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ORIGINAL PAPER

Glucose regulation is a repeatable trait affected by successive handling in zebra finches

Bibiana Montoya[1,2,3] · Michael Briga[2,4] · Blanca Jimeno[2,5] · Simon Verhulst[2]

Abstract
The capacity to adequately respond to (physiological) perturbations is a fundamental aspect of physiology, and may affect health and thereby Darwinian fitness. However, little is known of the degree of individual variation in this capacity in non-model organisms. The glucose tolerance test evaluates the individual's ability to regulate circulating glucose levels, and is a widely used tool in medicine and biomedical research, because glucose regulation is thought to play a role in the ageing process, among other reasons. Here, we developed an application of the intraperitoneal glucose tolerance test (IP-GTT) to be used in small birds, to test whether individuals can be characterized by their regulation of glucose levels and the effect of successive handling on such regulation. Since the IP-injection (intraperitoneal glucose injection), repeated handling and blood sampling may trigger a stress response, which involves a rise in glucose levels, we also evaluated the effects of handling protocols on glucose response. Blood glucose levels decreased immediately following an IP-injection, either vehicle or glucose loaded, and increased with successive blood sampling. Blood glucose levels peaked, on average, at 20 min post-injection (PI) and had not yet returned back to initial levels at 120 min PI. Glucose measurements taken during the IP-GTT were integrated to estimate magnitude of changes in glucose levels over time using the incremental area under the curve (AUC) up to 40 min PI. Glucose levels integrated in the AUC were significantly repeatable within individuals over months ($r = 50\%$; 95% CI 30–79%), showing that the ability to regulate glucose differs consistently between individuals.

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Introduction

Glucose is an important source of energy for several tissues. In vertebrates, its regulation involves the coordinated action of multiple organs and has an impact on protein and lipid metabolism (Hazelwood 2000; Bernard et al. 2003; Braun and Sweazea 2008). Thereby, glucose regulation is a life sustaining process and variations in this process may affect individual's performance (Hazelwood 2000; Bernard et al. 2003; Braun and Sweazea 2008; Scanes 2015; but see Ramenofsky 1990). An effective response of glucose regulation is recognized as a hallmark of health in humans and laboratory models, and its impairment has been linked to mechanisms associated with the ageing process (Gardner et al. 2005; Semba et al. 2010; Picard et al. 2014; Regan et al. 2020). Although glucose regulation is thought to be plastic (i.e. a single genotype may produce multiple phenotypes in response to environmental changes), the few studies available with non-model organisms suggest this trait to still be repeatable within individuals (Pratt et al. 2005; Bröjer

et al. 2013). However, further studies are required to better understand to what extent glucose regulation capacity is consistent throughout an individual's life, and thereby can systematically affect individual's physiological performance, and be considered an individual trait that is potentially a subject of selection.

The glucose tolerance test (GTT) is a widely used tool to investigate the capacity to regulate glucose in humans and laboratory animal models (Andrikopoulos et al. 2008; Babbar et al. 2018). Using repeated blood sampling, the GTT measures how fast an orally or injection administrated glucose dose is cleared from the blood stream, bringing glucose concentration back to pre-administration levels. Glucose regulation has been little studied in non-model organisms, and studies available on birds are mostly restricted to poultry (Heald et al. 1965; Boswell et al. 1997; Brzęk et al. 2010; Datar and Bhonde 2011; Sumners et al. 2014). Birds are interesting in this respect, because avian plasma glucose concentrations are considerably higher when compared to mammals of similar body mass (Braun and Sweazea 2008). These high levels are apparently maintained without compromising avian organismal functioning, as suggested by the longer lifespans of birds compared to mammals of similar body mass (Holmes et al. 2001; Scanes and Braun 2013). However, when comparing individuals, high baseline glucose levels are associated with shorter lifespans in zebra finches *Taenopygia guttata* (Montoya et al. 2018), collared flycatchers *Ficedula albicollis* (Récapet et al. 2016, glycated hemoglobin), and blue tit nestlings *Cyanistes caeruleus* (Kaliński et al. 2014); and with growing up in poor-quality habitats in blue tit nestlings (Kaliński et al. 2014). Therefore, studying the capacity to regulate glucose may be a useful tool to estimate health state, monitor the consequences of habitat degradation on free living birds, and help to gain insights on the ageing process.

We applied an intra-peritoneal glucose tolerance test (IP-GTT) to evaluate the repeatability of the ability to regulate glucose levels in zebra finches, and the effects of successive handling on this ability. Earlier studies have shown that blood glucose levels increase as part of the acute stress response (Remage-Healey and Romero 2001; Jimeno et al. 2018a). Hence, considering that IP-GTT involves repeated handling (i.e. successive blood sampling) that may trigger a stress response, we first evaluated the effect of handling on blood glucose levels by varying the number and timing of blood samples, and comparing effects of injections with and without a glucose load. Second, we estimated glucose levels at different time intervals after the intra-peritoneal injection loaded with different doses of glucose. A protocol that applies the IP-GTT to small birds was not available prior to our study, and hence we developed a protocol to suit small birds on the basis of protocol-directed experiments. Performance in a GTT can be characterized by integrating several glucose measurements at successive time intervals (i.e. glucose curve), an strategy to do this is calculating the area under the glucose curve (hereafter AUC) using the trapezoidal method, we implemented this common approach used when testing glucose tolerance (Fig S1; Le Floch et al. 1990; Andrikopoulos et al. 2008).

Methods

Housing

Individuals included in the study were randomly selected from the zebra finch colony of the University of Groningen, the Netherlands (53° 13′ 0″ N/6° 33′ 0″ E). Adult birds were housed in single sex outdoor aviaries (L × H × W: 310×210×150 cm) and individually identified with a numbered metal ring. Birds had ad libitum access to tropical seed mixture, cuttlebone, egg food, water and sand (Bogena, Hedel, The Netherlands). These housing conditions are similar to those in other facilities (e.g. Griffith et al. 2017).

Blood sampling

Data were collected between June 16th and September 4th 2014. Birds were captured using handheld nets, and individually placed in a box with a wire mesh top (L × W × H: 40×40×15 cm) without access to food or water. Individuals were then maintained in low-light conditions for 30 min prior to sampling (following Montoya et al. 2018). Blood (40 µL per sample) was taken from the brachial vein by puncturing the vein each time, starting from a distal position in the vein with respect to animal's body, and alternating between wings among samples. Blood was collected in heparinized microcapillary tubes, and immediately after sampling, was added to a heparin (500 IU/mL)—EDTA (0.01%) solution (760 µL per sample), and stored on ice for up to 40 min. All samples were then frozen at −20 °C for a maximum of 48 h before glucose measurement. We first performed a pilot test to evaluate whether glucose concentration in frozen plasma and frozen whole blood were correlated. The amount of blood taken per individual on a single day never exceeded 160 µL. After blood sampling, individuals were left undisturbed in the waiting box for an hour, at a warm place (near an incandescent light bulb) with ad libitum access to food and water, before being returned to their aviary.

Protocol development

A schematic overview of protocol-directed experiments, including specific aim and sample size, is presented in Table 1 in chronological order. The aim of these sequential experiments was to determine the optimal conditions for

Table 1 Description of aim, design and sample size of the different experiments performed in this study

Exp	Aims and predictions	Design	Sample sizes
1	Aim: To evaluate the effect of IP−injection on initial blood glucose levels Pred: Only glucose loaded IP−injection will increase initial blood glucose levels	Individuals received an IP−injection, either 20% glucose or saline loaded, immediately before (< 1 min; $n = 51$) or immediately after (< 1 min) the initial blood sampling (i.e. 30 min after capture; $n = 31$)	$n = 82$ (37 females and 45 males)
2	Aim: To test the effect of IP−injection timing on PI blood glucose levels Pred: Only glucose loaded IP−injection will increase PI blood glucose levels	Individuals received an IP−injection, either 20% glucose or saline loaded, before ($n = 22$) or after ($n = 34$) the initial sampling and were resampled T10, T20 and T40 min after injection	$n = 56$ (24 females and 32 males)
3	Aim: To explore the effect of successive handling on T40 min PI blood glucose levels Pred: Successive handling will increase T40min PI blood glucose levels	Individuals received an IP−injection, either 20% glucose or saline loaded, after the initial sampling and were resampled thrice (T10, T20 and T40 min PI; $n = 24$) or once (T40 min PI; $n = 20$) after the IP−injection	$n = 44$ (21 females and 23 males)
4	Aim: To assess the effect of the IP−injection glucose concentration on blood glucose levels after successive handling Pred: Individuals injected with higher glucose concentrations and successively handled will have higher blood glucose levels	Individuals received an IP−injection saline ($n = 26$), 20 ($n = 11$) or 30% ($n = 13$) glucose loaded, after the initial sampling and were resampled thrice (T10, T20 and T40 min PI) after the IP−injection	$n = 50$ (21 females and 29 males)
5	Aim: To describe the performance in the GTT under the parameters tested in the previous experiments Pred: No predictions	Individuals received an IP−injection 30% glucose loaded, after the initial sampling and were resampled thrice (T10, T20 and T40 min PI) after the IP−injection	$n = 24$ (9 females and 15 males)
6	Aim: To evaluate blood glucose levels at T120 min PI. within the GTT developed Pred: Blood glucose levels will have reached the initial concentration at T120 min PI	Individuals received an IP−injection 30% glucose loaded, after the initial sampling and were resampled thrice (T10, T20 and T120 min PI) after the IP−injection	$n = 22$ (10 females and 12 males)

Exp Experiment number, *Pred* Prediction, *IP* Intra peritoneal, *PI* Post−injection, *GTT* Glucose tolerance test

the IP-GTT in zebra finches, over which we calculated repeatability estimates. Administrating glucose through IP injection was chosen over an oral supplementation because in a pilot study using the oral version of the protocol, birds required longer to reach glucose peak values, making the whole protocol considerably longer (S. Moonen and S. Verhulst unpublished data). In experiment 1, we tested the effect of the IP-injection on initial glucose levels by sampling a subset of individuals immediately before or after an IP-injection that was loaded with either 20% glucose or saline solution. This experiment was performed over six days and included 82 birds. In experiment 2, we evaluated if the glucose trajectory, i.e. the glucose levels in the three subsequent post-injection (PI) measurements, was affected by the timing of the first sample (i.e. before or after IP-injection) and the load of the IP-injection (0 or 20% glucose); sampling sessions were performed over five days and 56 individuals were tested. In experiment 3, we assessed the effect of successive handling on glucose levels by dividing birds into two subsets: half of them had four blood samples of the GTT taken (T0, T10, T20 and T40 min PI), while the other half had only the first and the last sample of the GTT taken (T0 and T40 min PI). IP-injection was loaded with either 0 or 20% glucose, and the 44 birds included in the experiment were sampled within three days. In experiment 4, we selected the optimal glucose concentration for the IP-injection, by comparing the effect of 0, 20 and 30% glucose doses on blood glucose levels of individuals successively handled during the GTT (i.e. four samples taken). Tests were performed within five days and included 50 individuals. In experiment 5, we explored the performance of individuals using the final IP-GTT protocol developed through experiments 1–4; 24 individuals were tested. In experiment 6, we evaluated the effect of delaying the last sample of the GTT until T120 min PI on blood glucose levels, with 22 birds included in the experiment. Individual birds were used only once in each of the six experiments described above.

Glucose or saline solution (control) were always administrated intra-peritoneally. There is no standard dose for the GTT in birds, and previous studies have used a range from 1 to 80% glucose solution in oral and intravenous versions of the GTT (Muiruri et al. 1975; Ramachandran and Patel 1989; Joseph et al. 1996; Totzke et al. 1998). We injected 100 μL of a 20 or 30% D(+)-glucose monohydrate solution ($C_6H_{12}O_6$—saline solution, Merck KGaA, Darmstadt, Germany), kept on ice, on the midline abdomen (~0.25 cm to left side of the midline and ~0.5 cm above cloaca). This dosage closely corresponds to the optimal reported for mice during an IP-GTT, 2 gr/kg (Andrikopoulos et al. 2008). In all experiments, we used the sampling interval as reported by Brzęk et al. (2010), but blood sampling at T60, T120 and T180 min after injection was omitted due to limitations on

the blood volume that can be taken from birds as small as zebra finches (however, see experiment 6).

Glucose measurements

The Hoffman's ferricyanide method was used to determine glucose levels in whole blood (Hoffman 1937). Standard curve and samples were read in a Technicon autoanalyzer (Beckman Coulter LX20PRO). A standard glucose curve was included at the beginning and end of each assay session, and glucose values were estimated using the average values of the two curves. Blood samples were analyzed in duplicate, and statistical calculations were performed on the average of the two duplicates, of which the extrapolated repeatability was 86% (calculated following Eq. 37 in Nakagawa and Schielzeth 2010).

We used area under the glucose curve (AUC) to quantify the response to the glucose injection. We calculated the incremental AUC using the trapezoidal method (Tai 1994). The AUC was calculated from the glucose levels in four consecutive blood samples, just before glucose injection (T0-G0), at 10 min (T10-G10), 20 min (T20-G20) and 40 min (T40-G40) after glucose injection as shown in Figure S1. When G3 was below G0, which was unusual (6/56 cases), we estimated the time point at which initial glucose level (G0) was reached using the interpolation of the equation of the line G20 and G40, and taking the resulting time point to calculate area c in Figure S1. In addition to the AUC as described above, we also calculated the AUC up to the moment that, on average, the peak glucose level was reached (T20 min PI), and we denoted this as the AUCpeak.

Statistical analyses

We analyzed the data with linear mixed models using the function lmer of the package lme4 in R version 3.3.1 (Bates et al. 2015, R core team 2017). P values were obtained using the package lmerTest (Kuznetsova et al. 2017) and post-hoc tests were performed with the package emmeans (Lenth 2019). Unless mentioned otherwise, blood glucose level was the dependent variable in all models.

The effect of the IP-injection on initial glucose level (G0) (experiment 1) was tested in a model including IP-injection time (before or after initial sampling), injection load (0 or 20% glucose) and sex as fixed factors, and sampling time (hour of day) as a covariate. In this and subsequent analyses the inclusion of sex and sampling time in no case explained a significant part of the variance, and hence all results are reported without further reference to these variables.

The influence of IP-injection on glucose levels (experiment 2) was evaluated in a model that included as response variable the glucose levels obtained in the four samples taken (G0, G10, G20, G40), and as fixed factors the IP-injection

time (before or after initial sampling), the injection load (0 or 20% glucose), and the sample number (i.e. T0, T10, T20 or T40). Interactions of injection timing with sample number and injection load were also tested. Bird identity was included as a random effect.

The impact of successive handling on glucose levels (experiment 3) was explored fitting a model including the injection load (0 or 20% glucose), the sample number (T0, T10, T20 or T40), and the number of samples taken (either two samples, at T0 and T40 min PI, or four samples at T0, T10, T20 and T40 min PI) as fixed factors, and testing the interactions of number of samples taken (two or four) with injection load and with sample number (T0, T10, T20 or T40); bird identity was maintained as a random effect.

The effect of the glucose concentration of the IP-injection on blood glucose levels during successive handling (experiment 4) was evaluated including injection load (0, 20 or 30% glucose) and sample number (T0, T10, T20 or T40) as main effects, and the interaction between these two factors. Bird identity was included as a random effect.

The performance of the birds in the IP-GTT (i.e. under the conditions defined through experiments 1–4, experiment 5) was evaluated by including blood glucose levels at initial (G0) and all PI samples as response variable (G10, G20 and G40), and sample number (T0, T10, T20 or T40) as fixed factor. Bird identity was included as a random effect. The effect of delaying the final blood sample up to T120 min PI (experiment 6) was analyzed as described for experiment 5. Finally, we fitted a model including AUC as response variable, whether it was the first or second AUC measurement for a given bird, the time at which peak was attained and the glucose concentration at peak as fixed effects, and bird identity as a random effect. Repeatability was calculated by estimating the intra-class correlation coefficient associated with bird identity.

Results

Sample preparation

Glucose levels measured in plasma and whole blood frozen samples were moderately correlated ($r = 0.72$, $N = 8$, $P = 0.04$; Fig S2). It is worth noting that this correlation underestimates the true association between glucose estimates, because it was not corrected for measurement error. Correcting for measurement error on both axes following Muchinsky (1996), i.e. calculating the disattenuated correlation coefficient, yields a disattenuated correlation of $r = 0.99$. Therefore, glucose concentrations were analyzed in frozen whole blood samples to minimize sample handling, and to avoid variation among samples related to the degree of hemolysis induced by sample handling.

IP-injection timing

Sampling immediately before the IP-injection yielded substantially higher initial glucose levels than sampling immediately (within seconds) after IP-injection (mean ± s.e., before: 12.9 ± 0.35; after: 10.9 ± 0.21; $\beta = 1.98 \pm 0.38$ mM, $F_{1,80} = 26.52$, $P < 0.001$, Table S1; data experiment 1, Table 1). Whether the IP-injection was loaded with 0 or 20% glucose in interaction with sampling time (before or after injection) did not affect initial glucose levels (IP-load x sampling timing $F_{1,78} = 0.38$, $P = 0.54$), presumably because the interval between IP-injection and sampling was less than one minute.

The effect of sampling time relative to the IP-injection persisted over the subsequent time points, with individuals sampled immediately after the IP-injection having lower PI glucose levels than individuals sampled just before the IP-injection (Fig. 1; $\beta = -2.42 \pm 0.52$ mM, $F_{1,115.5} = 21.30$, $P < 0.001$, Table S1; data experiment 2, Table 1). This effect did not change significantly over successive samples as indicated by the non-significant interaction between initial sampling timing and the sample number ($F_{3,157.1} = 0.39$, $P = 0.76$). Furthermore, there was no interaction between IP-injection time and IP-injection load (i.e. 0 or 20% glucose) on blood glucose levels ($F_{1,71.0} = 0.27$, $P = 0.61$). Glucose levels increased over time in both 0 and 20% glucose IP-injected birds (Fig. 1; all pair-wise comparisons between initial and > 10 min PI levels, $P < 0.01$), yet the increment was more pronounced in glucose IP-injected birds ($F_{3,161.5} = 11.52$, $P < 0.001$; Table S1). Considering results
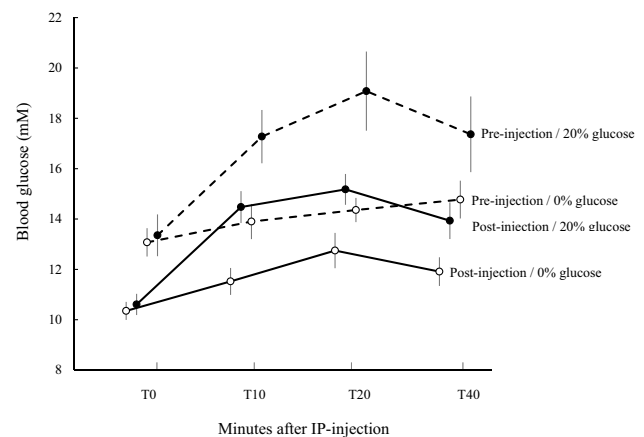

Fig. 1 Effect of timing of initial sampling and injection load on glucose levels. Initial glucose sample (T0) was taken after 30 min fasting and immediately before (dashed lines) or after (solid lines) a glucose injection (i.e. pre-injection or post-injection sample). Intra-peritoneal injection was loaded with either 0% (open dots) or 20% glucose (solid dots). Note that to avoid overlapping s.e.'s markers were slightly displaced along the *X*-axis. Data from experiment 2

of experiments 1 and 2 (Table 1), IP-injection was performed after initial sampling in all subsequent tests.

Sampling frequency

The observation in experiment 2 that glucose levels increased with successive sampling, also when only vehicle was injected, was reason to test the effect of sampling frequency on glucose levels directly in experiment 3 (Table 1). This revealed that birds sampled at T0 and T40 min PI only had lower glucose levels at T40 min PI compared to birds additionally sampled at intermediate time points T10 and T20 min PI (Fig. 2; sample $\times$ handling $F_{1,41.5} = 14.11$, $P < 0.001$, Table S1). In this experiment, blood glucose levels at T40 min PI were indistinguishable from initial glucose levels when no samples were taken at T10 and T20 min, even when birds were injected with 20% glucose (IP-glucose load $\times$ handling $F_{1,40.2} = 1.18$, $P = 0.28$, Table S1). Contrastingly, blood glucose was maintained elevated above initial glucose levels at T40 min PI, when birds were resampled between the initial and final sample.

Glucose doses

Considering that successive handling had a stronger effect on glucose levels than the 20% glucose load of the IP-injection (Fig. 2), in experiment 4 we tested whether increasing IP-injection load from 20 to 30% glucose would increase blood glucose levels further (Table 1). When comparing blood glucose levels over all time points (i.e. T0, T10, T20 and T40 min PI-samples) among birds IP-injected with 0, 20 or 30% glucose, we found a significant interaction between the IP-injection load and the sample number ($F_{6,128.6} = 19.85$,
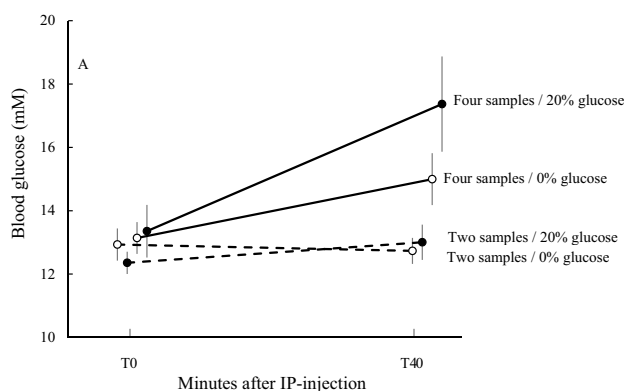
$P < 0.001$, Table S1). Blood glucose levels of birds IP-injected with 0 or 20% glucose differed significantly at T20 min PI only (post-hoc test $t_{67.4} = -3.40$, $P = 0.05$; at T10 and T40 min: $P > 0.66$, Table S1). In contrast, blood glucose levels of birds IP-injected with 30% glucose differed from 0 and 20% IP-injected birds at all PI time points (post-hoc tests: all $P < 0.001$; Fig. 3). Hence, IP-injection was loaded with 30% glucose in all subsequent trials.

Glucose tolerance test

In experiment 5, we performed the glucose tolerance test (GTT) following the conditions developed using results of experiments 1–4 (Table 1). PI glucose levels varied significantly among time points ($F_{3,67.0} = 40.52$, $P < 0.001$) and were elevated above initial levels at all PI time points (Fig. 4a; T10 min: $\beta = 9.98 \pm 1.11$ mM, T20 min: $\beta = 11.21 \pm 1.12$ mM, T40 min: $\beta = 7.01 \pm 1.14$ mM; all post-hoc tests $P < 0.001$). Glucose levels peaked on average at T20 min PI (mean $\pm$ S.E. mM, T0 min: 14.34 ± 0.53; T10 min PI: 24.19 ± 1.23; T20 min PI: 25.53 ± 0.77), and had not yet reached initial level at T40 min PI (21.40 ± 0.98 mM). Interestingly, there was inter-individual variation in the time at which glucose levels peaked: 37.5% of the individuals peaked at T10 min PI, 50% peaked at T20 min PI, and in the remaining 12.5% glucose peaked T40 min PI. The magnitude of AUC in tests performed over 40 min varied depending on the timing of the peak ($F_{2,84} = 3.31$, $P = 0.04$), being higher when the peak was reached at 20 min PI. compared to peaks at both T10 and T40 min PI. Moreover, the AUC increased with the magnitude of the glucose peak ($\beta = 37.59 \pm 4.15$ mM, $F_{1,84} = 92.33$, $P < 0.001$; Fig. 4a).
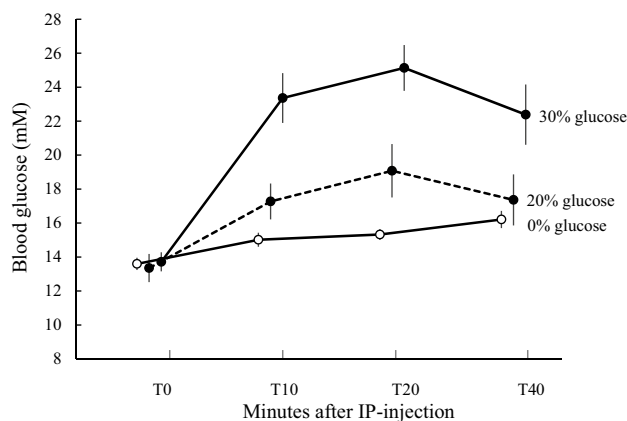

Fig. 2 Effect of sampling frequency and injection load on glucose levels (mean $\pm$ s.e.). Birds were sampled either two (i.e. T0 and T40 min PI; dashed lines) or four times (i.e. T0, T10, T20 and T40 min PI; solid lines); for clarity the results at T10 and T20 min PI are not shown. In all cases, first sample (T0) was taken after 30 min fasting. IP-injection was loaded with either 0% (open dots) or 20% glucose (solid dots). Data from experiment 3

Fig. 3 Effect of injection load on glucose levels (mean $\pm$ s.e.). Birds were IP-injected with 0% (open dots and solid line), 20% (closed dots and dashed line) or 30% glucose (closed dots and solid line). In all cases, first sample (T0) was taken after 30 min fasting. Note that to avoid overlapping s.e.'s markers were slightly displaced along the X-axis. Data from experiment 4, all individuals were blood sampled four times
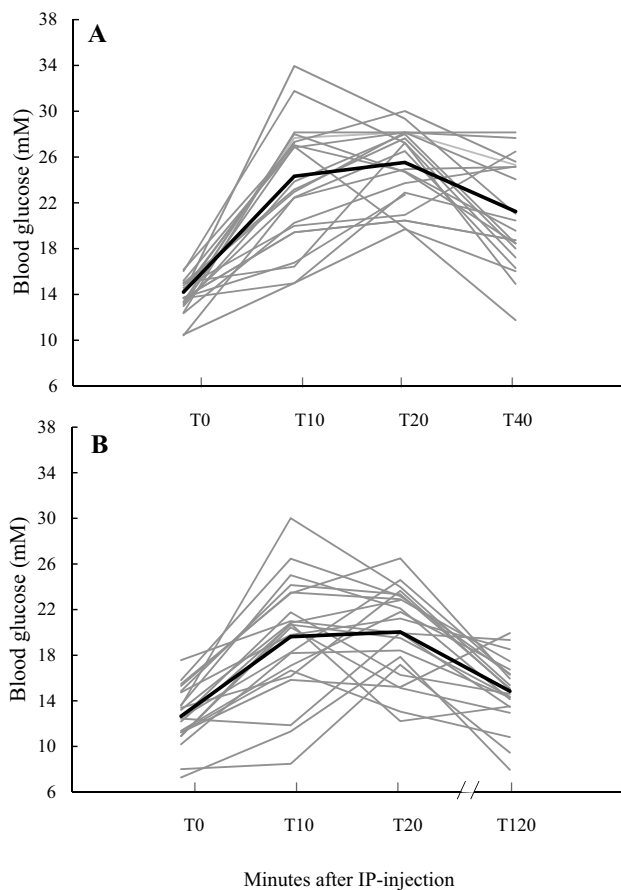
Fig. 4 Glucose levels shown by individual zebra finches during an IP-glucose tolerance test. Initial blood sample (T0) was taken seconds before the IP-injection, and IP-injection was loaded with a solution of 30% glucose. Panel A shows data from experiment 5 and corresponds to twenty-four individuals (9 females and 15 males) sampled for the last time at T40 min post injection. Panel B shows data from experiment 6 and comprises twenty-two individuals (10 females and 12 males) sampled for the last time at T120 min PI. In both panels, the black thick line represents mean values

Because only two out of the 24 individuals tested (experiment 5) reached initial glucose levels at T40 min PI, we repeated the test using a different set of individuals, but with the fourth sample taken at T120 min PI instead of T40 min PI (experiment 6). Blood glucose levels again differed among PI sampling points ($F_{3,62.22} = 38.05$, $P < 0.001$), and at T120 min PI blood glucose levels were still higher than initial levels (Fig. 4b, post-hoc test $t_{62.2} = -2.67$, $P < 0.05$). Only one out of 22 individuals had reached their initial level at T120 min PI. Therefore, to reduce the timespan of the protocol, which is desirable for practical and welfare reasons, the last sample in our final protocol was taken at T40 min PI.

Repeatability

A different set of birds (i.e. not used in experiments 1–6, $n = 28$, 15 females and 13 males) was tested twice with a two-month interval to estimate the repeatability of AUC under the IP-GTT developed through experiments 1–6. Repeatability of each time point of the glucose curve ranged from 37 to 63% (T0 min = 54%, CI = 23–76%; T10 min = 63%, CI = 35–81%; T20 min = 51%, CI = 18–74%; T40 min = 37%, CI = 1–65%). AUC repeatability was 50% (Fig. 5a; 95% C.I. 30–79%) calculated over all time points, while repeatability of the AUCpeak, including only sampling points up to average peak levels (T0, T10 and T20 min PI), was comparable at 47% (Fig. 5b; 95% C.I. 13–71%). Similar to results obtained in experiment 5, in tests performed to calculate repeatability estimates, we also found that AUC was higher when the peak was reached at T20 min PI ($F_{2,43.0} = 12.43$, $P < 0.001$), and when the magnitude of the glucose peak was more elevated ($\beta = 26.47 \pm 2.10$ mM, $F_{1,47.1} = 159.22$, $P < 0.001$).

Discussion

Our primary interest was to quantify variation among individuals in the ability to regulate blood glucose levels, and to this end we adapted the IP-GTT for application in the zebra finch. We estimated the extent of individual variation by calculating the repeatability of the performance of individuals in response to the test. At repeatabilities of 50% (AUC; 95% CI 30–79%), and 47% (AUCpeak; 95% CI 13–71%), the among-individual consistency in the response to a glucose challenge was substantial over months, and at a level comparable to the repeatability of 63–64% observed in horses (Bröjer et al. 2013; Pratt et al. 2005). We therefore conclude that glucose regulation can be considered an individual trait. The repeatability observed for the AUC was higher than the repeatability of 30% we reported previously for initial glucose levels in the same population (Montoya et al. 2018), but close to that reported for corticosterone response to dexamethasone in the same study population (Jimeno et al. 2018a), which can also be considered a test of the ability to recover stability after a challenge. Interestingly, in this study repeatability of initial glucose levels was higher at 54% than what we previously reported (30%, Montoya et al. 2018), and this could be associated with lower variability in the sampling conditions in the current study (i.e. all experiments were performed in the same year, individuals were sampled/resampled during the same season, and exactly the same protocol was always used). Hence, the GTT provides information about the potential of the individual for dealing with challenges while maintaining the functionality of vital physiological process (i.e. buffer the potential impacts of
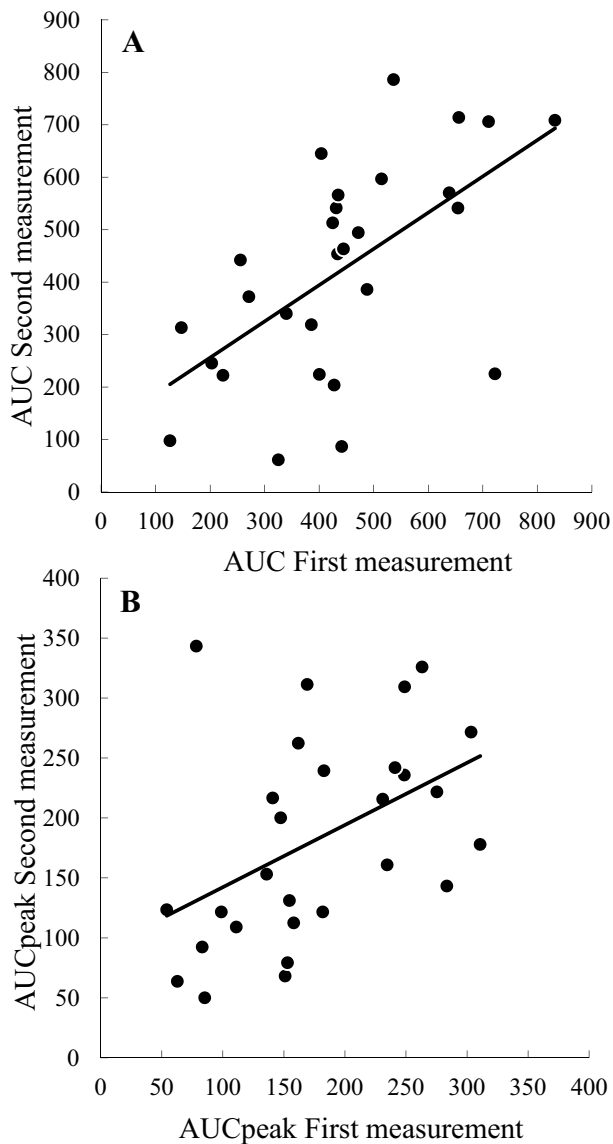
Fig. 5 Individual repeatability of the glucose regulation response in a GTT. Panel A, individual repeatability of the area under the curve of glucose (AUC×40 min; repeatability: 50%, 95% CI 30–79%) and panel B, individual repeatability of the area until reaching glucose peak (AUCpeak, up to and including T20 min post-injection; repeatability: 47%, 95% CI 13–71%). AUC and AUCpeak were measured in an intra-peritoneal glucose tolerance test. Twenty-eight individuals (15 females and 13 males) were tested with an interval of two months between the first and the second measurement

environmental fluctuations), which has been proposed as a good estimate of individual's condition (Hill 2011).

Glucose levels increase in response to various stressors, presumably through the action of glucocorticoids such as corticosterone (e.g. Jimeno et al. 2018b). Hence, it was not surprising that sampling by itself affected glucose levels (Fig. 2), and this result suggests that a GTT response might also provide information about individual's response

to challenging conditions. The number of samples taken therefore needs to be considered carefully when developing a GTT, maintaining a balance between disturbance of the animal and resolution desired for the test. On the one hand, the effect of successive sampling on glucose levels, as well as welfare considerations, are reasons to minimize the frequency of sampling. On the other hand, taking a larger number of samples allows a better description of the response to the glucose injection. This balance may be especially important when performing this test in the wild, where it may be particularly informative on the capacity of the individual to deal with disturbances at different life stages, but at the same time, the number of samples taken may potentially affect the individual's survival. Some alternatives to address this potential pitfall may be implementing the use of portable devices to measure glucose levels directly in the field, which requires small amounts of blood (see Tomasek et al. 2019), and/or reduce the extension of the protocol aiming to obtain only AUCpeak which is highly repeatable (47%) and requires half of the time (20 min) of the full protocol (40 min) to be completed. The fact that blood glucose did not return to initial levels 40 min after the IP-injection, which is likely due to the effect of successive handling, limits the use of this protocol to evaluate glucose regulation.

In contrast to the increase in glucose levels due to successive sampling, the IP-injection itself resulted in an immediate *decrease* of blood glucose levels (within seconds), and this effect was independent of whether the injection was saline or glucose loaded. Surprisingly, the effect of taking the initial sample before or after the IP-injection persisted throughout the GTT (Fig. 1). In captive starlings (*Sturnus vulgaris*), glucose levels increased after a subcutaneous saline injection (Remage-Healey and Romero 2001). However, that increase was only evident 40 min after the injection, which is consistent with the fact that stress-induced hyperglycemia is evident only 15–20 min after inducing an acute stress response (Remage-Healey and Romero 2000). We hypothesize that our finding of an immediate depletion of the circulating glucose after the IP-injection is due to the immediate skeletomuscular activation in response to the handling required for performing the IP-injection, but this explanation needs to be verified (e.g. by IP-injecting labeled glucose). Alternatively, the IP-injected liquid, which was colder than body's temperature, may have triggered a hypoglycemic response, as observed in birds acutely exposed to cold conditions (Thomas and George 1975; Parker and George 1976), but given the small volume of the injection (0.1 ml, less than 1% of body mass) this seems unlikely.

Glucose peaked at 25.53 and 20.04 mM on average (experiments 5 and 6 respectively). This large difference in peak values between experiments 5 and 6 suggests that there may be a seasonal effect on the magnitude of the response observed in a GTT, which is consistent with the

reported influence of ambient temperature on initial glucose levels in this species (Montoya et al. 2018). Indeed, experiments 5 and 6 only differed in the month in which they were performed, with some overlapping weeks. Remarkably, despite the fact that the performance in the IP-GTT can be affected by seasonal variation, the within-individual repeatability calculated over two months was moderate, which supports the idea that glucose regulation is an individual trait.

In the few other avian species in which a GTT was applied (two captive raptors' species and one free living sea bird), glucose peak after a glucose load of 0.18–5 gr/kg of glucose was reported to increase relative to initial glucose levels, reaching 6–30 mM above, and to reach the highest value between 5 and 90 min after glucose administration (Chieri et al. 1972; Minick 1978; Myers and Klasing 1999). This wide range of variation illustrates the need to establish and validate species-specific GTT protocols, such as the one we present here.

Results obtained here show that an individuals' capacity to regulate glucose levels after a glucose administration is repeatable, hence it is an individual trait. This finding raises the question of what causes such individual variation, which will be of particular interest when this capacity is indeed a measure of 'condition', but this remains to be established. Measuring capacity to regulate glucose levels using an IP-GTT differs from estimating initial glucose, in that initial glucose levels provide only a snapshot at a single time-point, whereas the IP-GTT measures the individual's dynamic capacity to deal with challenges, which can potentially be considered an estimate of condition (Hill 2011). However, there is still very little information about the links between initial glucose levels, performance in a GTT and advanced glycation end products (AGEs). It remains to be established whether individual variation in the capacity to reduce experimentally increased glucose levels, and return to initial levels, reflects a general capacity to deal with physiological challenges, or whether it is specific to the regulation of glucose.

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