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RESEARCH ARTICLE

Glucose Concentrations in Closely Related Titmice (*Baeolophus*) Species Linked to Regional Habitat Differences Across an Avian Hybrid Zone

Jennifer C. Vaughn[1,*], Gary Voelker[1] and J. Jill Heatley[2]

[1]*Department of Wildlife and Fisheries Sciences and Texas A&M Biodiversity and Research Collections, Texas A&M University, College Station, TX, USA*

[2]*Department of Small Animal Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, College Station, TX, USA*

Abstract:

Aims:

We used physiological data, in conjunction with habitat information, to elucidate the interactions between two hybridizing songbirds within a hybrid zone.

Background:

Hybrid zones are ideal regions to examine a variety of ecological, behavior, and evolutionary processes. In addition to genetics, behavior, and morphology, physiological differences may impact hybrid fitness, genetic introgression, and even the stability of a hybrid zone.

Objective:

To assess physiological differences in hybridizing species, we investigated selected venous blood analytes in two species of songbirds hybridizing along the Balcones Escarpment in central Texas.

Methods:

Using a portable blood analyzer, we assayed blood samples from Black-crested Titmouse (*Baeolophus atricristatus*) and Tufted Titmouse (*B. bicolor*) individuals along a longitudinal transect that included the contact zone. Ecologically, this transect varies from higher elevation semi-arid regions on the Balcones Escarpment (and west across the Edwards Plateau) to lower elevation mesic forests east of the escarpment.

Results:

As expected, several blood analytes differed with age, sex, and sedative administration; however, we observed relatively increased blood glucose concentrations in Black-crested Titmice, which occupy the semi-arid habitats of west Texas. Furthermore, glucose concentrations were further elevated following rainfall events. Blood glucose concentrations often increase during stressful conditions and or related to changes in diet.

Conclusion:

We suspect that Black-crested Titmice have relatively increased blood glucose concentrations as a product of living in a semi-arid environment that causes chronic stress from unpredictable food and water resources. The link between rainfall and glucose may be a result of the increased and greater diversity of food availability after rainfall. Although further research is needed, we suspect that habitat differences and associated lack of physiological adaptations may be a limiting factor in westward range expansion in the more aggressive Tufted Titmice.

Keywords: Blood gas, Passeriformes, Speciation, Habitat, Geography, Electrolytes, Biochemistry.

1. INTRODUCTION

Geographic regions in which species interbreed or hybridize, commonly known as hybrid zones, are unique locations to examine speciation and adaptation [1 - 3]. Hybrid

* Address correspondence to this author at the Department of Wildlife and Fisheries Sciences and Texas A&M Biodiversity and Research Collections, Texas A&M University, College Station, TX, USA; Tel: +1 214-908-3808; E-mail: jcary@tamu.edu

zones, in animals, often form *via* secondary contact of lineages that initially diverged in allopatry [4]. Beyond genetics, morphology and behavior are classically often used to assess hybridization, given their importance in species recognition and mate selection [5 - 8]. However, during isolation, sister lineages may also develop physiological adaptations to different environmental conditions [9, 10]. Post-isolation, populations and species expansion may be limited or affected

by new adaptations. As secondary contact zones usually occur at sharp ecological boundaries (ecotones), the impact of physiology on distribution may be a critical component to understanding species or populations interactions, especially hybridization of species [2, 11].

Physiology is known to play a role in limiting geographic range, but some hybrid zone studies have supported physiological differences as limiting factors to distribution [12]. Once range expansion or distribution is limited, genetic introgression, selection, and other evolutionary forces are impacted. For example, spatial distribution in two hybridizing swordtail fish was linked to differences in heat-tolerant gene expression in varying water temperatures [13]. In birds, hybrid tit-tyrant flycatchers in the Andes mountains are not found at the higher elevations preferred by one parental species because they are not physiologically adapted to high elevation living [14]. Thus, scientists must consider physiological adaptations in hybridization research.

In this study, we investigated physiological differences between two species of hybridizing songbirds in central Texas. Based on similar studies and new evidence, we suspect that differences are linked to regional habitats and/or climatic distinctions on either side of the hybrid zone.

1.1. Texas Hybrid Zone

In Texas, the Balcones Escarpment (BE) is a southwest-to-northcentral oriented inactive fault line that creates a strong west to east ecological boundary [15, 16]. West of the BE, the Edwards Plateau ranges in elevation from 180 m to nearly 900 m with a semi-arid climate dominated by juniper-oak vegetation, receiving an average of 380-860 mm of rainfall per year, with the majority occurring in May or June [17]. East of the BE, lower elevation (0-180 m) and a more temperate climate characterize the more mesic habitats of the Blackland Prairie and Post-Oak forests nearer the BE, and the Piney Woods of the interior coastal plains in eastern-most Texas [17, 18]. Rainfall in these ecoregions is fairly evenly distributed annually with an average of 700-1000 mm in the Post-Oak and Blackland Prairie regions and 900-1300 mm in the Piney Woods [17, 18].

With this change in elevation, vegetation and rainfall regimes on and east of the BE coincide with a longitudinal distributional barrier for numerous vertebrate taxa, including birds [19 - 21]. In addition to distributional range limits occurring at the escarpment, some species-pairs are thought to hybridize at this ecological interface [22, 23]. Avian species-pair recently confirmed to be hybridizing at this interface are the Black-crested Titmouse (*Baeolophus atricristatus*) and Tufted Titmouse (*B. bicolor*) [23]. Black-crested Titmice are primarily found in the more arid-scrubby habitat to the west and south (to include northern Mexico) of the BE ecotone but are occasionally found east of the BE [24, 25]. Tufted Titmice are found in the more mesic, deciduous forests east of the BE and throughout the eastern United States and Canada [25, 26].

While these non-migrating sister species share similar diet, breeding/nesting behavior, and general patterns of intraspecific communication, they differ in plumage, song, morphology and habitat preference [24, 25]. Currently ranked as separate species, their taxonomic status has fluctuated between species and subspecies because ornithologists have postulated hybridization [24, 25]. Using morphological measurements and crest plumage, Dixon identified several hybrid zones, with the primary one located in central Texas [26]. He estimated this zone to be approximately 50 km wide and 175 km long (Fig. **1**) [23, 26]. Curry and Patten recently investigated plumage and morphological variation within this central hybrid zone and their findings support Dixon's assessments of hybridization [27, 28]. In a related study, Vaughn and Voelker (in progress) provide genetic evidence of hybridization in birds captured near the BE.

Since this hybrid zone occurs at a strong ecotone, we aim to investigate which blood gases and electrolytes differ between Tufted and Black-crested Titmice and assess if the differences are linked to climate and/or habitat. In a previous study of a suite of passerines, including titmice, we observed differences in venous blood analytes between individuals sampled east of the BE, relative to those sampled west of the BE [29, 30]. We hypothesize that, between species, differences in blood analyte concentrations will be strongly correlated with habitat or climatic differences associated with the different ecoregions east and west of the BE. We also aim to report the role that other non-habitat variables (age, sex, and methodology) have on blood gas and electrolytes within two closely related species. The aim is to expand the knowledge of the variability of blood gas and electrolytes in wild passerines as well as provide a better understanding of ecological impacts on physiological differences between closely related, hybridizing species.

2. MATERIALS AND METHODS

2.1. Capture and Sampling

From March-August in 2010-2012, we captured 54 titmice (*Baeolophus spp*.) on private properties in central Texas (Fig. **1**). Most birds were collected *via* mist nests from 0630-1200 CST; two were caught between 1700-2000 CST. We collected 26 titmice west of the Balcones Escarpment; two of these were captured west of the contact zone (Table **1**) [26, 27]. East of the BE, we collected 28 titmice. In a related project, we determined that plumage is not a reliable indicator of hybridization. Therefore, for this study, we used mitochondrial DNA for species assignment for all individuals in this study.

After removal from the mist net, titmice were held in cloth bags for 5-15 min before blood collection. As part of a larger study, Titmice captured in 2012 (*n* = 26) were administered 0.01-0.03 mL midazolam (3.9 +/-1.8 mg kg^{-1}; 5mg ml^{-1} Injection USP Hospira, Inc.) intra-nasally prior to blood collection for sedation [29]. Midazolam is a safe and effective sedative of wild birds [31 - 33]. Clinical effects of sedation were observed and recorded on a sedation scale [29]. Following rest or sedation plus rest, we obtained blood samples (200-500 µL) *via* jugular venipuncture and immediately transferred to lithium heparin Microtainer tubes (Sarstedt Inc., USA) for anticoagulation prior to analysis.
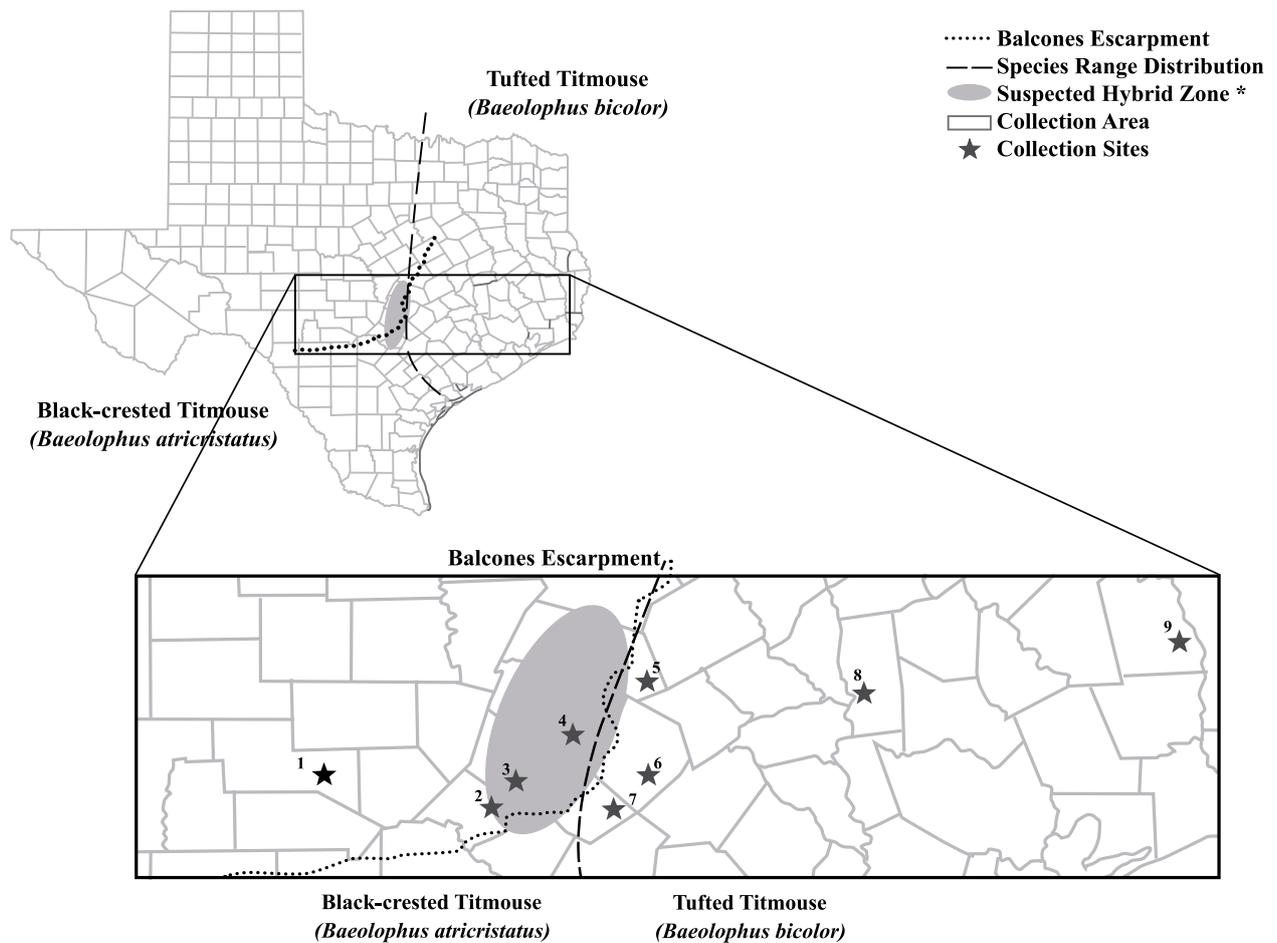
Fig. (1). Map of locations for sampling (square) of Tufted and Black-crested Titmice. Counties west of the Balcones Escarpment (dashed line) include Kerr (1), Comal (2), Hays (3), and Travis (4). Counties east of the Escarpment are Williamson (5), Bastrop (6), Caldwell (7), Grimes (8), and Tyler (9). The extent of Titmouse species' distribution range in Texas (dotted line) and central contact zone [26] (shaded oval) are also shown. Map modified from EnchantedLearning.com (2018).

Table 1. Titmice (*Baeolophus* spp.) based on species (mtDNA clade), age, sex, and capture location. Balcones Escarpment consists of "West" and "Contact zone."

-	West	Contact Zone	East	Total
Juvenile	0	11	11	**22**
Adults	2	13	17	**32**
Adult Male	1	10	16	**27**
Adult Female	1	3	1	**5**
Black-crested Titmouse	2	24	0	**26**
Tufted Titmouse	0	0	28	**28**

Samples were analyzed in the field using an i-STAT 1 system (Abbott Laboratories, USA), following standard field protocols [30]. Cartridges that evaluated blood gases were used first to minimize the change in analytes based on time-lapse from sampling [29]. Remaining cartridges were analyzed within 5 min of blood collection. Venous blood analytes measured by the i-STAT 1 system were pH, carbon dioxide partial pressure (pCO_2), oxygen partial pressure (pO_2), lactate,

ionized calcium (iCa^+), glucose, sodium (Na^+), potassium (K^+), chloride (Cl^-), blood urea nitrogen (BUN), and hematocrit (Hct). The i-STAT 1 system also determined, *via* calculations, bicarbonate (HCO_3), total carbon dioxide (tCO_2), base excess (BEecf), dissolved venous oxygen (sO_2), and hemoglobin (Hb) [34, 35]. Packed red blood cell volume (PCV) was also determined *via* standard centrifugal methods [30].

Following sampling, we humanely euthanized and

deposited birds as museum specimens at the Biodiversity Research and Teaching Collections (BRTC) at Texas A&M University (College Station, TX). Morphological and genetic information from these birds will be used in future studies. This research was conducted under required Texas Parks and Wildlife (permit# SPR-0209-016) and United States Fish and Wildlife Permits (permit #188 MB205752) and with approval of the Texas A&M University Institutional Animal Care and Use Committee (AUP# 2012-6).

2.2. Statistical Analysis

All venous blood analytes (n = 16) were assessed for normality using a Shapiro-Wilk Goodness of Fit Test (α = 0.05). We transformed analytes, which were not normally distributed and tested for, and removed, any outliers. Thus, for all subsequent analyses, we used the log of lactate and tCO_2, the reciprocal of Hct, and the reciprocal of the exponent of pH. As obtaining the reciprocal square root failed to normalize BUN data, we categorized this analyte as less than 3 mg/dL or greater or equal to 3 mg/dL or greater. We performed non-parametric tests for further statistical analysis of this analyte.

We further explored possible confounding factors. We tested all analytes against age (juvenile, adult), sex (male, female), and administration of midazolam (yes, no) as well as differences between populations based upon capture locality (west, contact zone, east) and location relative to BE (east, west) (Fig. **1**, Table **1**). For each analyte and factor, heteroscedasticity was tested using a 2-sided, 2 factor F Test and the 3 factor Bartlett test. If variances were unequal, as in Hct, we utilized Welch's test to assess significant differences between means, and we weighted models to account for heteroscedasticity. For variables with equal variance, we used a one-way analysis of variance (ANOVA) tests and paired T-tests to compare population differences and the effect of sample size (α = 0.05).

We further tested related or similar variables using correlation analysis and Bland Altman plots to assess interactions between multiple variables for those analytes showing significance. The i-STAT 1 system calculates sO_2 from measurements of pO_2, pH, and HCO_3. The matched-paired analysis confirmed a correlation of sO_2 and pO_2, as well as sO_2 with pH and HCO_3 [35]. Therefore, we opted not to analyze sO_2 in future analyses and focus on the measured value of pO_2. The i-STAT 1 system calculates hemoglobin by using the measured hematocrit and multiplying by a factor of 0.34, creating a manufactured value [34]. We confirmed the correlation between Hct and Hgb (0.925, $p <0.001$) and then focused on Hct differences across variables. Hematocrit (Hct) from the i-STAT 1 system and PCV determined *via* centrifuge were compared *via* the Tukey Mean Difference plot. We incorporated rainfall data and geographic location in two-way ANOVAs or linear regressions to determine if there was a biological reason for those analytes showing statistical significance. All values in the results section are means with standard error, unless reported otherwise. All significance tests set alpha at 0.05.

3. RESULTS

3.1. Sample Collection

We analyzed venous blood samples from 54 titmice (*Baeolophus* spp.) (22 juveniles, 33 adults) (Table **1**). In the field, using plumage, 27 were identified as Black-crested Titmice (*Baeolophus atricristatus*), 23 as Tufted Titmice (*B. bicolor*), and four as hybrids. Mitochondrial DNA, from a related genetic study, identified the four phenotypic hybrids as one Black-crested and three Tufted Titmice (Vaughn and Voelker, in progress). Two phenotypic Black-crested Titmice captured east of the BE (Grimes and Williamson counties) actually classified as Tufted Titmice from mtDNA [24].

3.2. Age, Sex, and Midazolam

Sex and age of titmice affected relatively few analytes. Females had increased potassium concentrations compared to males (ANOVA, $F_2 = 4.77$, $p = 0.03$, Table **2**). Hematocrit, as measured with an i-STAT 1 system (Hct), failed to agree with the traditional centrifuge method (PCV) (Tukey Mean Difference -12.6, SE 0.9, $p <0.001$) (Table **2**). We confirmed the correlation of Hct and Hb (0.93, $p <0.001$) and thus focused on Hct differences based on age and sex. Hematocrit (Hct) was higher in adult titmice compared to juveniles (Transformed 1/Hct, Welch's Test, $t_1 = 2.03$, $p = 0.05$) and concentrations of hemoglobin (Hb) showed a similar trend (Welch's Test, $t_1 = 1.97$, $p = 0.05$) (means, SE in Table **2**).

Table 2. Venous blood analyte values from collected Titmice based on age, sex, and midazolam administration.

Means, standard error, and sample size of untransformed values reported for reference purposes. Probability values from means' significance testing provided with two degrees of freedom. Significant values (α ≤ 0.05) are shown in bold.						
a. Age						
Analyte	**Units**	**n**	**Juvenile**	**n**	**Adult**	***p***
pH^{NT}	at 37°C	21	7.56 ±0.01	29	7.59 ±0.01	0.09
pO_2	mmHg	21	34.5 ±1.1	30	35.2 ±0.8	0.61
sO_2	%	21	75 ±2	29	78 ±1	0.13
pCO_2	mmHg	21	28.7 ±1.2	29	28.2 ±1.1	0.75
HCO_3	mmol L^{-1}	21	25.0 ±0.9	29	27.0 ±0.8	0.09
tCO_2^{NT}	mmol L^{-1}	21	26.1 ±0.8	29	27.8 ±0.8	0.16
BEecf	mmol L^{-1}	21	3.2 ±0.9	29	5.3 ±0.9	0.10
$Lactate^{NT}$	mmol L^{-1}	16	3.8 ±0.3	26	3.8 ±0.2	0.98

(Table 2) cont.....

Analyte	Units	n	Juvenile	n	Adult	p
Glucose	mg dL^{-1}	20	339.4 ±13.5	28	322.3 ±12.2	0.36
Na$^+$	mmol L^{-1}	20	158.5 ±1.0	28	158.7 ±0.7	0.80
K$^+$	mmol L^{-1}	20	3.7 ±0.1, 20	28	3.7 ±0.1	0.99
Cl$^-$	mmol L^{-1}	15	119.6 ±1.1	25	119.0 ±0.9	0.67
BUN^T	mg dL^{-1}	15	1.8 ±0.2	25	1.6 ±0.2	0.61^M
PCV	%	17	58 ±2	31	58 ±1	0.87
Hct^{NT}	% PCV	20	42 ±1	28	44 ±1	0.07^W
Hb^{NT}	g dL^{-1}	20	14.0 ±0.2	28	15.0 ±0.3	0.06^W
b. Sex						
Analyte	Units	n	Female	n	Male	p
pH^{NT}	at 37°C	18	7.60 ±0.01	31	7.59 ±0.01	0.15
pO$_2$	mmHg	18	33.6 ±1.0	31	35.5 ±0.8	0.16
sO$_2$	%	18	74 ±2	31	78 ±1	0.06
pCO$_2$	mmHg	18	28.6 ±1.1	31	28.9 ±1.1	0.95
HCO$_3$	mmol L^{-1}	18	25.5 ±0.8	31	27.4 ±0.9	0.23
tCO$_2$^{NT}	mmol L^{-1}	18	25.8 ±1.0	31	27.7 ±0.8	0.13
BEecf	mmol L^{-1}	18	3.0 ±0.8	31	5.2 ±0.9	0.10
Lactate^{NT}	mmol L^{-1}	13	3.9 ±0.3	28	3.8 ±0.2	0.77
Glucose	mg dL^{-1}	16	349.6 ±15.4	31	317.4 ±11.1	0.10
Na$^+$	mmol L^{-1}	16	157.8 ±1.1	31	159.1 ±0.6	0.25
K$^+$	mmol L^{-1}	16	**3.9 ±0.2**	31	**3.6 ±0.1**	**0.03**
Cl$^-$	mmol L^{-1}	12	120.8 ±1.1	27	118.7 ±0.8	0.14
BUN^T	mg dL^{-1}	12	2.4 ±0.4	27	1.5 ±0.1	0.14^M
PCV	%	15	58 ±2	32	59 ±1	0.77
Hct^{NT}	% PCV	16	**42 ±1**	31	**44 ±1**	**0.04**
Hb^{NT}	g dL^{-1}	16	14.3 ±0.2	31	14.97 ±0.2	0.06
c. Midazolam						
Analyte	Units	n	Not Administered	n	Administered	p
pH^{NT}	at 37°C	28	7.59 ±0.02	22	7.56 ±0.01	0.14^W
pO$_2$	mmHg	28	**36.4 ±0.8**	23	**33.2 ±0.9**	**0.01**
sO$_2$	%	28	**80 ±1**	22	**72.9 ±1.7**	**0.00**
pCO$_2$	mmHg	30	**25.8 ±1.1**	22	**31.9 ±0.7**	**<0.001^W**
HCO$_3$	mmol L^{-1}	28	**24.3 ±0.7**	22	**28.5 ±0.8**	**<0.001**
tCO$_2$^{NT}	mmol L^{-1}	28	**25.3 ±0.7**	22	**29.4 ±0.8**	**<0.001**
BEecf	mmol L^{-1}	28	**3.0 ±0.8**	22	**6.3 ±1.0**	**0.01**
Lactate^{NT}	mmol L^{-1}	19	**4.8 ±0.2**	23	**3.0 ±0.1**	**<0.001**
Glucose	mg dL^{-1}	25	**310.0 ±11.6**	23	**350.4 ±12.8**	**0.02**
Na$^+$	mmol L^{-1}	25	**157.5 ±0.7**	23	**159.8 ±0.7**	**0.02**
K$^+$	mmol L^{-1}	25	**3.8 ±0.1**	23	**3.5 ±0.1**	**0.02**
Cl$^-$	mmol L^{-1}	17	**121.3 ±1.0**	23	**117.7 ±0.8**	**0.01**
BUN^T	mg dL^{-1}	17	**2.3 ±0.2**	23	**1.3 ±0.2**	**<0.001^M**
PCV	%	30	58 ±1	18	58 ±1	0.92
Hct^{NT}	% PCV	25	43 ±1	23	44.0 ±0.9	0.49
Hb^{NT}	g dL^{-1}	25	14.6 ±0.2	23	14.8 ±0.3	0.61

^{N}Variable not normally distributed, tested with Shapiro-Wilk Goodness of Fit test for normality.

^{T}Variable transformed to normalize data. 1/exp(x): pH, Log(x): tCO2, Lactate, 1/sqrt(x): BUN, 1/x: Hct, Hb

^{W} Means have unequal variance, p-value provided from Welch's 2 sided F-test

^{M}Variable unable to reach normal distribution, Mann-Whitney ranked score test performed (Mean scores and Z probability provided)

Abbreviations: pO$_2$: partial pressure oxygen, sO$_2$: saturated oxygen, pCO$_2$: partial pressure carbon dioxide, HCO$_3$: bicarbonate, tCO$_2$: total carbon dioxide, BEecf: base excess, Na$^+$: sodium, K$^+$: potassium, Cl$^-$: chloride, BUN: blood urea nitrogen, Hct: hematocrit, Hb: hemoglobin

Midazolam administration affected all blood analytes except pH, Hct, PCV, and Hb (Table **3**). Midazolam administration was associated with increased values of pCO_2, bicarbonate, tCO_2, base excess, glucose, and sodium but lower values of pO_2, sO_2, lactate, potassium, chloride, and blood urea nitrogen. Midazolam administration was associated with relatively decreased pO_2 in juveniles compared with adults receiving midazolam, while pO_2 was comparably lower in adults than juveniles, regardless of the administration of midazolam (ANOVA, $F_3 = 6.47$, $p < 0.001$), (Table **4**, Fig. **2**).
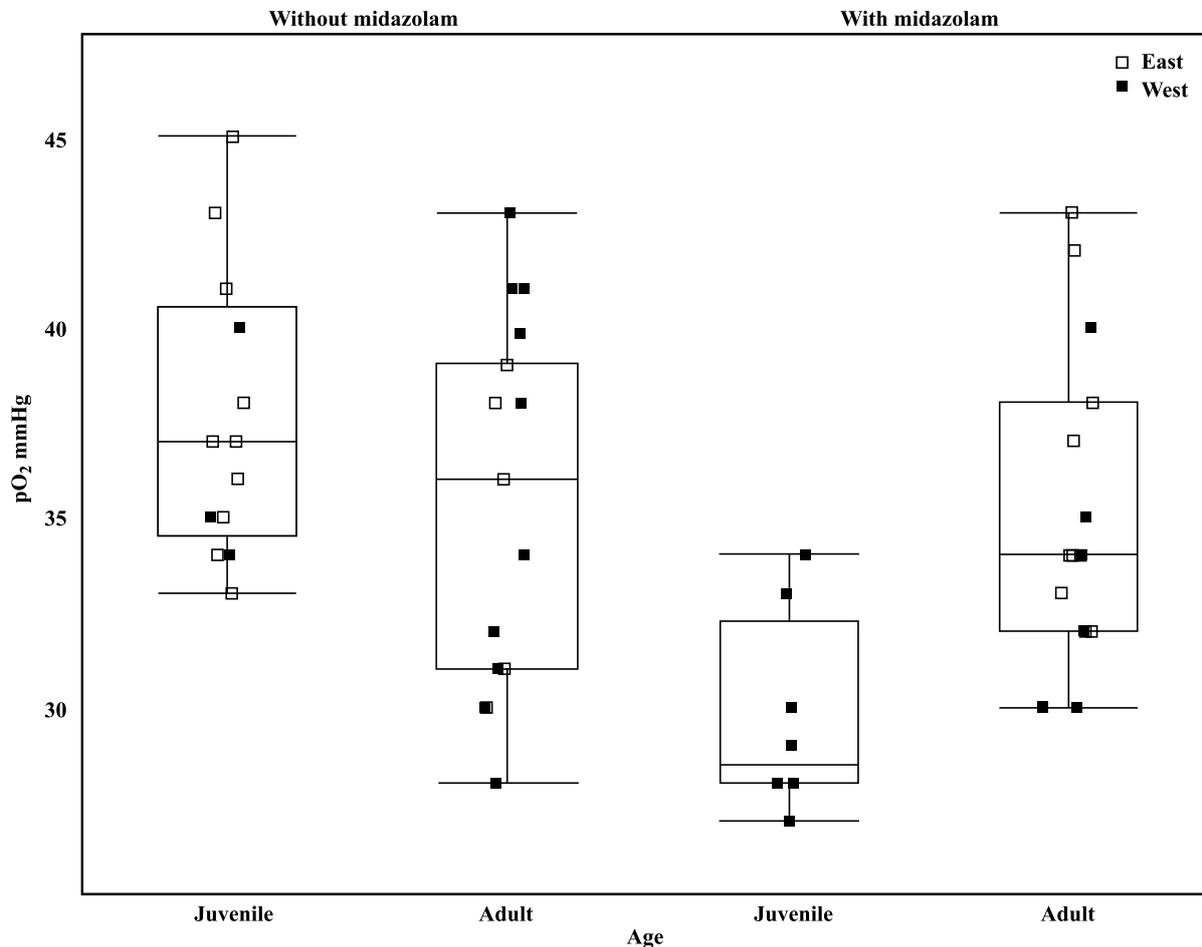
Fig. (2). Venous partial pressure of oxygen (pO_2) from different ages of Titmice (*Baeolophus* spp.) both east (open squares) and west (filled squares) of the Balcones Escarpment and with and without administration of Midazolam. Juvenile titmice had lower pO_2 values after administration of midazolam ($p = 0.01$), based on an interaction of midazolam and age ($p = 0.01$, adj $R^2 = 0.23$).

Table 3. Venous blood analyte values from collected Titmice based on capture location and location relative to Balcones Escarpment.

Mean, standard error, and sample size provided using untransformed values for reference purposes. Probability values from means' significance testing provided with 3 degrees of freedom for capture location and 2 for location relative to Balcones Escarpment. Significant values ($\alpha \leq 0.05$) are shown in bold.							
a. Capture Location							
Analyte	**Units**	**n**	**West**	**n**	**Contact Zone**	**n**	**East**	***p***
pH[NT]	at 37°C	2	7.59 ±0.12	23	7.58 ±0.02	25	7.58 ±0.01	0.98
pO_2	mmHg	2	36.5 ±6.5	23	**33.2[†] ±0.1**	26	**36.3[†] ±0.8**	**0.06**
sO_2	%	2	80 ±3	23	**74[†] ±1**	25	**79[†] ±1**	0.10
pCO_2	mmHg	2	28.9 ±0.8	23	28.6 ±1.4	25	28.2 ±1.0	0.97
HCO_3	mmol L^{-1}	2	28.5 ±6.75	23	26.3 ±0.8	25	25.8 ±0.9	0.68

(Table 3) cont.....

Analyte	Units	n	West	n	Contact Zone	n	East	*p*
tCO$_2$[NT]	mmol L^{-1}	2	29.5 ±6.5	23	27.0 ±0.8	25	26.9 ±0.8	0.71
BEecf	mmol L^{-1}	2	6.5 ±8.5	23	4.4 ±0.8	25	4.2 ±0.9	0.80
Lactate[NT]	mmol L^{-1}	1	3.5 ±1	19	3.5 ±0.3	22	4.7 ±0.3	0.41
Glucose	mg dL^{-1}	2	**348.0 ±25.0**	21	**369.0[†] ±13.6**	25	**294.7[†] ±8.3**	**<0.001**
Na$^+$	mmol L^{-1}	2	156.0 ±3.0	21	158.4 ±0.8	25	159.0 ±0.7	0.52
K$^+$	mmol L^{-1}	2	4.1 ±0.6	21	3.7 ±0.1	25	3.6 ±0.1	0.39
Cl$^-$	mmol L^{-1}	2	118.5 ±1.5	17	119.4 ±1.2	21	119.1 ±0.9	0.95
BUN[T]	mg dL^{-1}	2	1.5 ±0.5	17	1.8 ±0.3	21	1.7 ±0.1	0.70[C]
PCV	%	2	64 ±4	19	56 ±2	27	59 ±1	0.22
Hct[NT]	%PCV	2	41 ±2	21	44 ±1	25	44 ±1	0.47
Hb[NT]	g dL^{-1}	2	13.8 ±0.5	21	14.9 ±0.3	25	14.7 ±0.3	0.42
b. Location relative to Balcones Escarpment								
Analyte	Units	n	West	n	East		*p*	
pH[NT]	at 37°C	25	7.58 ±0.02	25	7.58 ±0.01		0.87	
pO$_2$	mmHg	25	**33.5 ±0.9**	26	**36.3 ±0.8**		**0.03**	
sO$_2$	%	25	**75 ±2**	25	**79 ±1**		**0.06**	
pCO$_2$	mmHg	25	28.6 ±1.3	25	28.2 ±1.0		0.81	
HCO$_3$	mmol L^{-1}	25	26.5 ±0.9	25	25.8 ±0.9		0.59	
tCO$_2$[NT]	mmol L^{-1}	25	27.2 ±0.9	25	26.9 ±0.8		0.79	
BEecf	mmol L^{-1}	25	4.6 ±0.9	25	4.2 ±0.9		0.78	
Lactate[NT]	mmol L^{-1}	20	3.5 ±0.3	22	4.1 ±0.3		0.18	
Glucose	mg dL^{-1}	23	**367.1 ±12.6**	25	**294.7 ±8.3**		**<0.001**	
Na$^+$	mmol L^{-1}	23	158.3 ±0.8	25	159.0 ±0.7		0.48	
K$^+$	mmol L^{-1}	23	3.7 ±0.1	25	3.6 ±0.1		0.37	
Cl$^-$	mmol L^{-1}	19	119.3 ±1.1	21	119.1 ±0.9		0.90	
BUN[T]	mg dL^{-1}	19	1.7 ±0.3	21	1.7 ±0.1		0.42[M]	
PCV	%	21	57 ±2	27	59 ±1		0.40	
Hct[NT]	%PCV	23	44 ±1	25	44 ±0.8		0.95	
Hb[NT]	g dL^{-1}	23	14.8 ±0.2	25	14.7 ±0.3		0.67	

[N]Variable not normally distributed, tested with Shapiro-Wilk Goodness of Fit test for normality.

[T]Variable transformed to normalize data. 1/exp(x): pH, Log(x): tCO2, Lactate, 1/sqrt(x): BUN, 1/x: Hct, Hb

[†]Statistical significance between each pair t-test (alpha=0.05), p-value provided on second row

[C]Variable failed to reach normal distribution, Chi-square value and probability provided

[M]Variable unable to reach normal distribution, Mann-Whitney ranked score test performed *PCV measured from centrifuge, Hct measured by iSTAT machine

Abbreviations: pO$_2$: partial pressure oxygen, sO$_2$: saturated oxygen, pCO$_2$: partial pressure carbon dioxide, HCO$_3$: bicarbonate, tCO$_2$: total carbon dioxide, BEecf: base excess, Na$^+$: sodium, K$^+$: potassium, Cl$^-$: chloride, BUN: blood urea nitrogen, Hct: hematocrit, Hb: hemoglobin

Table 4. Effect of multiple variables on blood venous analytes collected from Tufted and Black-crested Titmice.

Analysis of Variance (ANOVA), Analysis of Covariance (ANCOVA), and linear regression. Variable interactions represented by asterisk (*) and bold values indicate single variables or full models that showed significance (*p*<0.05).				
Model Components		-	**Full Model Results**	
Analytes	Factors	*p*-value	adj R^2	Prob>F
Hct	Age	0.60	-	-
-	Sex	0.15	-	-
-	Age*Sex	0.53	0.04	0.19
Potassium (K$^+$)	Sex	0.09	-	-
-	Midazolam	0.11	-	-
-	Sex*Midazolam	0.94	0.10	0.060

(Table 4) cont.....

Analytes	Factors	*p*-value	adj R^2	Prob>F
K^+ With Midazolam	Sex	-	0.03	0.200
K^+ No Midazolam	Sex	-	0.02	0.240
pO_2	Midazolam	**0.02**	-	-
-	Balcones Escarpment (BE)	**0.04**	-	-
-	Midazolam*BE	0.33	0.15	**0.010**
pO_2	Midazolam	**0.00**	-	-
-	Age	0.17	-	-
-	Midazolam*Age	**0.00**	0.25	**<0.001**
Glucose	Midazolam	0.05	-	-
-	Balcones Escarpment (BE)	**<0.001**	-	-
-	Midazolam*BE	0.54	0.36	**<0.001**
Glucose	Balcones Escarpment	**<0.001**	-	-
-	Relative Rainfall	0.34	-	-
-	Relative Rainfall*BE	**0.03**	0.38	**<0.001**
Glucose	Balcones Escarpment	**<0.001**	-	-
-	Days since last rain	**0.02**	0.39	**<0.001**

3.3. Population and Balcones Escarpment

Blood glucose concentrations of titmice differed significantly based on population (west, contact zone, east) (Table **3**). Titmice captured within the contact zone had the highest glucose concentrations, followed by western and then eastern populations (Table **3**). Administration of the sedative midazolam also increased glucose concentrations, but the location of capture was a stronger influence of glucose than sedative (east *vs.* west of BE) (2-way ANOVA, Table **4**).

Blood glucose concentrations were related to relative rainfall (higher or lower) and "days since rainfall" (Table **4**). For titmice captured west of the BE, blood glucose concentrations were increased in months of high rainfall. However, for birds captured east of the BE, no difference of blood glucose was noted in association with levels of monthly rainfall (Fig. **3**). In titmice captured west of the BE, length of time since rainfall was positively correlated with higher blood glucose concentrations occurring after 12-25 days after rainfall (Linear Regression R^2 = 0.40, ANOVA, F_3 = 9.83, *p* <0.001) (Table **4**, Fig. **4**).

Other venous blood analytes that varied significantly based on population, primarily between the contact zone and eastern populations included pO_2, sO_2, pH and HCO_3. Both pO_2 and sO_2 values were significantly lower in individuals within the contact zone compared to the eastern population (Table **3**). Although the western population had the highest values for pO_2 and sO_2, these did not differ significantly from contact zone or eastern population values (Table **3**). Midazolam administration resulted in decreased pO_2 values, with a more pronounced effect in Titmice west of the BE (ANOVA, F_1 = 5.10, *p* =0.03, Table **3**). No interaction of capture location and midazolam administration was apparent (ANOVA, t_2 = -0.99, *p* = 0.33, Table **4**).

4. DISCUSSION

Venous blood glucose concentrations of titmice differed east and west of the BE and were also relatively increased in birds captured west of the BE, especially during months with higher rainfall.

4.1. Location and Rainfall Increase Venous Blood Glucose Concentrations

Of 16 blood venous blood analytes assessed in two parapatric species of titmice, only blood glucose concentrations were statistically significantly different east and west of the BE, with relatively increased values west of the BE. A small sample size from the western population and inherent analyte variability likely prevented statistical distinctions between the western and hybrid zone populations (Table **3**). The difference in glucose east and west of the BE was also observed in our related studies of multiple Texas passerine species [29, 30]. Although pO_2 and sO_2 also differed between titmice east and west of the BE, differences resulted from age, sex, and/or midazolam administration.

In general, within bird species, glucose can vary by species, age, time of year, or can be elevated by physiological or psychological stress [36 - 39]. In birds, blood glucose concentrations are modulated by Glucocorticoid (GC) production pathways *via* two different receptors. Baseline GC production is mediated by the mineralocorticoid receptor from which increased production can occur following an increase in energetic demands, thermoregulatory changes, or even a period of low food abundance [37 - 40]. Stress-induced GC production, however, is mediated through glucocorticoid receptors that upregulate gluconeogenesis based on 2-10 fold higher concentration of glucocorticoid than baseline.
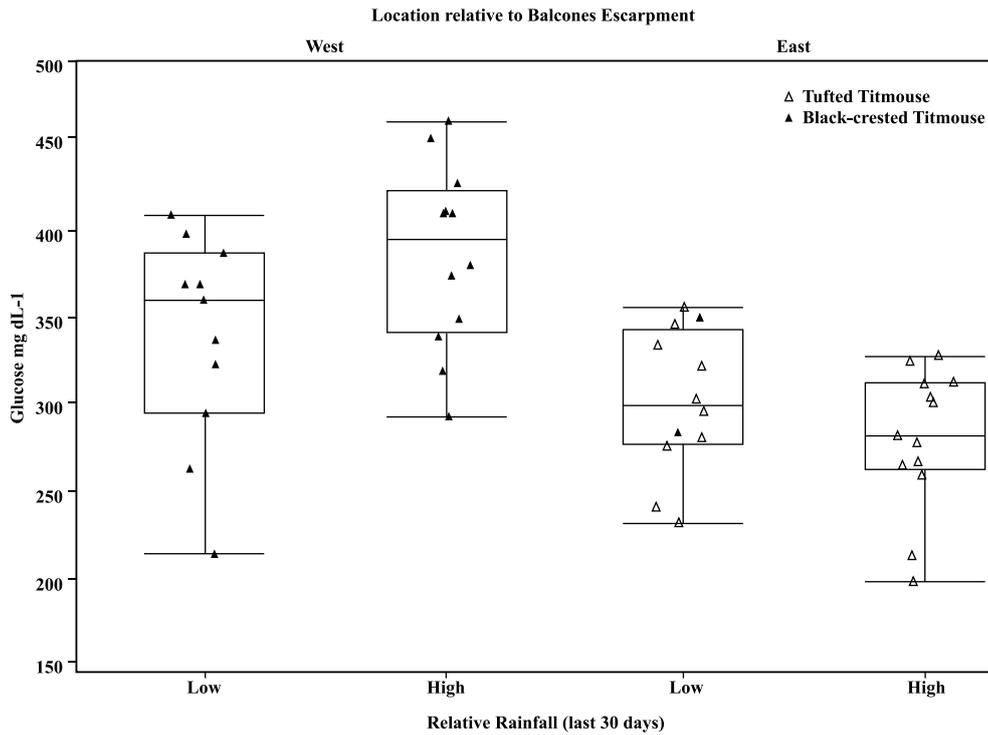
Location relative to Balcones Escarpment

Fig. (3). Venous blood glucose concentrations (mg dL^{-1}) of Titmice captured west and east of the Balcones Escarpment (BE) based on relative monthly rainfall data. Black-crested Titmice are represented by solid triangles and Tufted Titmice represented with open triangles. Titmice captured west of the BE had relatively increased concentrations of glucose than those captured east (R^2=0.33, p<0.001). Concentrations were further elevated west of the escarpment during months of relatively high rainfall (BE p<0.001, relative rainfall p = 0.34, BE*relative rainfall p = 0.03; whole model R^2 = 0.38, p<0.001; Table **4**).
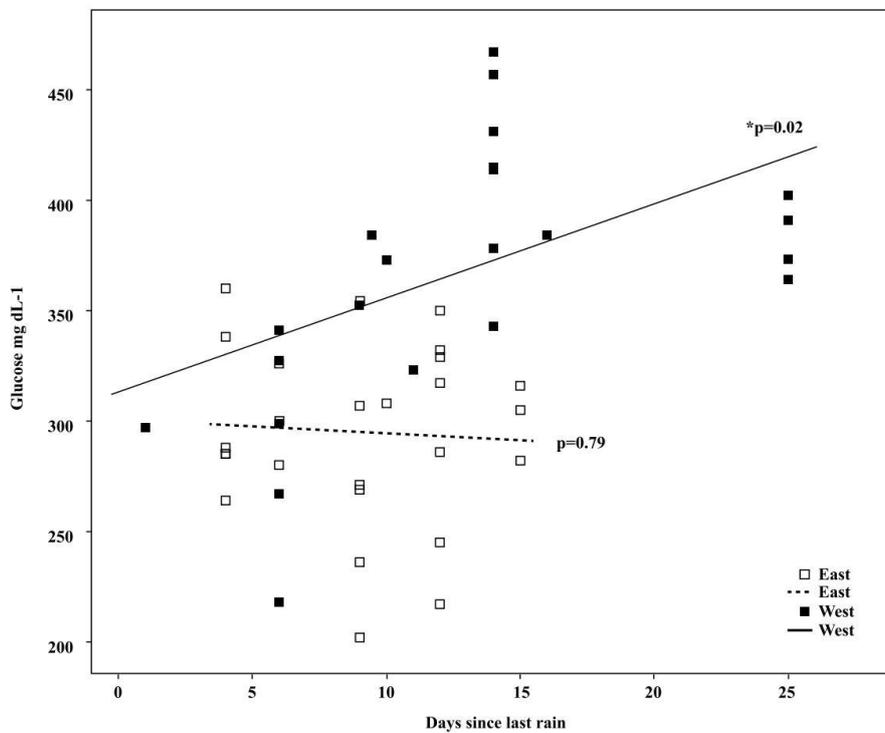
Fig. (4). Venous blood glucose concentrations (mg dL^{-1}) of Titmice captured east (open square, dashed fit line) and west (filled square, solid fit line) of the Balcones Escarpment and the number of days since the last rain event. Titmice captured west of the Balcones Escarpment had relatively increased blood glucose concentrations during periods of prolonged dryness (Adj. R^2 east = 0.00, Adj. R^2 west = 0.24; whole model Adj. R^2 = 0.30, p <0.001).

Titmice captured west of the Balcones Escarpment have relatively increased glucose values compared with that east of the BE (Tufted Titmice), irrespective of sedative administration. Black-crested and Tufted Titmice share many life history similarities but differ in their habitat preference [24, 25]. Black-crested resides in the semi-arid scrub habitat of west Texas where precipitation is more sporadic, a factor that can lead to unpredictable food resources and induce physiological stress [17, 41 - 43]. Low food abundance can increase baseline glucose concentrations. Survival and adaptation to a continually altered physiological state, due to constant stress, is known as the Chronic Stress Hypothesis [44 - 46]. In an arid habitat, chronic stress may be due to scarce or unpredictable food or water sources and, over the long term, such stresses can induce adaptations to increase survivability [44, 46, 47].

Evidence for a an increased natural glucose concentration based on intermittent food/water resources is shown by the further increase in Black-crested glucose concentration following rain events, a pattern not observed in Tufted Titmice. In arid and semi-arid regions, rainfall amplifies primary productivity and triggers an increase in insect and fruit abundance, stimulating foraging by secondary and tertiary consumers [43, 48, 49]. We suspect that sporadic rain showers temporarily increase food resources, causing hyperphagia or an increase in metabolic activity (a known correlate of glucose concentrations). A rise in blood glucose has been observed in organisms exposed to unpredictable or pulse food resources as a result of a change in diet (*e.g.*, sugar-filled berries) or a product of hyperphagia [46]. We observed blood glucose concentrations were highest in birds captured 12-25 days following rain, likely a result of the time lag between rainfall and pulse food resources from increased primary productivity [38, 47, 48]. Glucose concentrations in Tufted Titmice, on the other hand, did not increase following rain events, lending support to the idea that these species, living in mesic habitats, have a continual food source year round and, thus, are not prone to infrequent bouts of low food sources. It is interesting to note that the two phenotypically (plumage) Black-crested Titmice captured east of the BE did not express similar glucose concentrations to those captured west. The adult male Black-crested Titmouse (Williamson county) had a glucose concentrations slightly less than the average of birds west of the BE (354 mg/dL, x = 367 mg/dL), but the juvenile male Black-crested (Grimes county) had a glucose value less than the average of birds east of the BE (288 mg/dL, x = 294 mg/dL). Both birds received a sedative and were captured in late spring; however, the adult male was captured just east of the BE (closer to the hybrid zone). Therefore, though these birds look Black-crested and have Tufted Titmouse DNA, their glucose values appear to match with longitude/habitat.

If habitat drives glucose, as our data suggests, it may play an important role in species distribution and reduce genetic introgression across the hybrid zone. Black-crested Titmice have been observed east of the BE, but Tufted Titmice are rarely observed west of the BE [24, 25]. Tufted Titmice may have low survival west of the BE due to physiological intolerance to unpredictable food and water resources. Therefore, the physiology of glucose metabolism could act as a limiting factor to the colonization west of the BE by the Tufted Titmouse.

4.2. Influence of Age and Sex

Other venous blood analytes were influenced by age, sex, season, and sedative administration. Although saturated oxygen and base excess were higher in males, especially those administered midazolam, we suspect that the sex difference is an artificial finding based on sample size and influence of midazolam.

Both sex and midazolam administration influenced venous blood potassium concentrations. Adult females showed relatively increased venous blood potassium concentrations compared to other groups. The most common cause of hyperkalemia in human and veterinary medicine is red blood cell lysis [50]. However, in avian species, the effect of hemolysis on potassium concentrations has not been evaluated to our knowledge. In this study, however, no adult female received the sedative midazolam. Thus, adult females may have undergone more stress and struggle during sampling resulting in a sample with relatively increased cellular lysis prior to analysis. During stress or strenuous exercise, increased blood pressure and muscular contractions trigger the kidneys to release potassium to ensure adequate sodium-potassium pump activity [51, 52]. Furthermore, as all females were captured during nesting or breeding season, females could have experienced greater energetic demands, and handling and blood sampling may have been more problematic without sedation. The capture of both sexes outside the breeding season could confirm if the increased potassium concentrations in female Titmice are consistently present, or they result from increased energetic demands related to breeding. Recording a hemolysis score for the blood samples could have facilitated the determination of the effect of hemolysis, if any, upon potassium concentrations in our samples [53].

As found in our study, juvenile passerine birds of many species often have a relatively decreased hematocrit compared to adults [54]. Hematocrit's difference between ages is likely a product of body size [55]. The relationship was strongest with males, as expected in Titmice, since males are larger than females [25, 27]. Since hematocrit varies greatly between individuals due to health, hydration, life history, and more, the unexplained high variance is unsurprising. The observed difference of Hct, between sexes, might have been stronger with a larger sample size of adult females and less disparity of the ratio of juveniles and adults.

Similar to other studies, we also found a discrepancy between PCV and measured Hct, with PCV values consistently greater than Hct [30, 34]. The discrepancy of methods for determination avian hematocrit is likely explained by the relative difference in the avian red blood cell shape, volume and retained nucleus, compared to the human red blood cell [29, 56, 57]. Packed cell volume (as determined by centrifugation), rather than Hct (as determined by electrical resistance *via* the i-STAT 1), provides consistently lower results and should remain the gold standard for use in passerine species. Packed cell volume (PCV or Hct), the ratio of red blood cells to total blood volume expressed as a percent, can indicate dehydration or fitness based on an individual's ability to transport oxygen [34]. Larger organisms usually have increased metabolic demands based on increased body and

organ size and greater demand for oxygen transport [58, 59]. In addition, adult birds and those with androgens, rather than estrogens, also tend toward relatively increased red blood cell mass with resulting increased PCV [60, 61]. However, Hct should be further evaluated in these species with a larger adult sample size and with individuals from across the full distribution of the species to ensure other factors, such as latitude or elevation, are not influencing Hct or PCV.

4.3. Effect of Midazolam and Handling Upon Venous Blood Analytes

Administration of midazolam affected many blood gas, and electrolyte values often used to determine health in wild birds. Interpretation of avian health in wild birds is inherently complicated as capture, handling, and blood collection are stressful events for free-living birds [62 - 64]. Physicians and dentists have used midazolam for decades to reduce procedural stress in children [65, 66]. Midazolam is a benzodiazepine that boosts inhibitory neurotransmitters, decreases neuron excitability and results in sedation, anxiolysis, and/or hypnosis depending upon the dose administered [65]. However, hypoxia, reduced respiration, reduced memory, and stimulation of a stress endocrine response may also occur [29, 65]. In multiple avian species, the intranasal administration of midazolam has been shown to reduce the stress of handling and venipuncture [67].

In our study, midazolam administration influenced all analytes we measured in Titmice except pH, Hct, and Hb. For both species, irrespective of geographic location, Titmice given midazolam had, on average, higher pCO_2, HCO_3, tCO_2, base excess, glucose, and sodium values. Midazolam lowered the venous partial pressure of oxygen (pO_2), and oxygen saturation (sO_2), as well as concentrations of lactate, potassium, chloride, and BUN. Except for glucose, changes in all analytes paralleled those seen following the administration of midazolam to humans [68].

While the location of capture was a stronger predictor of blood glucose concentrations than sedative administration, glucose concentrations were unexpectedly higher in birds receiving midazolam. Gluconeogenesis occurs near the end of the stress response pathway to meet the increased energetic demands of the stressor [51, 69]. Production of glucose is a vital component of the negative feedback mechanism regulating the sympathetic (aka "fight or flight") nervous system. Even after a stress stimulus is removed, glucose concentrations may continue to rise based on the time lag between neuroendocrine communication and the hepatic metabolic response [51, 70]. Mist-net capture, handling, and blood sampling each induce stress on wild birds, so increased glucose concentrations were expected in birds [64, 71, 72]. The apparent ineffectiveness of midazolam to reduce glucose concentrations is likely a product of the lag time between capture until midazolam administration. Although we obtained blood 0-5 min following sedation, a stressful event (mist-net capture) has already occurred prior to anxiolytic adminis-tration. Further stress, from handling, during sedative admin-istration, could also cause blood glucose concentrations to continue increasing during blood collection despite sedative administration. To better assess the efficacy of midazolam for stress reduction in captured birds, future studies could measure blood glucose concentrations and or corticosterone at various time intervals (*e.g.*, 15-60 min) following capture handling and midazolam administration. Although midazolam changed multiple venous blood analytes similarly to humans experiencing anxiolysis, this sedative's impact on blood gases and electrolytes in passerine birds needs further investigation.

We administrated midazolam to titmice in 2012 to reduce stress and facilitate blood collection while assessing health. Providing a rest period after capture and before blood collection may also reduce stress, as indicated by blood gases and other variables [71, 72]. Blood samples from Mourning Doves (*Zenaida macroura*), Boat-tailed Grackles (*Quiscalus major*), and House Sparrows (*Passer domesticus*), immediately after capture or after a 45-60 min delay, lacked differences in pH for any species, while blood gas values (pCO_2, pO_2, and HCO_3^-) and PCV varied based on species. However, lactate significantly decreased based on a rest period in all species. We found similar results in Titmice when comparing birds receiving a sedative to those without the sedative. However, this comparison should be reviewed with caution, as the authors studied two Passeriformes species and a Colum-biformes species that were larger than the Titmice of this study [72].

CONCLUSION

Titmice living west of the Balcones Escarpment have higher venous blood glucose concentrations than individuals east of the BE. Glucose values were further elevated following rainfall west of the BE. We suspect that naturally increased glucose values are due to chronic stress from unpredictable food sources in a semi-arid environment. Naturally higher glucose concentrations, in western birds, are supported by a further increase in their glucose concentrations following rainfall, an event representing a change in their environment. In arid and semi-arid environments, such as the BE, sporadic rainfall stimulates primary productivity, which can induce hyperphagia and/or increase metabolic activity, both of which are known causes of increased glucose. We recommend controlled experiments to investigate the behavior, physiology, and fitness of both species in opposing natural habitats. Such studies could provide evidence of hybrid zone reinforcement and possible hybrid zone movement or range expansion. Until data collection and knowledge advance to provide complete physiological reference data for appropriate indicator species of study, we, as wildlife biologists, must strive to continue increasing our understanding of avian physiology through comparative studies between species, updating handling protocols to include use of sedatives, understand and include environmental conditions in our study design, as well as investigating the impact of physiological differences between populations and species, especially as it relates to habitat effects.

LIST OF ABBREVIATIONS

BE	=	Balcones Escarpment
PCV	=	Packed Cell Volume

Hct	=	Hematocrit
Hb	=	Hemoglobin
ANOVA	=	Analysis of Variance

AUTHORS' CONTRIBUTIONS

J.C.V and G.V. conceived the idea, design, and experiment; J.C.V and J.J.H. collected data, conducted research; J.C.V. wrote the paper; J.C.V. and J.J.H. designed the methods; J.C.V. analyzed the data; J.J.H. and G.V. contributed materials, resources, funding, and edited the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTI-CIPATE

This research was conducted under required Texas Parks and Wildlife (permit# SPR-0209-016) and United States Fish and Wildlife Permits (permit# 188 MB205752) and with approval of the Texas A&M University Institutional Animal Care and Use Committee (AUP# 2012-6).

HUMAN AND ANIMAL RIGHTS

No humans were used for studies that are the basis of this research. All animal experiments were conducted in accordance with the Guidelines for euthanasia and ethical authorization of the study, all got approved from the Board of the Texas A&M University Institutional Animal Care and Use Committee (AUP# 2012-6).

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available at [https://vetmed.tamu.edu/schubot/research/publications/], reference number [publication # SC 161].

CONFLICT OF INTEREST

J.C.V.: none; G.V.: none; J.J.H.: none

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