Path analysis reveals that corticosterone mediates gluconeogenesis from fat-derived substrates during acute stress in songbirds.

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Abstract

Glucocorticoids (e.g., corticosterone or CORT in birds) mobilize energy reserves during stress to aid survival. Stress liberates glucose (GLU) by glycogenolysis, but with glycogen depletion, gluconeogenesis of fat and protein sources predominates. Songbirds have higher metabolic rates and GLU concentrations than mammals and likely rely more on fat and protein stores during stress. We tested this hypothesis in four songbird species using path analysis to model the interrelationships between CORT and energy metabolites both at baseline and after acute stress. Individuals in better condition had higher triglyceride and CORT levels at baseline than individuals in poor body condition, and these differences became more pronounced with stress. Free CORT (the fraction unbound to circulating proteins) was associated with more GLU and free glycerol at baseline, but the former relationship was lost after acute stress. This suggests a shift from a combination of glycogenolysis and gluconeogenesis to solely the latter with acute stress. Glucose levels were also associated with uric acid indicating that birds obtain GLU during stress from gluconeogenesis of mostly fat-derived substrates. This provides a previously elusive functional link between body condition and the stress response, and suggests songbirds are more susceptible to stress challenges during energy-limiting conditions than mammals.

Introduction

An organism's response to stress (i.e., a challenge to homeostasis) requires the ability to mobilize energy. In vertebrates, a hallmark of the stress response is the secretion of glucocorticoids (e.g., corticosterone or CORT in birds) from the adrenal glands. During acute stress under normal metabolic conditions or during the early stages of fasting (phase I), glucocorticoids stimulate glycogenolysis in concert with catecholamines, glucagon, and growth hormone (reviewed in [1]) and induce lipolysis (e.g., triglyceride (TRIG) breakdown), which results in the release of free glycerol (FG) and free fatty acids (FFAs) ([2]. Free glycerol serves as a substrate for hepatic gluconeogenesis, thus facilitating hyperglycemia after glycogen depletion [2], although glucocorticoids can also promote glycogen deposition when in an absorptive metabolic state [3,4,5]. Basal (i.e., non-stress-associated) glucocorticoids also induce feeding behavior [6,7,8,9,10], but elevated levels suppress appetite [11]. The hyperphagic action of glucocorticoids often elevates plasma TRIG [12] and can counteract the depletion of fat stores due to lipolysis [1]. The state-dependent actions (basal vs. stress) of glucocorticoids are mediated by two distinct glucocorticoid receptor types [13,14,15,16].

During fasting, glucocorticoids stimulate gluconeogenesis and vertebrates rely increasingly on lipid (phase II fasting) and protein catabolism to gain energy, with the latter becoming prominent as fat stores are used up (phase III fasting) [17,18]. Increased amino acid availability due to protein catabolism promotes hepatic enzyme synthesis and glycogen production [19]. Protein catabolism also elevates plasma uric acid (URIC), the predominant nitrogenous end product of protein metabolism in birds and an important antioxidant [20,21]. Sustained and severe fasting, however, elevates the risk of exhausting GLU reserves. This risk is mitigated by promoting the conversion of FFAs to ketones such as β-hydroxybutyrate (β -OHB), that serve as an alternative energy source, particularly to sustain the activity of the nervous system [22,23,24]. Investigating simultaneous changes in plasma concentrations of multiple metabolites during acute stress enables us to infer the energy sources (GLU, lipid, or protein) that animals preferentially use during short-term challenges to homeostasis.

Birds are exceptional models for understanding metabolic changes during acute stress. Compared to size-matched mammals, birds have higher metabolic rates and elevated blood GLU and FFA levels [17], and the energetic demands of flight is fueled by an increased reliance on lipid oxidation for energy [25]. Thus birds respond to a short-term decrease in energy availability faster and with greater loses of body mass than comparably sized mammals [26]. Plasma metabolite profiles track changes in body mass and fluctuate during periods of fasting and energy mobilization [27,28]. The amount of stored energy reserves (i.e., body condition) may, therefore, predict the substrates used during an acute stress response.

Subtle shifts in complex metabolic pathways are difficult to detect using univariate analyses due to intricate and often poorly understood relationships between variables of interest. This issue can be addressed using path analysis, which tests associations between variables based on a hypothetical framework of cause and effect interactions [29,30]. This approach separates the direct and indirect associations between variables and can inform on the specific pathways involved [31,32]. Based on our current understanding of mammalian metabolic pathways, we used path analysis to model how plasma CORT levels alter energy usage, as measured through changes in plasma metabolite (GLU, TRIG, FG, β -OHB, and URIC) levels in four free-living songbird species under both normal (*hereafter* baseline) conditions and after 30 minutes of restraint stress (*hereafter* acute stress), when CORT has increased in circulation.

We tested the hypothesis that the increased energy demands of songbirds during short-term acute stress drive them to primarily mobilize fat and protein stores, thus providing a functional link between CORT and body condition. We studied free-living individuals of four species: two pairs of closely related species from the family Mimidae that are both insectivorous and frugivorous: the Northern Mockingbird (*Mimus polyglottos*) and the Curve-billed Thrasher (*Toxostoma curvirostre*), and two granivorous species, the Abert's Towhee (*Melozone aberti*) and the House Sparrow (*Passer domesticus*). Besides having different diets, these species may differ with respect to the degree to which excess energy is stored as subcutaneous adipose tissue: mockingbirds and

sparrows tend to have greater furcular fat deposits than either thrashers or towhees (HBF, *personal observations*). Birds with smaller subcutaneous fat deposits may rely primarily on lean muscle mass for energy. However, there is limited understanding of the degree to which furcular fat deposits reflect whole body fat reserves in sedentary species that have a limited capacity for subcutaneous fat storage. In migratory species or those from wintering latitudes, furcular fat scores may predict total reserves except, when fat score is close to zero [33,34]. To consider how fat storage may influence metabolite use during acute stress, we also assessed how path models may differ between species that accumulate fat deposits and those that do not. We hypothesized that birds with relatively large fat deposits rely more on fat-derived energy during acute stress than birds with small fat stores.

Based on this hypothesis several predictions can be made concerning the use of TRIG and URIC during stress, the sources of GLU production, and how body condition may be related to circulating GLU and CORT levels. First, songbirds spend a substantial portion of the morning feeding to recover from their overnight fast [35,36] and during this time they are in an absorptive state when anabolism exceeds catabolism. Thus we predict that under baseline conditions and as time of day progresses, increased feeding elevates plasma TRIG levels, which are deposited as fat stores and in turn increases body condition. By contrast, during acute stress TRIG are catabolized to FFAs which are oxidized to produce ketones (i.e., β-OHB) for energy. Furthermore, these relationships are predicted to be stronger in species with high (sparrows and mockingbirds) than low (thrashers and towhees) amounts of furcular fat. Second, baseline plasma CORT may maintain glycemia *via* glycogen stores through interactions with other hormones, but gluconeogenesis is predominant during an acute stress response. Thus we predict that baseline plasma CORT has a direct effect on GLU levels, but this effect decreases during acute stress because CORT mobilizes FG for gluconeogenesis and this effect is predicted to be larger in species with more fat deposition (sparrows, mockingbirds). A further prediction is that body condition impacts the change in plasma GLU either by increasing CORT secretion (CORT-mediated) or by increasing FG levels (fat-mediated) during

acute stress. Finally, previous studies found changes in URIC levels during acute stress [37] but whether this stems from protein catabolism for energy or depletion with oxidative stress, a byproduct of GLU production, is uncertain. We predict that if the former, URIC levels would be directly related to CORT and would be stronger in species with low amounts of fat deposition (thrashers, towhees). The latter would be demonstrated by a direct association between GLU and URIC and this association would increase in strength during acute stress.

The present study uses novel statistic approaches to investigate metabolic processes in free-living animals in a manner consistent with the complex "network" structure of metabolic processes. Understanding the interaction of body condition and acute stress is vital as environmental change alters food availability and the high energetic demands of birds make them susceptible to the energy-depleting effects of environmental stressors.

Results

Species differences in furcular fat deposits

As predicted, species differed in their furcular fat scores with towhees and thrashers having lower fat scores than sparrows and mockingbirds (Kruskal-Wallis H: = 5.7283, p = 0.001; Fig 1). Both mockingbird and thrasher data contained outlying points, however these were retained in the analysis, as there was no basis for exclusion. Furcular fat scores were not correlated to time of day nor sampling date for any species (all $p \geq$ 0.092).

Baseline and stress-induced plasma hormone and metabolite concentrations

Acute stress increased plasma total and free CORT in all species (Table 1). Acute stress decreased plasma TRIG only in the mockingbird, but plasma FG decreased in three species: towhees, thrashers, and mockingbirds (Table 1). Plasma GLU levels increased with stress in thrashers and mockingbirds, but not in the other two species (Table 1). URIC levels in plasma decreased with stress in all species (Table 1), whereas β-OHB

increased with stress only in the towhee (Table 1). Stress did not influence plasma OSMO in any species (Table 1). Sampling date had no statistical effect on plasma CORT or any metabolite in any species (Appendix 1).

Model building

Several hypothetical models were tested in each species (Table 2). Major differences between models included the presence or absence of direct relationships between: 1) TRIG and β-OHB; 2) body condition and URIC; 3) time of day and total and free CORT levels; and 4) TRIG and URIC. As these models were tested, non-significant relationships or those with high VIFs (> 10) were removed until the best models were successfully generated for each species and under both baseline and acute stress-induced conditions (Table 2). These best models were then compared using the evaluation statistics described above. Standardized path coefficients are shown for all direct relationships tested across all models and species in Appendix 1. All joint probabilities for the best-supported model family error rates were less than 0.10, suggesting less than a 10% probability of type 1 error.

Model of baseline interactions

As time of day progressed, baseline TRIG concentrations increase in towhees, sparrows, and thrashers, but not in mockingbirds (Fig 2). Plasma TRIG are positively associated with body condition in all four species and with β-OHB in the thrasher, mockingbird, and sparrow (Fig 2). Body condition is also associated with plasma total, but not free CORT in all four species (Fig 2; Appendix 1). Plasma total CORT is directly associated with FG in three species, and directly with GLU in thrashers and mockingbirds (Fig 2). Similarly free CORT is directly associated with both FG and GLU (Fig 2). The associations are stronger for free than total CORT, with respect to both FG and GLU (Fig 2). URIC levels are negatively associated with plasma GLU in every species (Fig 2).

Models of stress-induced changes

Stress-induced TRIG concentrations are positively associated with body condition in all species except towhees (Fig 3). TRIG are also positively associated with β-OHB in sparrows, thrashers, and mockingbirds but this relationship is negative for towhees (Fig 3). Body condition is again positively associated with total, but not free CORT in all four species (Fig 3; Appendix 1). Total CORT is associated with FG negatively (towhees and thrashers) or positively (sparrows and mockingbirds; Fig 3). These relationships are also observed between free CORT and FG concentrations, but the strengths of these path coefficients are greater than for total CORT (Fig 3). Only in mockingbirds do we observe a direct association between CORT (total or free) and GLU levels (Fig 3). Interestingly, significant direct negative associations between plasma FG and GLU concentrations are observed only in thrashers and mockingbirds (Fig 3). FG concentration is also positively associated with β-OHB in towhees and thrashers (Fig 3). URIC levels are again negatively associated with plasma GLU in three species (Fig 3).

Discussion

We used path analysis models to test the hypothesis that during acute stress songbirds derive energy mainly from fat and protein stores. Several models were developed and tested. The most parsimonious models were similar between the four species studied, although some variation in the relative strength and directionality of specific relationships were observed. Thus the metabolic pathways proposed here appear evolutionarily conserved among passerines and they suggest that songbirds increasingly rely on fat, but not protein, for energy during the stress response. Several observations support this conclusion: 1) direct associations between body condition and CORT, 2) a direct association between CORT and GLU present mostly at baseline, 3) switching to associations between CORT and FG during acute stress, 4) a lack of direct associations between CORT or body condition with URIC, and 5) enhanced associations between TRIG and β-OHB with stress in two of four species. Overall this study provides strong precedence for a functional link between body condition and the ability to mount a

stress response. Furthermore it provides promise for the use of path analyses to identify general patterns of energy use during complex metabolic events.

As time of day progressed, baseline plasma TRIG levels increased in three species, likely resulting from its accumulation with daily foraging [28,53,54]. Higher plasma TRIG levels indicate fat deposition, which presumably translates to a positive association between TRIG and body condition, as was observed in all species. Despite the increased associations between time of day, condition and TRIG, there was no apparent change in furcular fat score with date or time of day. Furcular fat depots are thought to assist as energy storage sites during migratory flights and for overnight survival, particularly in colder climates [60,61,62]. One interpretation is that furcular fat depots may represent storage sites for fat accumulated at an excess (i.e., well above the required amounts to maintain homeostasis), and these reserves do not change with short-term and small-scale adjustments in body condition. Thus using TRIG levels, amounts of muscle, or a morphometry-based condition index may be better predictors of small-scale metabolic changes in body condition. Interestingly, time of day did not influence baseline TRIG levels in the mockingbird, despite this species having a large furcular fat score. Furthermore, the time of day was not related to TRIG levels during acute stress for any species, suggesting that short-term changes in circulating TRIG levels induced by the stress response are derived from stored sources and not from recently consumed food. Catabolism of these TRIG can provide FG and FFAs as substrates for oxidation to gain energy [25].

All species (except towhee) showed a positive association between baseline TRIG and β-OHB levels, and furthermore all species showed this association during acute stress. Interestingly, in the towhee during stress this association was negative, unlike in other species. Ketones generated by FFA oxidation provide alternative energy substrates for muscle and nervous tissue [24,63]. Higher circulating TRIG may increase background FFA oxidation and thus β-OHB levels, and together may indicate a reliance on ketones as an energy source to supply these tissues [64,65,66]. In two species (thrashers and sparrows), stress increased the strength of the association between TRIG

and β-OHB, suggesting greater FFA oxidation as a pathway for supplying energy rapidly. However in towhees, stress generated a negative association between TRIG and β-OHB, whereas in mockingbirds the strength of the association declined with stress. These observations suggest that FFA may not be oxidized during the acute stress response in these species. Alternatively, FFAs in towhees and mockingbirds may increase with stress but are used up prior to the 30 minute sampling point, resulting in no net increase in the concentration of these metabolites being detected. Little is understood concerning the metabolism of ketones in non-mammalian species and birds may serve as fruitful models for future studies on this subject.

We attempted to differentiate between the short-term metabolic effects of plasma total and free CORT. In all species body condition was positively associated with total but not free CORT at baseline and during stress. However, free CORT was in all cases more strongly related (i.e., larger path coefficients) to FG than total CORT. In addition, in all species stress resulted in a stronger association between these variables, although the direction of the relationship differed across species. Free CORT refers to the portion of the circulating steroid capable of diffusing into cells and binding to intracellular receptors [47,67]. There remains considerable debate concerning the relative biological importance of plasma free vs. total CORT [68]. Most studies attempting to differentiate the effects of free and total CORT investigate chronic (days) rather than acute (minutes) roles of CORT [68,69,70,71]. It has been suggested that plasma CBG decreases during acute stress and this decrease could elevate plasma free CORT levels [67]. In previous studies we did not, however, observe such a decrease in any species studied here [39]. Although total and free CORT are correlated, the present observations support the free hormone hypothesis [48] and suggest that short-term changes in free CORT influence the catabolism of TRIG into FG components for gluconeogenesis. Thus, free CORT may reflect the role that this hormone plays in mediating acute metabolic changes during stress more reliably than does total CORT.

In all species at baseline free CORT levels were positively related to FG, but not necessarily to TRIG. In mammals, baseline glucocorticoids mediate fat synthesis and

glycogen formation by interacting with insulin in hepatic and adipose tissues [9,72,73]. The consistent relationship between free CORT and FG in this study may reflect a "background" level of CORT-dependent TRIG catabolism that may be necessary to fuel gluconeogenesis in order to maintain baseline blood GLU levels. During acute stress, plasma FG concentrations declined in two species (towhees and thrashers), an observation consistent with previous bird research [28,53]. Other studies, however, reported either an increase [32] or no change [12], and both negative (thrashers and towhees) and positive (mockingbirds and sparrows) associations between stress-induced CORT and FG levels were observed in this study. As the most closely related species (thrashers and mockingbirds) showed opposite relationships between CORT and FG, these results are unlikely explained by phylogeny. In contrast to Sonoran Desert-dwelling towhees and thrashers, wide-ranging and cosmopolitan mockingbirds and sparrows had larger furcular fat stores. In these species, these fat deposits may sustain FG levels during stress resulting in net increases in circulating levels. By contrast, birds with smaller fat reserves (thrashers and towhees) may show a net depletion of plasma FG during stress. This depletion may be exacerbated by the rapid uptake of glycerol by the liver and kidneys, the major avian gluconeogenic sites [74]. However, the degree to which peripheral (e.g., furcular) fat stores sources vs. hepatic TRIG stores are utilized to fuel gluconeogenesis remains unstudied in birds.

Baseline plasma CORT was directly related to GLU levels (i.e., bypassing FG) in all species. At baseline, CORT assists in the regulation of GLU levels by encouraging glycogenolysis [9,72,73]. In response to acute stress, mockingbirds, but not the other species, retained a direct relationship between CORT and GLU. The uncoupling of GLU and CORT supports the hypothesis that stress-induced increases in GLU result from gluconeogenesis with FG as a primary energetic substrate.

However, during stress only two species (mockingbirds and thrashers) demonstrated negative associations between FG and GLU with declines in the former, associated with increases in the latter. In contrast to these two related species, sparrows and towhees showed no significant associations between FG and GLU. GLU did not

increase with stress in these species, unlike in thrashers and mockingbirds. One explanation for a lack of hyperglycemia with stress may be the 30 min time frame not being long enough for gluconeogenesis to increase GLU levels. Supporting this contention, plasma GLU in towhees is elevated after one hour, but not after only 30 min of acute stress (Davies and Deviche, *unpublished data*). Alternatively, gluconeogenesis and GLU utilization may occur concurrently, resulting in no net change in circulating GLU levels.

In fasted mammals plasma GLU increases during stress as a result of numerous concurrent effects of glucocorticoids (reviewed in [75]). Data on this subject for birds are less consistent than for mammals. For example, continuous infusion and bolus injections to induce physiological blood CORT levels did not elevate plasma GLU during a 5 hour interval in the turkey (*Meleagris gallapavo*) and produced sporadic increases in plasma GLU in the chicken (*Gallus domesticus*) but only after several hours [76]. In European Starlings (*Sturnus vulgaris*), GLU also did not change with repeated acute stress despite fluctuations in plasma CORT [77], but in another study exogenous CORT induced hyperglycemia during the day but not at night [78]. By contrast, an earlier study on this species demonstrated a hyperglycemic effect of acute stress during the night (when GLU is low) but not during the day (when GLU is high) [79]. Recent food intake may also influence plasma GLU levels. In the present study, birds captured in the morning were likely foraging in an effort to replenish energy reserves used up throughout the night [28]. Birds captured later in the day (i.e., having had more time available to forage and being in an absorptive state) may have higher GLU levels than those captured earlier, but this was not observed. Time of day was associated with increased TRIG (i.e., likely indicating feeding) and thus plasma GLU levels are unlikely to reflect recent feeding activity.

Besides fat stores, birds can also use muscle proteins as substrates for gluconeogenesis [80] as well as for powering flight [81]. We measured changes in levels of URIC, which is derived from the degradation of amino and nucleic acids and can reflect protein (esp. purine) catabolism [82] at baseline and after acute stress. In free-

living migrating and captive birds, plasma URIC levels increase when body mass declines [12,83]. However, we did not observe a direct relationship between body condition and URIC in this study for any species. Furthermore, URIC decreased with stress in all species and was negatively associated only with GLU in all models, with the exception of stressed towhees. Decreased URIC levels during acute stress may result from excretion of this metabolite, which is the primary nitrogenous waste product in birds. However, in this study most birds did not appear to have excreted during the 30 min restraint period (*personal observation*), although feces may be retained in the cloaca and not visible to the researcher. Thus circulating URIC levels likely do not reflect mobilization of proteins during stress.

URIC also acts as a potent antioxidant to quench free radicals generated during gluconeogenesis [84,85,86,87] and previous studies have reported on its antioxidant properties in birds [21,37,88]. Birds have higher circulating URIC concentrations than similar-sized mammals [17,84], which may explain their decreased susceptibility to oxidative stress despite having higher GLU levels [89,90]. In a study of 57 bird species, the majority demonstrated a decline in plasma URIC after one hour of acute stress [88]. A rapid stress-associated increase in plasma GLU due to gluconeogenesis may induce oxidative stress which may deplete URIC to prevent oxidative damage to tissue. This hypothesis is speculative but the antioxidant properties of URIC are not well understood and thus deserve further experimental study.

Due largely to their increased energy demands, songbirds respond metabolically to acute stress to a greater degree than mammals. Their physiological responses to energy limitation and stress are more robust than the modest responses of mammals and avian species can, therefore, serve as excellent models to investigate the metabolic effects of stress. In addition, the ability of birds to rapidly mobilize fat resources to maintain glucose levels during stress makes them particularly susceptible to stress when their body condition is already compromised. The use of modeling to assess changes in metabolites may help assess health condition at the individual and population levels.

Materials and methods

All procedures were pre-approved by the Arizona State University Institutional Animal Care and Use Committee and were conducted with appropriate permits from the Arizona Game and Fish Department, Bureau of Land Management, United States Fish and Wildlife Service, United States Geological Survey's Bird Banding Laboratory, and the Parks and Recreation Departments for the cities of Phoenix, Tempe, Scottsdale, and Laveen. Also all private landowners first granted us permission to access their lands for sampling. No endangered or protected species were used in this study

Sampling locations

Birds were sampled in and around Phoenix, Arizona (USA) and locations varied between species, but included a mix of urban-suburban (all species), and either farmlands (sparrows) or undeveloped Sonoran Desert habitats (mockingbird, thrasher, and towhee) with all locations no more than 25 km apart. Detailed information on study sites is found in [38,39]. Previous research suggested that urban birds are often in better body condition and have greater CORT responses to stress than conspecifics from desert areas [38,39]. Individuals from all sampling locations were analyzed together to provide data over a wide range of body conditions.

Field data collection

Birds were caught using mist nets and either passively (sparrows) or with conspecific song playback recordings (other species), and within 5 hours of sunrise. Previous research demonstrated no effect of song playback on CORT levels [40]. Sample sizes were as follows: mockingbirds: $N = 25$; thrashers: $N = 61$; towhees: $N = 46$; and House sparrows: $N = 41$. Sampling occurred between March and June 2006 and between March and May 2007. These periods coincided with the breeding season of all birds ranging from incubation to chick-rearing. Previous research did not demonstrate changes in plasma CORT within these periods [39]. Nonetheless sampling date was included in model building to test for seasonal changes in metabolite levels. The time taken to

capture the bird may impact circulating CORT levels and this can potentially alter the metabolic profile. However previous research in these species did not show any relationship between capture time and plasma CORT concentrations in either thrashers or towhees [40] and this was unquantifiable in sparrows that were passively netted. Furthermore, sample size limits the number of variables that can be tested in a path model at one time, thus time of capture was not included in the model [29,30]. Upon capture a blood sample (~200 µl) was taken within 3 min from the right jugular vein using a heparinized 0.3 ml syringe and a 29.5 gauge needle. Plasma CORT did not increase significantly during the first 3 minutes following capture (*data not shown*), although such an increase was previously observed in House Sparrows [41]. These samples were defined as pre-stress (i.e., baseline) samples. Birds were then held in a cloth bag for 30 min and a second blood sample (~200 µl) was collected in the same fashion. This capture and restraint protocol is commonly used to induce an acute stress response [42,43]. Blood samples were kept on ice until centrifuged to separate formed elements from plasma, which was stored at −80 °C until assayed.

Only adult males in breeding condition were used and age, sex, and breeding status were established using plumage characteristics [44] and unilateral laparotomy after the second blood sample was collected [38,39]. Body mass ($\pm$ 0.1 g) and wing chord ($\pm$ 1 mm) were measured to generate body condition indices. To assess differences in subcutaneous fat stores between species, we scored furcular fat (scale of 1 to 5) according to [44,45,46]. Each bird received a uniquely numbered aluminum leg band and was released at the capture location.

Plasma CORT Assays

Most plasma CORT circulates bound to CORT binding globulins (CBG) [47] and it is suggested that only the unbound fraction of hormone (*hereafter* free CORT) binds to intracellular receptors in target cells [48,49]. Thus both total (i.e., bound and unbound) and free (i.e., unbound) CORT was measured. Plasma total CORT was measured using commercial competitive enzyme-linked immunoassays (ELISA; Assay Designs Inc., Ann

Arbor, Michigan, USA) as described and validated by [39]. Samples were assayed in duplicate and with baseline and stress-induced samples from the same individual assayed on the same plate. The sensitivity of the assay ranged from 5.8 - 18.1 pg/ml depending on the plate and the mean intra- and inter-assay coefficients of variation were 8.6%, and 14.7%, respectively. Radioligand binding assays were used to estimate plasma CBG binding capacity according to [50,51] with minor modifications specified in [39]. Previously described equilibrium dissociation constants for CORT binding to CBG for each species [39] were used to estimate plasma concentrations of free and CBG-bound CORT according to the equation of [52].

Plasma Metabolite Assays

Plasma FG and TRIG were measured using a sequential color endpoint assay (Sigma-Aldrich, reagents F6428 and T2449) modified for small plasma volumes (5 µl) and described in [28,53]. Plasma GLU and β-OHB were measured using colorimetric enzyme endpoint assays (Cayman Chemical Co. Ann Arbor, Michigan, USA; Cat No. 10009582 and 700190, respectively). Plasma URIC was also measured using a colorimetric assay (Biovision Research, Mountain View, California, USA; Cat No. K608-100). Previous studies have validated these assays for use in several songbird species [2,27,28,54]. Samples were assayed in duplicate and in random order, and all concentrations are expressed in the same unit (mM) to facilitate comparisons. Assay sensitivities and mean intra- and inter-assay coefficients of variation are as follows: FG: 0.05 – 6.3 mM, 6.5% and 13.0%; TRIG: 0.07 – 11.4 mM, 7.2% and 10.1%; GLU: 2.5 – 28.36 mM, 3.3% and 11.9%; β-OHB: 0.01 – 4.8 mM, 5.5% and 14.0%; and URIC: 0.01 – 6.1 mM, 3.7% and 9.8%.

To determine whether stress-induced biochemical changes alter plasma solute concentration, plasma osmolality (OSMO: mOsm/l) was measured using a vapor pressure osmometer (Model 5500XR, Wescor Inc. Logan, Utah, USA) with 10 µl samples assayed in duplicate. The osmometer was calibrated to known concentration standards (290 and 1000 mOSm/kg) before use, and intra-assay sensitivity was 3.4%.

Path analysis

Pairwise differences between baseline and stress-induced concentrations of metabolites and CORT were assessed using paired Student's *t*-tests. Linear regressions of body mass on wing chord provided standardized residuals for use as body condition indices for each species. Species differences in furcular fat scores were assessed using Kruskal-Wallis analysis of variance (ANOVA), and effects of time of day and date were assessed using Pearson correlations. When necessary, continuous data were natural logarithm (ln) transformed to satisfy normality assumptions. Path analysis enables testing of an *a priori* hypothesis of relationships generated on the basis of theory and the results of Pearson's correlation analysis between variables. The direction and strength of relationships between each variable was quantified using path (β) coefficients calculated using the maximum likelihood method [30]. The effects of factors outside the model, such as measurement errors, were represented by residual error terms (i.e., unexplained variation) in the model. Predictor variables that are highly correlated (i.e., collinear) can inflate the variance of β coefficients and decrease their accuracy from the "true" value [55]. The effects of collinearity on β coefficients is difficult to detect and can be estimated by examining variance inflation factors (VIFs) for each predictor variable. A VIF of less than 10 is generally considered acceptable [56]. Hypothetical models (or representations) were compared to observed data to determine the goodness of fit. Non-significant relationships ($P \geq 0.05$) between variables were removed from the path diagrams, except where necessary to maintain connectivity, and until the most parsimonious and best-supported model was attained.

The multiple comparisons associated with path analytical model building increase the likelihood of type 1 errors, but conventional multiple comparison corrections (e.g., Bonferroni) would be far too conservative to enable model development. Instead the joint probability, a multiple comparison significance test specifically designed for path analysis, was calculated [57]. This method provides the probability of a type 1 error being committed using all significant tests in a model (i.e., family error rate) or in specific number of comparisons using critical alpha values

generated by the path model.

Evaluation of alternative models involved several approaches. The hypothetical models were tested using a χ^2 goodness of fit test statistic, in which a smaller value indicates better consistency with observed data. The root mean square error of approximation (RMSEA), which estimates the amount by which estimated values differ from actual values, was also used for model comparison. A RMSEA $\leq$ 0.05 is usually considered to indicate a 'close fit' to the observed data [58]. To eliminate spurious relationships, the hypothetical model was tested against a saturated model in which all variables are directly connected to each other, and against an independent model (equivalent to a traditional multiple regression) in which no connection exists between variables. The Normed Fit Index (NFI) compares the hypothetical model to both the saturated and independent models. Larger NFI values are preferred and values > 0.9 are generally considered adequate [59]. The Akaike information criterion (AIC) also distinguishes between models derived from the maximum likelihood with the most parsimonious model being associated with the smallest AIC value. Together, these tests provide a rigorous assessment and comparison of different models. For brevity, only model-building results for the top three parsimonious models are presented for each species. Statistical analyses were performed using SPSS Version 13.0 (2004) with the AMOS 7.0 extension for path analysis.

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List of Figures

Table 1. Plasma concentrations of corticosterone (CORT), metabolites, and osmolality (OSMO) for four songbird species at baseline and after 30 minutes of capture and restraint stress. All values indicate mean ± SEM and asterisks indicates significant differences between baseline and stress levels (* $p \leq 0.05$, and ** Bonferroni corrected $p \leq 0.0125$).

	House Sparrow ($N = 40$)			Abert's Towhee ($N = 46$)			Curve-billed Thrasher ($N = 61$)			Northern Mockingbird ($N = 25$)		
	Baseline	Stress		Baseline	Stress		Baseline	Stress		Baseline	Stress	
Total CORT (ng/ml)	4.16 ± 1.62	36.72 ± 6.39	**	12.45 ± 1.35	37.22 ± 2.11	**	11.98 ± 2.53	57.07 ± 7.97	**	7.63 ± 1.32	37.84 ± 8.43	**
Free CORT (ng/ml)	0.06 ± 0.01	0.64 ± 0.10	**	0.19 ± 0.03	0.56 ± 0.03	**	0.25 ± 0.07	0.93 ± 0.10	**	0.26 ± 0.03	0.51 ± 0.11	*
TRIG (mM)	1.99 ± 0.10	1.69 ± 0.16		2.01 ± 0.19	1.53 ± 0.16		2.89 ± 0.65	2.30 ± 0.53		3.02 ± 0.39	1.84 ± 0.28	*
FG (mM)	0.76 ± 0.06	0.71 ± 0.11		0.86 ± 0.10	0.61 ± 0.06	*	0.81 ± 0.13	0.42 ± 0.06	**	0.97 ± 0.18	0.51 ± 0.28	*
GLU (mM)	21.39 ± 3.9	20.18 ± 2.62		19.56 ± 0.71	18.72 ± 0.87		15.33 ± 0.46	18.14 ± 0.68	*	17.61 ± 1.21	20.08 ± 1.63	*
URIC (mM)	0.37 ± 0.08	0.20 ± 0.05	*	0.69 ± 0.07	0.46 ± 0.05	*	1.03 ± 0.15	0.52 ± 0.06	**	0.39 ± 0.05	0.24 ± 0.05	*
β-OHB (mM)	0.14 ± 0.02	0.17 ± 0.03		0.11 ± 0.01	0.66 ± 0.02	**	0.12 ± 0.02	0.14 ± 0.02		0.07 ± 0.01	0.07 ± 0.02	
OSMO (mOsm/kg)	302.69 ± 7.65	299.71 ± 4.79		332.50 ± 4.60	331.06 ± 4.67		297.62 ± 4.39	299.99 ± 4.97		326.70 ± 5.02	319.22 ± 5.67	

Table 2. Model fit parameters for the three best-supported path analyses models relating time of day, body condition, corticosterone and plasma metabolite levels at baseline and after 30 min of acute stress for four songbird species. Model generally fits to observed data if: 1) $\chi 2$ is not-significant, 2) RMSEA is ≤ 0.05; 3) NFI is > 0.9; and 4) the model has a large AIC value.

Species (*No. birds, models tested*)	χ^2	**df**	***p***	**RMSEA**	**NFI**	**AIC**
House Sparrow (40, 5)						
Baseline *(accepted)*	8.46	8	0.391	0.04	0.91	74.92
2nd	9.73	8	0.275	0.04	0.80	64.01
3rd	9.88	7	0.302	0.05	0.78	51.30
Stress-induced *(accepted)*	6.11	7	0.525	0.03	0.88	62.47
2nd	7.91	6	0.472	0.04	0.85	58.38
3rd	8.03	5	0.451	0.05	0.83	60.32
Abert's Towhee (46, 4)						
Baseline *(accepted)*	7.74	7	0.531	0.04	0.89	68.37
2nd	7.32	7	0.579	0.05	0.89	68.14
3rd	8.50	7	0.310	0.06	0.83	63.96
Stress-induced *(accepted)*	11.02	5	0.107	0.05	0.85	80.28
2nd	12.75	5	0.082	0.07	0.84	78.93
3rd	10.65	6	0.134	0.06	0.92	69.23
Curve-billed Thrasher (61, 7)						
Baseline *(accepted)*	11.27	8	0.406	0.05	0.95	88.17
2nd	11.01	8	0.316	0.04	0.94	85.06
3rd	8.69	7	0.260	0.06	0.95	81.35
Stress-induced *(accepted)*	11.22	7	0.116	0.06	0.87	87.53
2nd	11.19	7	0.116	0.06	0.87	85.29
3rd	13.42	7	0.089	0.07	0.82	84.15
Northern Mockingbird (25, 5)						
Baseline *(accepted)*	12.19	7	0.247	0.06	0.97	82.13
2nd	10.04	6	0.144	0.05	0.95	86.19
3rd	9.55	6	0.101	0.04	0.90	83.16
Stress-induced *(accepted)*	10.41	7	0.238	0.06	0.98	83.11
2nd	13.06	6	0.064	0.07	0.88	62.51
3rd	15.32	5	0.028	0.08	0.73	58.39

Appendix 1. Relationships tested using path analysis between time of day, date, and plasma levels of metabolites and corticosterone (CORT) in four songbird species at baseline and after 30 mins of capture and restraint stress. Shown are standardized path coefficients (β) and *P*-values. Significant relationships ($p \leq 0.05$) are shown in **bold**.

			Baseline		Stress-induced	
Variable		**Path**	**β**	***p***	**β**	***p***
Curve-billed Thrasher						
Date	→	TRIG	0.11	0.484	0.03	0.606
	→	Total CORT	0.00	0.874	0.05	0.281
	→	Free CORT	-0.08	0.511	-0.21	0.390
	→	Body Condition	0.04	0.166	0.15	0.067
Time of Day	→	TRIG	**0.46**	**0.008**	0.08	0.243
	→	Body Condition	0.00	0.933	0.18	0.409
	→	Total CORT	0.00	0.979	0.00	0.808
	→	Free CORT	0.00	0.899	-0.01	0.672
TRIG	→	Body Condition	**0.55**	**0.006**	**0.30**	**0.016**
	→	Total CORT	0.00	0.997	0.13	0.453
	→	Free CORT	0.00	0.999	0.04	0.145
	→	FG	**0.70**	**0.001**	**0.52**	**0.001**
	→	β-OHB	**0.29**	**0.038**	**0.46**	**0.001**
	→	GLU	0.00	0.999	0.00	0.843
Body Condition	→	Total CORT	**0.72**	**0.001**	**0.57**	**0.001**
	→	Free CORT	0.14	0.096	0.01	0.730
	→	β-OHB	0.05	0.583	0.11	0.484
	→	FG	-0.03	0.327	0.00	0.779
	→	GLU	-0.08	0.228	0.04	0.286
Total CORT	→	Free CORT	**0.90**	**0.001**	**0.69**	**0.001**
	→	β-OHB	0.13	0.095	0.00	0.777
	→	FG	**0.65**	**0.004**	**-0.67**	**0.001**
	→	GLU	**0.41**	**0.008**	0.04	0.272
	→	URIC	0.11	0.073	-0.04	0.184
Free CORT	→	β-OHB	0.01	0.688	0.00	0.178
	→	FG	**0.87**	**0.001**	**-0.58**	**0.008**
	→	GLU	**0.52**	**0.007**	0.03	0.106
	→	URIC	0.00	0.810	-0.04	0.185

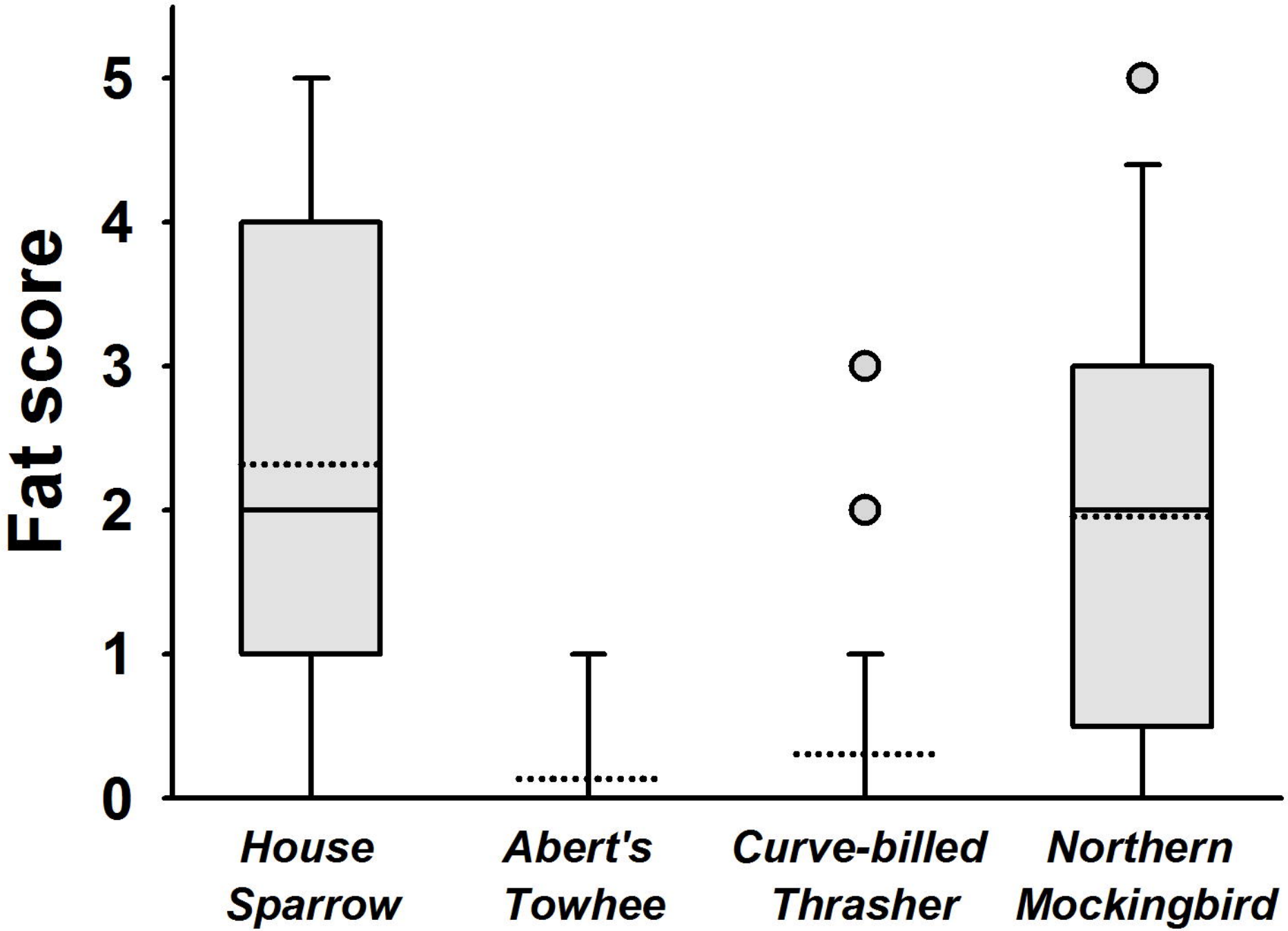
FG	→	β-OHB	-0.05	0.134	**0.25**	**0.032**
	→	GLU	**-0.21**	**0.027**	**-0.73**	**0.001**
	→	URIC	-0.04	0.177	0.05	0.581
GLU	→	URIC	**-0.32**	**0.019**	**-0.66**	**0.001**

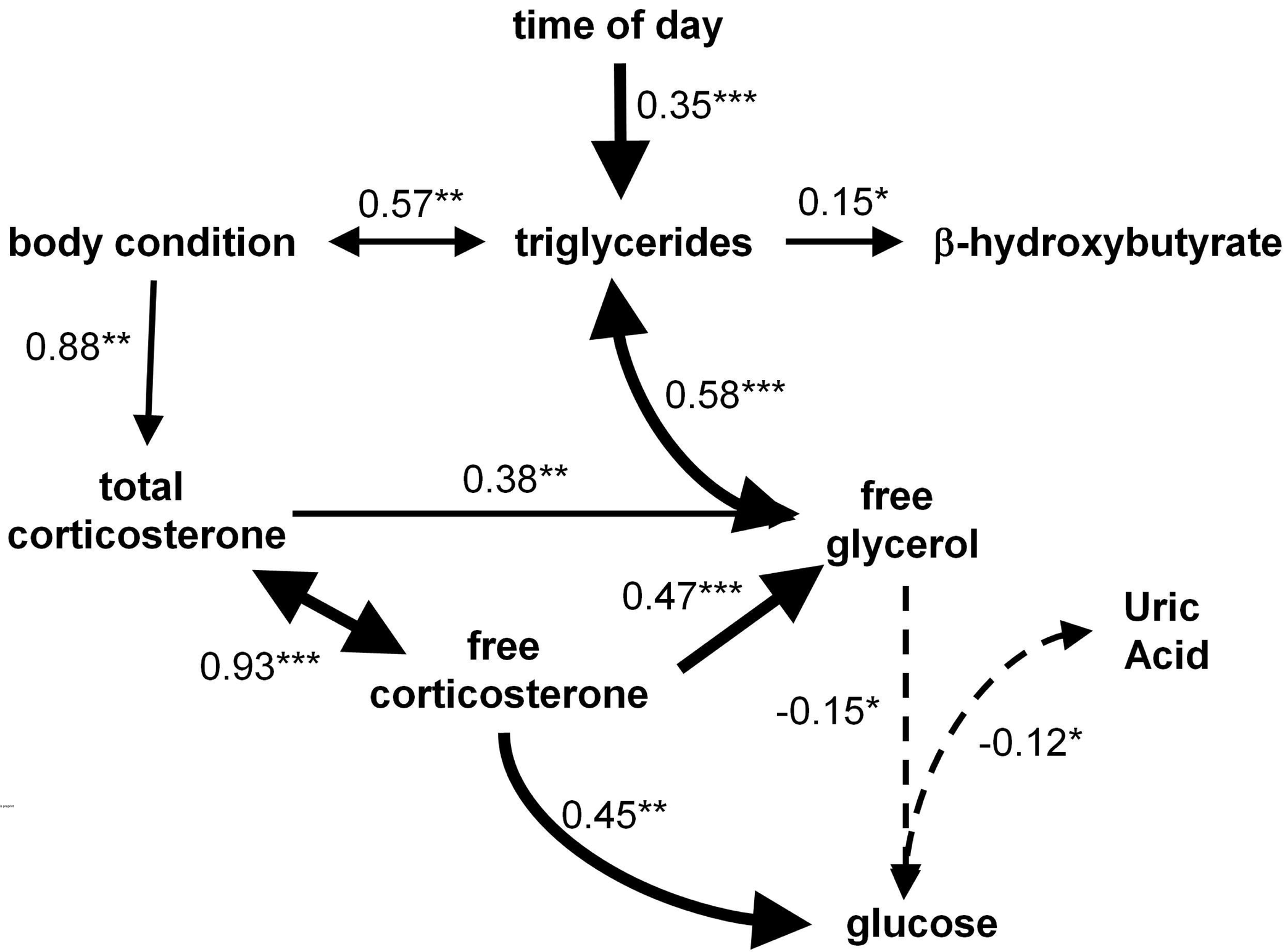
Northern Mockingbird

Date	→	TRIG	0.08	0.280	0.11	0.095
	→	Total CORT	0.06	0.561	0.02	0.294
	→	Free CORT	-0.10	0.345	-0.11	0.287
	→	Body Condition	0.11	0.214	0.02	0.638
Time of Day	→	TRIG	0.13	0.450	0.08	0.175
	→	Body Condition	0.02	0.644	0.03	0.189
	→	Total CORT	0.05	0.577	-0.18	0.408
	→	Free CORT	0.02	0.600	0.09	0.470
TRIG	→	Body Condition	**0.32**	**0.007**	**0.22**	**0.037**
	→	Total CORT	0.01	0.707	0.02	0.636
	→	Free CORT	0.00	0.973	0.09	0.121
	→	FG	**0.61**	**0.001**	**0.70**	**0.001**
	→	β-OHB	**0.53**	**0.001**	**0.29**	**0.031**
	→	GLU	0.11	0.484	0.00	0.779
Body Condition	→	Total CORT	**0.19**	**0.046**	**0.32**	**0.006**
	→	Free CORT	0.06	0.169	0.07	0.226
	→	β-OHB	0.08	0.286	0.00	0.869
	→	FG	0.00	0.808	-0.10	0.507
	→	GLU	0.01	0.682	0.00	0.793
Total CORT	→	Free CORT	**0.74**	**0.001**	**0.73**	**0.001**
	→	β-OHB	0.00	0.926	-0.09	0.281
	→	FG	0.12	0.084	-0.19	0.383
	→	GLU	**0.24**	**0.039**	**0.41**	**0.001**
	→	URIC	0.09	0.243	0.00	0.866
Free CORT	→	β-OHB	0.18	0.409	0.18	0.410
	→	FG	**0.42**	**0.008**	**0.67**	**0.001**
	→	GLU	**0.56**	**0.001**	**0.47**	**0.006**
	→	URIC	-0.13	0.453	-0.18	0.406
FG	→	β-OHB	-0.06	0.170	0.11	0.071
	→	GLU	**-0.16**	**0.046**	**-0.33**	**0.004**
	→	URIC	0.03	0.629	0.03	0.606
GLU	→	URIC	**-0.27**	**0.003**	**-0.17**	**0.043**

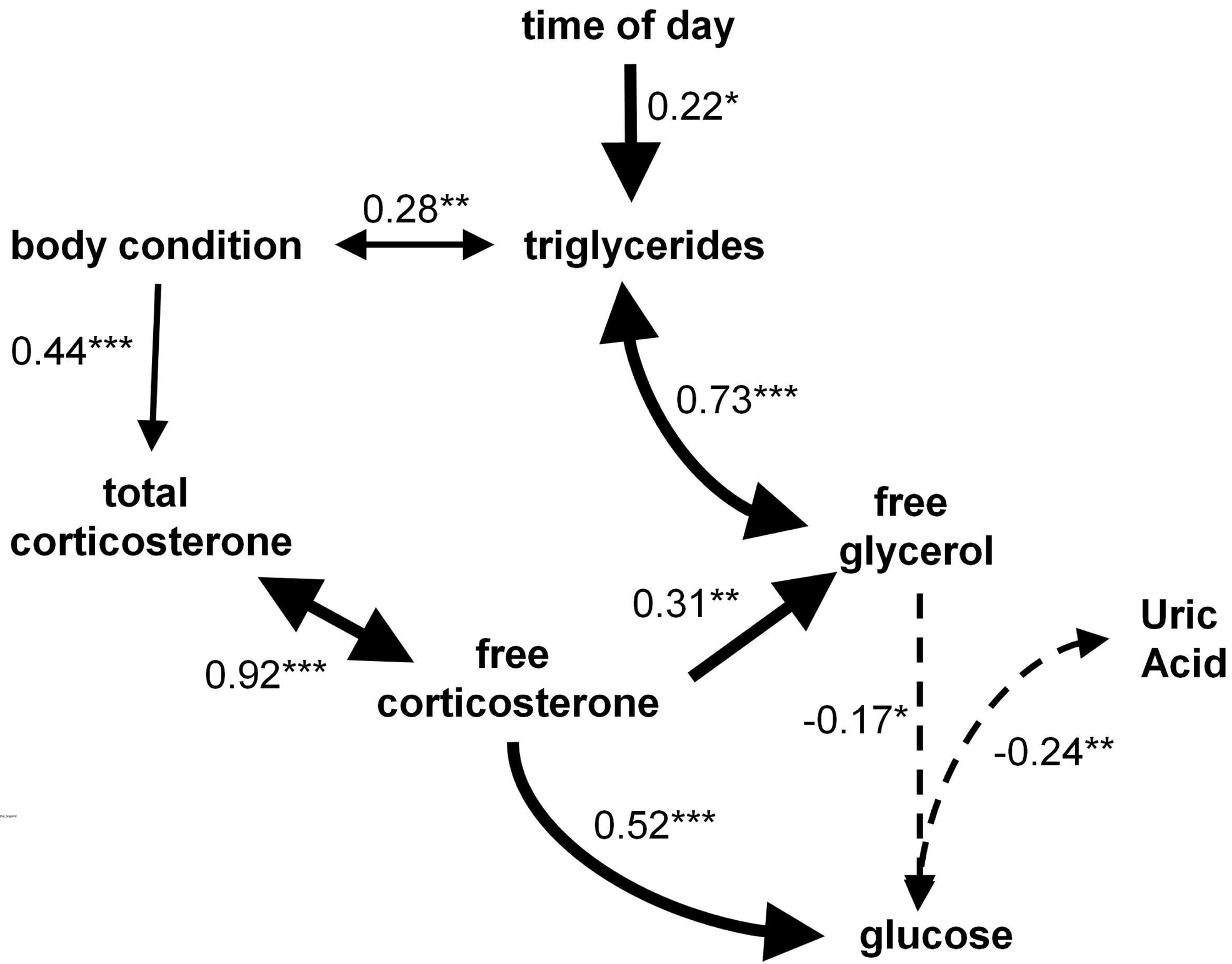
House Sparrow						
Date	→	TRIG	0.03	0.189	0.12	0.066
	→	Total CORT	0.08	0.210	0.11	0.097
	→	Free CORT	0.00	0.765	-0.04	0.144
	→	Body Condition	0.11	0.477	0.11	0.211
Time of Day	→	TRIG	**0.35**	**0.001**	0.02	0.309
	→	Body Condition	-0.09	0.292	0.15	0.448
	→	Total CORT	0.07	0.175	0.02	0.640
	→	Free CORT	0.11	0.102	0.00	0.887
TRIG	→	Body Condition	**0.57**	**0.003**	**0.38**	**0.029**
	→	Total CORT	0.04	0.159	0.01	0.752
	→	Free CORT	0.14	0.069	0.01	0.678
	→	FG	**0.58**	**0.001**	**0.91**	**0.001**
	→	β-OHB	**0.15**	**0.047**	**0.55**	**0.002**
	→	GLU	0.00	0.804	0.02	0.323
Body Condition	→	Total CORT	**0.88**	**0.002**	**0.62**	**0.001**
	→	Free CORT	0.03	0.182	0.08	0.229
	→	β-OHB	-0.01	0.679	0.07	0.193
	→	FG	-0.12	0.463	-0.20	0.373
	→	GLU	0.00	0.825	0.18	0.410
Total CORT	→	Free CORT	**0.93**	**0.001**	**0.70**	**0.001**
	→	β-OHB	-0.03	0.181	0.03	0.607
	→	FG	**0.38**	**0.008**	**0.65**	**0.002**
	→	GLU	0.01	0.703	0.05	0.151
	→	URIC	0.00	0.979	0.07	0.236
Free CORT	→	β-OHB	0.04	0.161	0.01	0.754
	→	FG	**0.47**	**0.001**	**0.92**	**0.001**
	→	GLU	**0.45**	**0.004**	0.00	0.835
	→	URIC	0.05	0.588	0.00	0.870
FG	→	β-OHB	-0.20	0.368	0.00	0.975
	→	GLU	**-0.15**	**0.044**	-0.08	0.280
	→	URIC	-0.03	0.626	0.06	0.561
GLU	→	URIC	**-0.12**	**0.050**	**-0.19**	**0.031**
Abert's Towhee						
Date	→	TRIG	0.15	0.362	0.02	0.624
	→	Total CORT	0.06	0.562	0.06	0.585

	→	Free CORT	0.00	0.786	0.15	0.137
	→	Body Condition	0.02	0.667	-0.04	0.439
Time of Day	→	TRIG	**0.22**	**0.007**	0.02	0.326
	→	Body Condition	0.00	0.911	0.00	0.931
	→	Total CORT	0.06	0.562	0.02	0.341
	→	Free CORT	0.20	0.374	0.05	0.451
TRIG	→	Body Condition	**0.28**	**0.004**	0.06	0.547
	→	Total CORT	0.11	0.483	0.01	0.663
	→	Free CORT	0.01	0.729	0.01	0.611
	→	FG	**0.73**	**0.001**	**0.95**	**0.001**
	→	β-OHB	0.02	0.667	**0.91**	**0.001**
	→	GLU	0.03	0.624	0.07	0.236
Body Condition	→	Total CORT	**0.44**	**0.001**	**0.48**	**0.007**
	→	Free CORT	0.00	0.770	0.00	0.844
	→	β-OHB	0.08	0.259	0.07	0.482
	→	FG	0.04	0.198	0.03	0.315
	→	GLU	0.05	0.151	0.05	0.476
Total CORT	→	Free CORT	**0.92**	**0.001**	**0.94**	**0.001**
	→	β-OHB	-0.08	0.202	-0.04	0.416
	→	FG	0.10	0.513	**-0.67**	**0.001**
	→	GLU	0.00	0.891	0.09	0.104
	→	URIC	0.10	0.509	0.10	0.124
Free CORT	→	β-OHB	0.02	0.639	0.00	0.917
	→	FG	**0.31**	**0.002**	**-0.81**	**0.001**
	→	GLU	**0.52**	**0.001**	0.00	0.797
	→	URIC	0.07	0.357	0.00	0.868
FG	→	β-OHB	0.09	0.273	**0.25**	**0.037**
	→	GLU	**-0.17**	**0.037**	0.01	0.388
	→	URIC	0.08	0.268	0.06	0.527
GLU	→	URIC	**-0.24**	**0.006**	-0.06	0.312

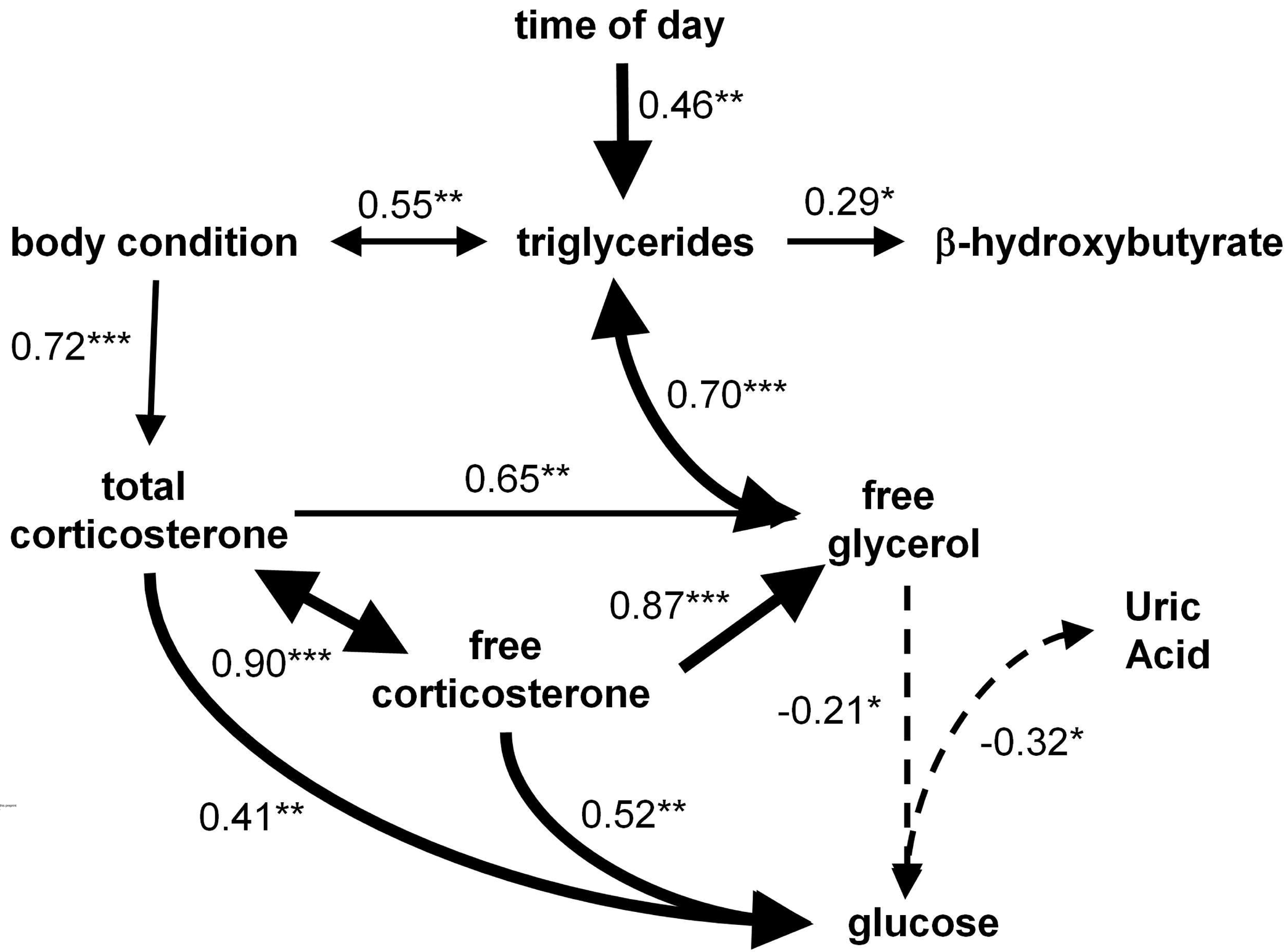

Fat score
5
4
3
2
1
0
House Sparrow
Abert's Towhee
Curve-billed Thrasher
Northern Mockingbird



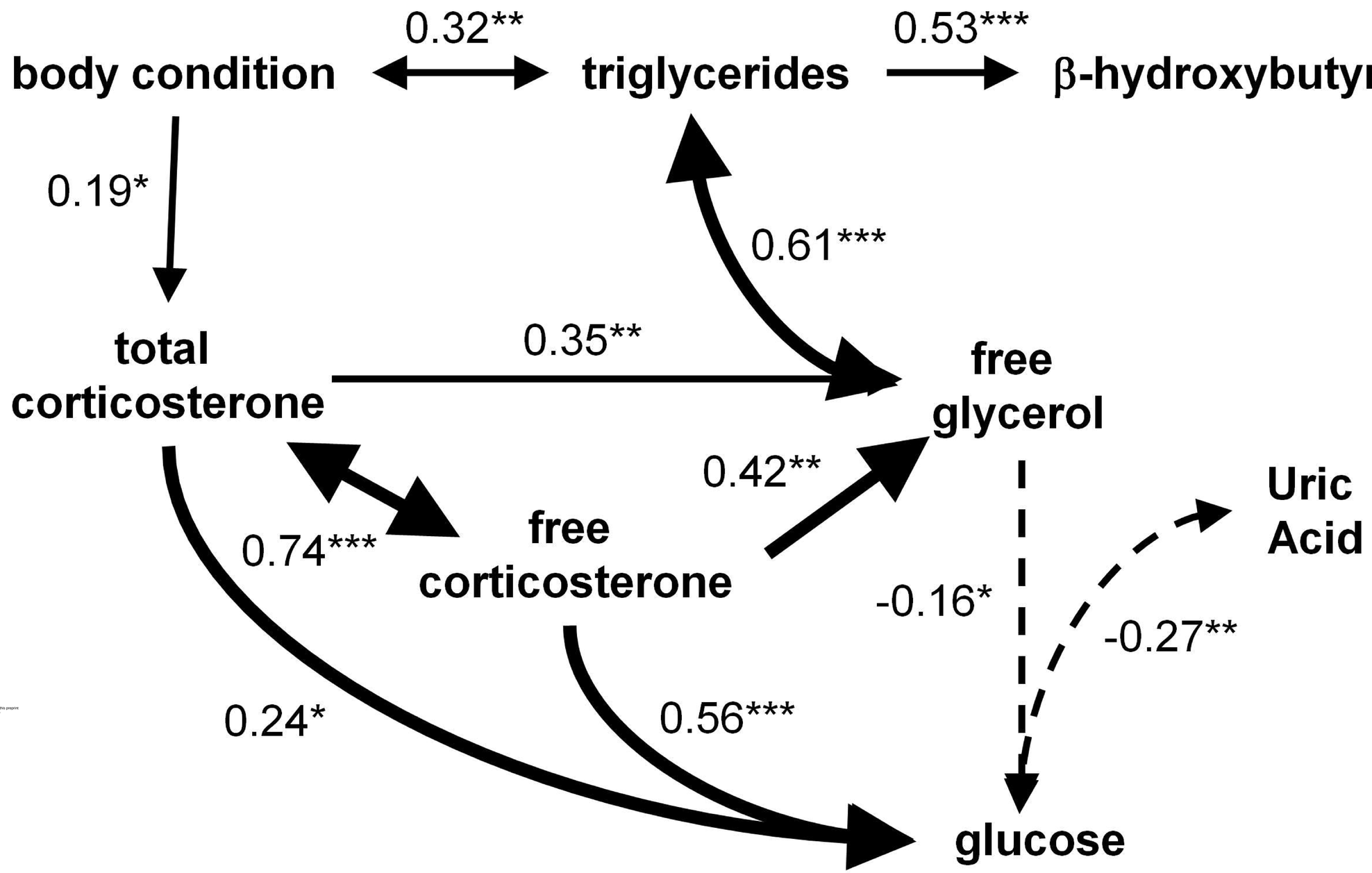
House Sparrow (Baseline: ≤ 3 min)



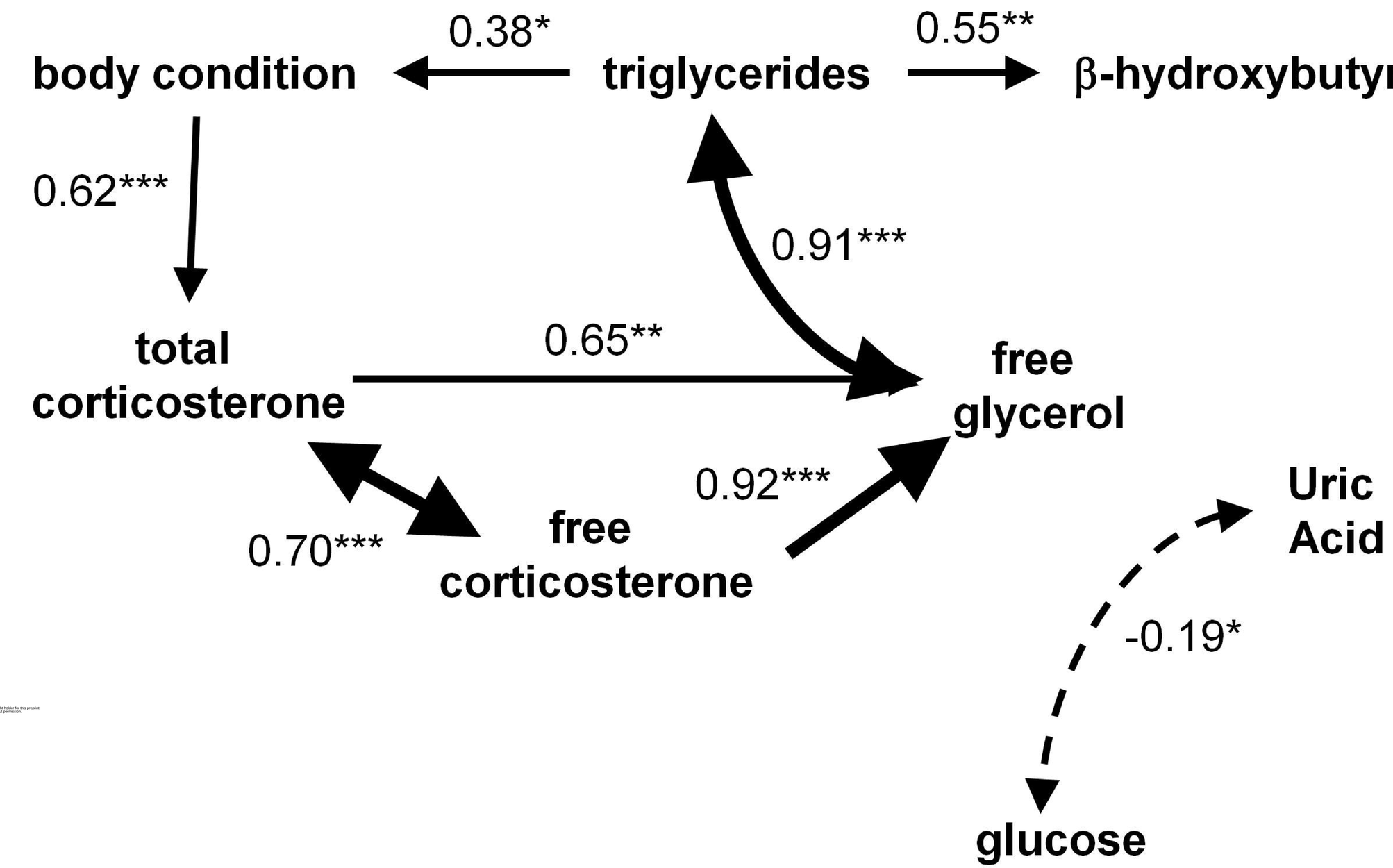
Abert's Towhee (Baseline: ≤ 3 min)



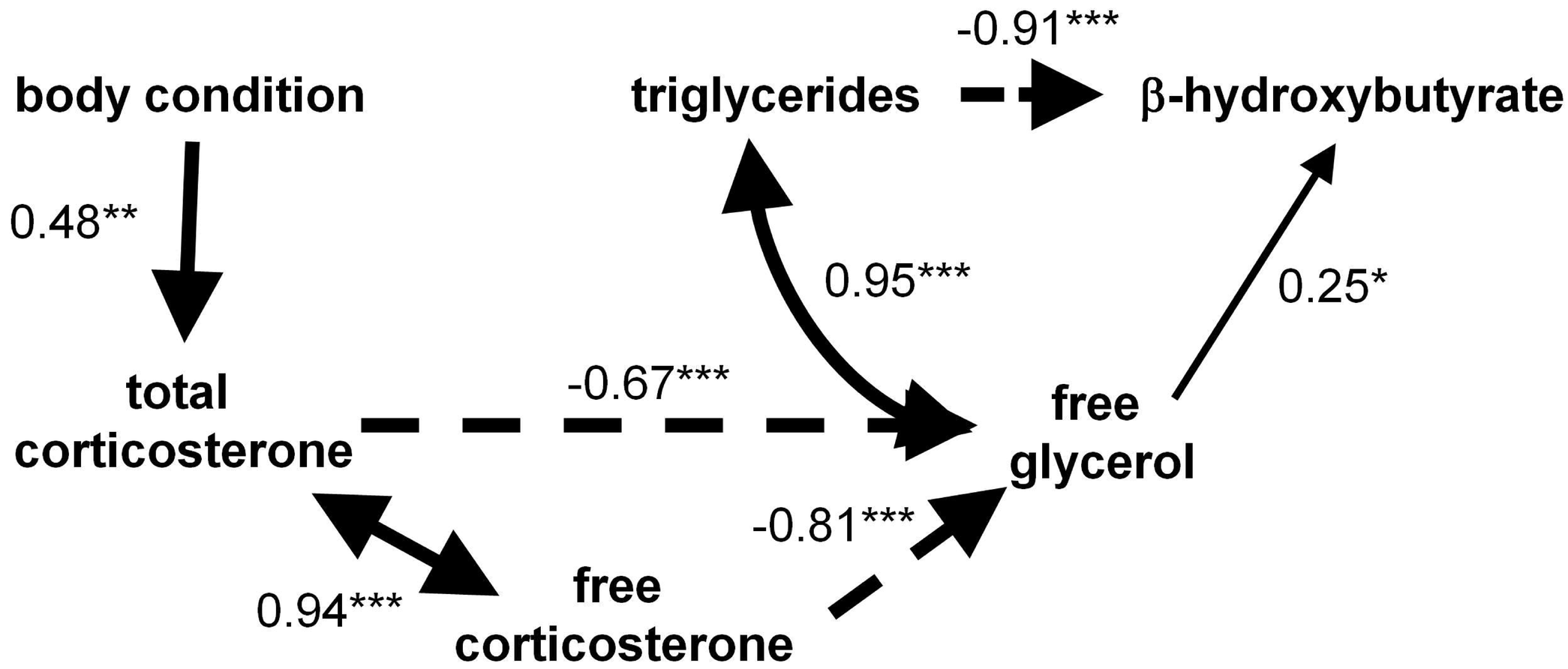
Curve-billed Thrasher (Baseline: ≤ 3 min)



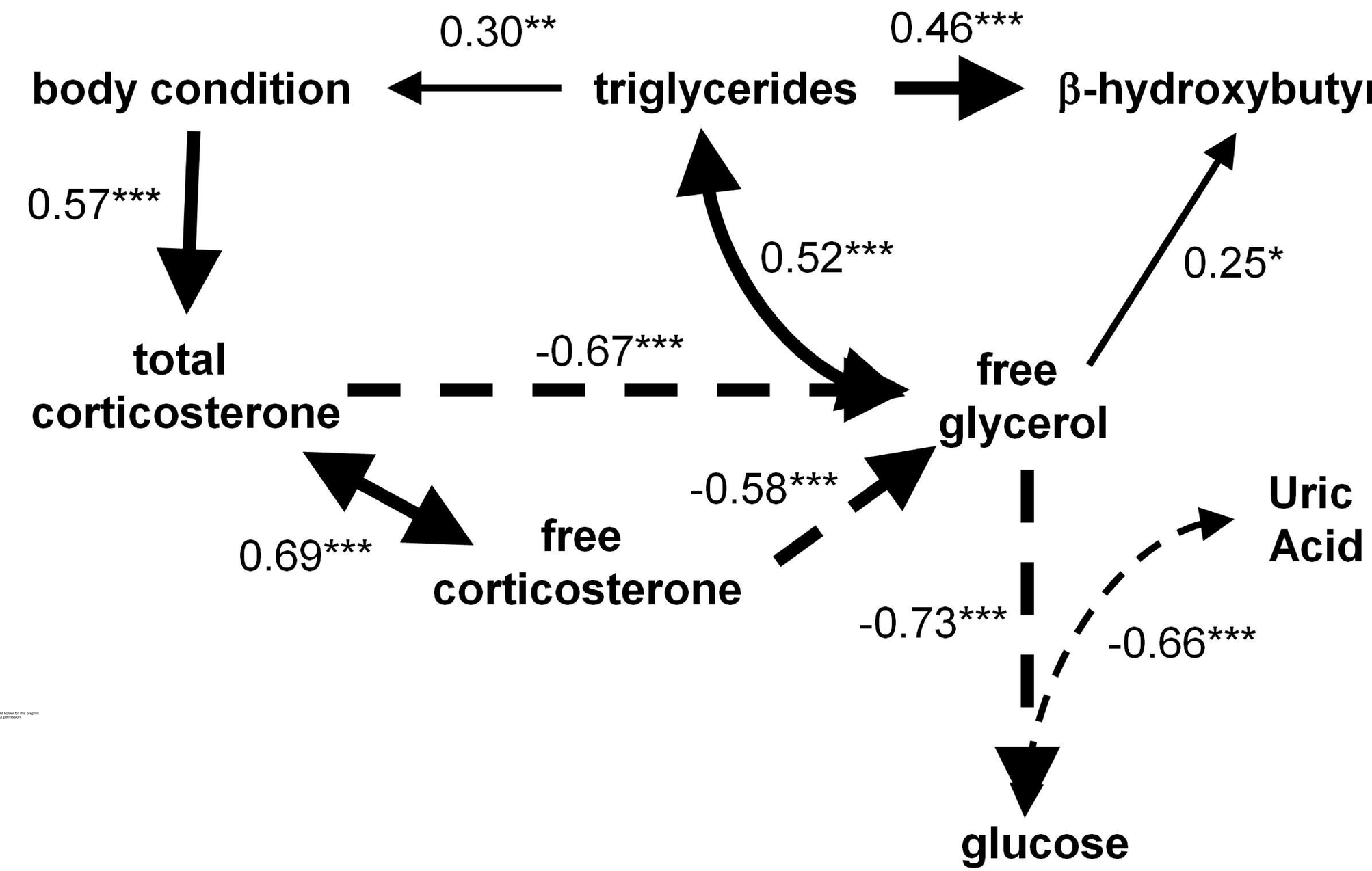
Northern Mockingbird (Baseline: ≤ 3 min)



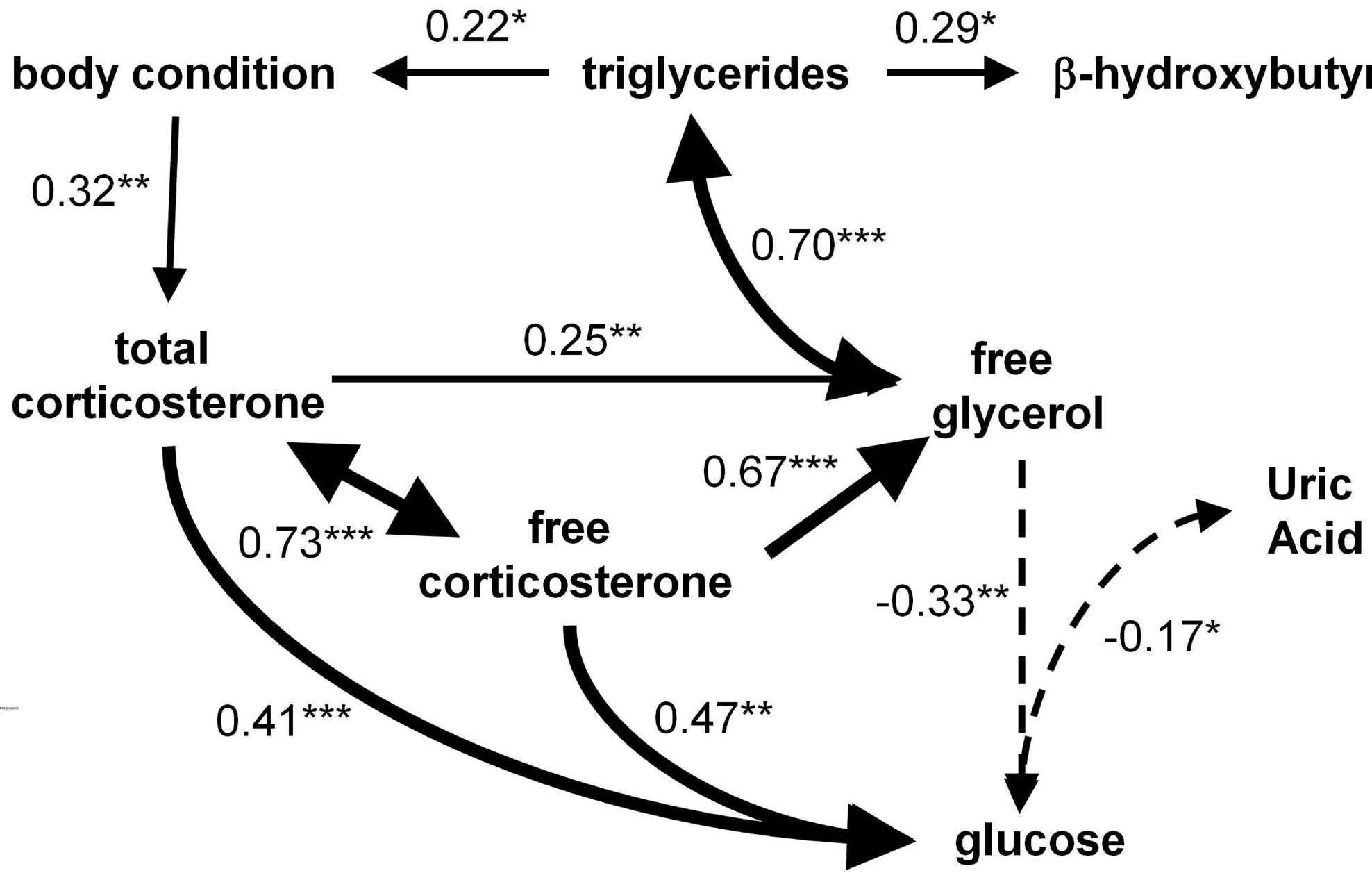
House Sparrow (Stress-induced: 30 min)



Abert's Towhee (Stress-induced: 30 min)



Curve-billed Thrasher (Stress-induced: 30 min)



Northern Mockingbird (Stress-induced: 30 min)