR signaling, such as the inability to cope with the energy expenditure of the body when food is scarce.

14. L 562: please insert ‘can/could’ or something along that line before ‘utilize’. You have not tested whether constitutively active GCGR are involved in flying or migration. Also, again, bats can also fly and migrate.

R: Thanks for reminding us of the important point.

On line 403, we revised as:

In contrast, birds can utilize the energy support of constitutively active GCGR for flight and presumably evolved other molecular and behavioral strategies (including migration) to cope with environmental changes[56].

15. L 636: please add ‘may’ here.

R: On line 456, we revised as:

In conclusion, constitutively active GCGR with high hepatic expression may underly sustained high blood glucose in birds and is the consequence of a “switchless gene” that contributed, among many other adaptations and exaptations, to the evolution of flight in birds and their ancestors.

16. L 882ff: Please add a statement to the legend for Suppl Fig 1 to make the reader aware that that the scale of the y-axes differ for panels a-i.

R: Thanks for your suggestion.

On line 607, we revised as:

Due to differences in constitutive activity across the receptors, the y-axis scales in the **Extended Data Fig. 1a-i** are different.

17. Legends for Supplementary Tables: Thank you for now providing titles for the supplementary tables. However, more information is required. For example, for Suppl. Table1 the statistical measure of the variance is not stated. Are the data provided as means ± std errors, or standard deviation or confidence intervals…? Please add such information to all Tables (where it applies).

R: Thanks for your suggestion.

We have reorganized our supplementary tables, adding the corresponding analysis methods, sample sizes, and p-values for all data. We have also listed all the animals, instruments, software, and

antibodies used. Additionally, we have written a supplementary table guide.

Referee #3 (Remarks to the Author):

I have no further comments. The manuscript improved significantly during the revision and I think that it is an important manuscript

R: Many thanks for your encouraging support.