



Comparison of metabolic substrates in alligators and several birds of prey

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ABSTRACT

On average, avian blood glucose concentrations are 1.5–2 times those of mammals of similar mass and high concentrations of insulin are required to lower blood glucose. Whereas considerable data exist for granivorous species, few data are available for plasma metabolic substrate and glucoregulatory hormone concentrations for carnivorous birds and alligators. Birds and mammals with carnivorous diets have higher metabolic rates than animals consuming diets with less protein whereas alligators have low metabolic rates. Therefore, the present study was designed to compare substrate and glucoregulatory hormone concentrations in several birds of prey and a phylogenetically close relative of birds, the alligator. The hypothesis was that the combination of carnivorous diets and high metabolic rates favored the evolution of greater protein and fatty acid utilization leading to insulin resistance and high plasma glucose concentrations in carnivorous birds. In contrast, it was hypothesized that alligators would have low substrate utilization attributable to a low metabolic rate. Fasting plasma substrate and glucoregulatory hormone concentrations were compared for bald eagles (*Haliaeetus leucocephalus*), great horned owls (*Bubo virginianus*), red-tailed hawks (*Buteo jamaicensis*), and American alligators (*Alligator mississippiensis*). Avian species had high circulating β -hydroxybutyrate (10–21 mg/dl) compared to alligators (2.81 ± 0.16 mg/dl). In mammals high concentrations of this byproduct of fatty acid utilization are correlated with insulin resistance. Fasting glucose and insulin concentrations were positively correlated in eagles whereas no relationship was found between these variables for owls, hawks or alligators. Additionally, β -hydroxybutyrate concentrations were low in alligators. Similar to carnivorous mammals, ingestion of a high protein diet may have favored the utilization of fatty acids and protein for energy thereby promoting the development of insulin resistance and gluconeogenesis-induced high plasma glucose concentrations during periods of fasting in birds of prey.

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1. Introduction

Avian flight relies primarily on the metabolism of fatty acids for energy (Rothe et al., 1987; Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002). Despite this reliance on fatty acids, birds maintain relatively high plasma glucose concentrations and are resistant to the glucose-lowering effects of insulin such that very

high doses of insulin are required to lower plasma glucose concentrations (Chen et al., 1945; Braun and Sweazea, 2008). Similarly, very high concentrations of glucose are necessary to stimulate the release of insulin from pancreatic β -cells in chickens, *Gallus domesticus* (King and Hazelwood, 1976; Naber and Hazelwood, 1977; Colca and Hazelwood, 1981). Data for plasma glucose concentrations in birds with various dietary habits (Braun and Sweazea, 2008) as well as carnivorous birds specifically (Table 1) are widely available in the literature. Moreover, significant attention has focused on glucose regulation in domestic species (Braun and Sweazea, 2008). For example, studies have shown that administration of exogenous insulin (50 μ g/kg body mass) to chickens results in an immediate (2–5 min) increase in plasma free fatty acids and uric acid with a

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Table 1
Plasma glucose and triglyceride concentrations for some carnivorous avian species and Crocodylia.

Animal	Glucose range (mM/l)	Average triglycerides (mM/l)	References
Golden eagle (<i>Aquila chrysaetos</i>)	14.1–20.5	0.55–2.04	Balasch et al. (1976), O'Donnell et al. (1978), Polo et al. (1992), Nazifi et al. (2008)
Spanish imperial eagle (<i>Aquila adalberti</i>)	13.6–25.6	0.51–1.19	Ferrer et al. (1987), Polo et al. (1992)
Eastern imperial eagle (<i>Aquila heliaca</i>)	10.0	NA	Balasch et al. (1976)
Booted eagle (<i>Aquila pennata</i>)	15.2–16.9	NA	Ferrer et al. (1987)
Bonelli's eagle (<i>Aquila fasciata</i>)	17.1–21.6	NA	Ferrer et al. (1987)
Andean condor (<i>Vultur gryphus</i>)	12.2–21.9	0.85–1.9	Balasch et al. (1976), Gee et al. (1981)
Peregrine falcon (<i>Falco peregrinus</i>)	16.1–18.7	NA	Gee et al. (1981)
Prairie falcon (<i>Falco mexicanus</i>)	23.0 ± 0.08	NA	O'Donnell et al. (1978)
Red-tailed hawk (<i>Buteo jamaicensis</i>)	17.0–31.9	NA	O'Donnell et al. (1978), Stein et al. (1998)
Marsh hawk (<i>Circus cyaneus</i>)	20.5 ± 0.81	NA	O'Donnell et al. (1978)
Common buzzard (<i>Buteo buteo</i>)	15.4–28.0	NA	Balasch et al. (1976), Ferrer et al. (1987)
American kestrel (<i>Falco sparverius</i>)	18.3–34.3	NA	Stein et al. (1998)
Common kestrel (<i>Falco tinnunculus</i>)	18.1–26.4	NA	Ferrer et al. (1987)
Black kite (<i>Milvus migrans</i>)	12.0–23.2	NA	Balasch et al. (1976), Ferrer et al. (1987)
South American snail kite (<i>Rostrhamus sociabilis</i>)	18.6–18.7	1.4–2.1	Gee et al. (1981)
Black vulture (<i>Aegypius monachus</i>)	16.1–24.5	0.03–0.95	Villegas et al. (2002)
Egyptian vulture (<i>Neophron percnopterus</i>)	14.2–24.9	0.75–1.86	Polo et al. (1992)
Griffon vulture (<i>Gyps fulvus</i>)	11.6–22.2	0.56–1.90	Balasch et al. (1976), Ferrer et al. (1987), Polo et al. (1992)
King vulture (<i>Sarcoramphus papa</i>)	12.4 ± 2.84	NA	Balasch et al. (1976)
Waved albatross (<i>Phoebastria irrorata</i>)	9.8–19.2	NA	Padilla et al. (2003)
Eurasian eagle owl (<i>Bubo bubo</i>)	18.2–24.7	NA	Ferrer et al. (1987)
Great horned owl (<i>Bubo virginianus</i>)	20.8 ± 0.72*	NA	O'Donnell et al. (1978)
Barn owl (<i>Tyto alba</i>)	11.2–16.7	NA	Ferrer et al. (1987)
Mugger crocodile (<i>Crocodylus palustris</i>)	2.7–5.4	36–1331	Stacy and Whitaker (2000)
Saltwater crocodile (<i>Crocodylus porosus</i>)	4.5–12.1	NA	Padilla et al. (2011)
Morelet's crocodile (<i>Crocodylus moreletii</i>)	2.9–4.2	NA	Padilla et al. (2011)
Nile crocodile (<i>Crocodylus niloticus</i>)	1.8–4.8	NA	Lovely et al. (2007), Padilla et al. (2011)
American alligator (<i>Alligator mississippiensis</i>)	5.1–5.5	NA	Coulson et al. (1950), Padilla et al. (2011)

* Fasting value; NA: data not available.

relatively delayed (15–30 min) decrease in plasma glucose concentrations (Hazelwood and Langslow, 1978). Interestingly, glucagon (50 µg/kg body mass) likewise increases plasma free fatty acids within 2–5 min, whereas uric acid and glucose concentrations were elevated 15 min later (Hazelwood and Langslow, 1978). These findings show that both insulin and glucagon rapidly augment plasma free fatty acid concentrations, the primary fuel source for avian flight muscles, in a granivorous species, although free fatty acids increased more in response to glucagon as compared with insulin (Hazelwood and Langslow, 1978; Kuzmiak et al., 2012). Similar to chickens, administration of insulin induces hypoglycemia in great horned owls (*Bubo virginianus*) (Grande, 1970) and adult male Embden domestic geese (*Anser anser domesticus emdben*) (Grande,

1969), whereas glucagon increases both free fatty acid and glucose concentrations in the plasma of great horned owls (Grande, 1970). In contrast to chickens, plasma free fatty acid concentrations are not increased in owls that are administered insulin (Grande, 1970), suggestive of differences in the insulin-mediated regulation of substrate metabolism between owls and chickens.

Although plasma glucose concentrations are available in the literature for many carnivorous species of birds (Table 1), very few investigators have examined indices of free fatty acid utilization (beta-hydroxybutyrate, triglycerides) or glucoregulatory hormones for birds with primarily carnivorous diets. In one study, healthy captive red-tailed hawks (*Buteo jamaicensis*) were found to have similar plasma insulin (171.3 ± 13.5 pg/ml) and glucagon

(198 ± 7.3 pg/ml) concentrations (Minick et al., 1996). In contrast, data for recently injured but otherwise healthy bald eagles (*Haliaeetus leucocephalus*) showed that plasma insulin concentrations (132 ± 10.7 pg/ml) were lower than glucagon concentrations (534 ± 53.9 pg/ml) (Minick and Duke, 1991). However, recently injured great horned owls (*Bubo virginianus*) have approximately half the concentration of plasma insulin (68 ± 9.7 pg/ml) as compared to hawks or eagles (Minick, 1986; glucagon data not available). Since substrate metabolism and glucoregulatory hormones can be affected by stress, our study was designed to provide additional information regarding fatty acid utilization and glucoregulatory hormones for healthy red-tailed hawks, bald eagles and great horned owls.

The divergence between avian and crocodylian lineages is estimated to have occurred roughly 254 million years ago, during the Middle Triassic (Janke and Arnason, 1997; Mindell et al., 1999). Despite the long separation, researchers examining the amino acid sequence for insulin have concluded that chickens are more closely related to the American alligator (*Alligator mississippiensis*) than mammals (Larhammar and Milner, 1989). Like birds, alligators preferentially utilize free fatty acids for energy (Black et al., 1963). For these reasons we chose to compare the selected carnivorous avian species with the American alligator. Fasting (72 h) plasma glucose concentrations of juvenile American alligators are low (7.44 ± 0.28 mM/l) and the animals are insensitive to the blood glucose-lowering effects of alligator insulin (Lance et al., 1993). This insulin insensitivity was hypothesized to result from stress-induced hyperglycemia that may have masked the hypoglycemic effects of insulin (Lance et al., 1993). In fact, stress-induced hyperglycemia has been documented in free-living Australian freshwater crocodiles, *Crocodylus johnstoni* (Jessop et al., 2003). However, when administered mammalian insulin, plasma glucose concentrations significantly decrease in American alligators (Lance et al., 1993) while tissue free glucose concentrations (indicative of uptake) increase (Stevenson et al., 1957). As in alligators, the absence of native insulin-mediated glucose disposal has also been observed in several domestic species of birds (reviewed in Braun and Sweazea, 2008). Administration of glucagon to an American alligator increases blood glucose, a response similar to those of birds and mammals (Stevenson et al., 1957). Removal of the pancreas (i.e., pancreatectomy) results in eventual death for alligators from ketosis (Penhos et al., 1967), great horned owls from hyperglycemia (Nelson et al., 1942), and causes glycosuria in hawks, falcons, buzzards and ravens, although pancreatectomy does not result in death for these latter avian species (Nelson et al., 1942). These findings suggest that glucose regulation by pancreatic hormones varies for alligators, great horned owls and other raptors; an observation that warrants further examination.

Birds, on average, have higher metabolic rates than crocodylians. For example, the standard metabolic rate (SMR) for great horned owls (*Bubo virginianus*) ranges from 184 to 314 kJ/kg/d (Lasiewski and Dawson, 1967; Pakpahan et al., 1989) and for male red-tailed hawks (*Buteo jamaicensis*) from 125.5 to 376.6 kJ/kg/d (Wasser, 1986; Pakpahan et al., 1989). In contrast, the SMR of crocodiles is 33.9 kJ/kg/d (Hulbert et al., 2002), and alligators have an even lower SMR of 4.3 kJ/kg/d (Coulson et al., 1989). Studies of 58 species of mammalian *Carnivora* show animals that consume primarily carnivorous diets have higher basal metabolic rates compared to those that consume vegetarian diets (Muñoz-García and Williams, 2005). Similarly, a review by Nagy et al. (1999) provides evidence that carnivorous, insectivorous and nectarivorous birds have higher field metabolic rates than omnivores or granivores. As vertebrate metabolic rate is positively correlated with blood glucose (Polakof et al., 2011), and carnivorous birds have high rates of gluconeogenesis (Migliorini et al., 1973), carnivorous birds would be expected to have higher blood glucose levels than

birds consuming non-carnivorous diets. Similarly, plasma glucose concentrations and metabolic rates are low in alligators (Lance et al., 1993). In fact, alligators can reportedly survive aglycemia for up to 24 h before succumbing to shock (Stevenson et al., 1957).

The purpose of the present study was to compare plasma substrate and glucoregulatory hormone concentrations for several species of birds with carnivorous diets to assess whether their fat- and protein-rich diets may have led to the evolution of decreased glucose sensitivity and resultant high plasma glucose concentrations. In mammals, ingestion of a low-carbohydrate, high-fat diet results in reduced glucose tolerance and insulin resistance (Bielohuby et al., 2013). Ingestion of a high-protein, low-carbohydrate diet similarly promotes insulin resistance (Brand-Miller and Colagiuri, 1999). Carnivorous birds also have higher glucose tolerance than omnivorous birds (Polakof et al., 2011). On the other hand, non-avian carnivorous species such as cats (*Felis catus*), white sturgeon (*Acipenser transmontanus*), rainbow trout (*Oncorhynchus mykiss*), and alligators are glucose intolerant (Myers and Klasing, 1999). In fact, several days are required for blood glucose concentrations to return to baseline following an intravenous glucose challenge in alligators (Myers and Klasing, 1999). As a result of high protein intake, amino acid gluconeogenesis occurs continuously during both fed and fasting states in birds as very little glucose is ingested (Pollock, 2006). In fact, Migliorini et al. (1973) showed that carnivorous birds are able to produce twice the concentration of glucose compared with other birds. For example, black vultures (*Coragyps atratus*) have higher liver gluconeogenic enzyme concentrations and greater gluconeogenic capacities as compared to chickens, which protects the animals from fasting-induced hypoglycemia (Migliorini et al., 1973; Veiga et al., 1978). For barn owls (*Tyto alba*), high gluconeogenic rates and low hepatic glucokinase activities promote sustained hyperglycemia (3.5 h) following an intravenous glucose challenge, whereas plasma glucose concentrations returned to baseline in chickens within 60 min (Myers and Klasing, 1999). Myers and Klasing (1999) postulated that glucose intolerance evolved in the owls as a result of their high-protein diet (primarily carnivorous) with little glucose ingestion.

In the present study, concentrations of metabolic substrates and glucoregulatory hormones were quantified across several species of birds of prey as well as alligators. The comparison and analyses of these variables may provide insight into the evolution of substrate utilization by several birds of prey. Our hypothesis was that the combination of carnivorous diets and high metabolic rates favored the evolution of greater protein and fatty acid utilization leading to insulin resistance and high plasma glucose concentrations in red-tailed hawks, bald eagles and great horned owls. In contrast, we hypothesized that alligators would have low substrate utilization attributable to a low metabolic rate.

2. Materials and methods

2.1. Experimental animals

Blood samples from bald eagles ($n = 4$), great horned owls ($n = 6$), and red-tailed hawks ($n = 5$) of both sexes were collected the morning following a 24 h fast at The Raptor Center (St. Paul, MN, USA) during the summer months. All birds at The Raptor Center are maintained under approved Institutional Animal Care and Use Committee (IACUC) protocols.

Raptors were typically fed once daily with a mixed diet of laboratory mice, rats and chickens (per availability) in the late afternoon. Postprandial uric acid concentrations typically take approximately 15 h to return to fasting concentrations. Therefore, 24 h fasts are routinely used at The Raptor Center when evaluating fasting blood

Table 2
Summary of insulin amino acid sequence homologies.

Species	Accession No.	% Sequence identity with alligator
American alligator (<i>Alligator mississippiensis</i>)	P12703	
Ostrich (<i>Struthio camelus</i>)	P67969	94
Turkey (<i>Meleagris gallopavo</i>)	P67968	94
Western graylag goose (<i>Anser anser anser</i>)	P68245	90
Muscovy duck (<i>Cairina moschata</i>)	P68243	90

chemistry. This fasting duration is similar to that used in other studies of raptors (Minick, 1986; Minick and Duke, 1991; Minick et al., 1996).

Samples of blood from captive juvenile American alligators of both sexes (72.4–97.8 cm in length, $n=8$) were collected during November. However, animals were housed in temperature-controlled tanks (31.1–31.7°C) to mimic spring and summer conditions when alligators feed. Alligators were maintained on a commercially available dry alligator ration as previously described (Else and Trosclair, 2008). After a 16-day fast, blood was collected mid-morning from the spinal vein (Zippel et al., 2003) by the biological staff at the Louisiana Department of Wildlife and Fisheries Rockefeller Wildlife Refuge (Grand Chenier, LA, USA). 10–19 days of fasting was used to ensure the animals were in a post-absorptive state (i.e., fasted) as described by others (Gatten, 1980; Lewis and Gatten, 1985).

2.2. Measurement of plasma substrates

Plasma glucose, triglyceride, and ketone body concentrations were analyzed using PTS Panels glucose and ketone test strips (specific for β -hydroxybutyrate) and read with a CardioChek PA meter (Polymer Technology Systems, Indianapolis, IN, USA). Capillary glucose concentrations as measured using meters are strongly correlated ($r=0.98$; $p<0.0001$) with venous plasma glucose concentrations measured using spectrophotometric methods (Guerci et al., 2003). Similarly, capillary ketone body concentrations measured using a meter are strongly correlated ($r=0.94$; $p<0.0001$) with venous measurements (Guerci et al., 2003).

2.3. Measurement of plasma glucoregulatory hormones

Plasma insulin was determined by homologous radioimmunoassay using antisera to chicken insulin as previously described (McMurtry et al., 1983). Alignments of published insulin sequences for alligators and birds indicate a high degree of conservation across species (Table 2 and Fig. 1).

Plasma glucagon concentrations were determined by a radioimmunoassay kit using antisera to rat glucagon following the

Table 3
Summary of glucagon amino acid sequence homologies.

Species	Accession No.	% Sequence identity with alligator
American alligator (<i>Alligator mississippiensis</i>)	P68954	
Ostrich (<i>Struthio camelus</i>)	P68953	100
Mallard duck (<i>Anas platyrhynchos</i>)	P68952	100
Turkey (<i>Meleagris gallopavo</i>)	P68260	96

manufacturer's protocol (Millipore, Billerica, MA, USA). Glucagon is highly conserved across species. In fact, protein sequence analyses demonstrated 93% homology between rat (accession no. 711649A) and alligator (accession no. P68954) as well as 96% between rat and chicken (accession no. 63750) glucagon. Alignments of published sequences show a high degree of conservation between alligators and birds as well (Table 3 and Fig. 1).

All hormone assays were performed in duplicate in one assay to avoid inter-assay variation. The intra-assay coefficient of variation was 2.4% for insulin and 3.3% for glucagon.

2.4. Alignment of published insulin and glucagon sequences

Protein sequences were obtained from the National Center for Biotechnology Information (NCBI) by performing a BLAST search for avian and alligator insulin and glucagon protein sequences (Tables 2 and 3). These sequences were then uploaded into ClustalW2 (European Molecular Biology Laboratory, Hinxton, UK) and aligned. Sequence similarities were determined by the program. Sequence information was not available for all species examined in this study.

2.5. Statistical analyses

Data were analyzed using one-way ANOVA (non-parametric). When significance was indicated, Dunn's or Tukey post hoc analyses were used. Data are expressed as means \pm SEM. Regression analyses comparing glucose, β -hydroxybutyrate, insulin and glucagon concentrations were performed. All statistical analyses were conducted using SigmaStat 3.0 software (SPSS Inc., Chicago, IL, USA). Significance was accepted if $p \leq 0.05$.

3. Results

3.1. Measurement of plasma substrates

Plasma glucose concentrations were significantly greater ($H=15.97$, $df=3$, $p=0.001$) in all birds examined (in mM/l: owls: 20.0 ± 0.66 ; eagles: 18.0 ± 0.93 ; hawks: 19.7 ± 0.99) compared to

Ostrich insulin	AANQHLCGSHLVEALYLVCGERGFFYSPKAGIVEQCCHNTCSLYQLENYCN	51
Turkey insulin	AANQHLCGSHLVEALYLVCGERGFFYSPKAGIVEQCCHNTCSLYQLENYCN	51
Goose insulin	AANQHLCGSHLVEALYLVCGERGFFYSPKAGIVEQCCHNTCSLYQLENYCN	51
Duck insulin	AANQHLCGSHLVEALYLVCGERGFFYSPKAGIVEQCCHNTCSLYQLENYCN	51
Alligator insulin	AANQRLCGSHLVDALYLVCGERGFFYSPKGGIVEQCCHNTCSLYQLENYCN	51
	:**:***** ***** *_*****	
Alligator glucagon	HSQGTFTSDYSKYLDRRAQDFVQWLMT	29
Ostrich glucagon	HSQGTFTSDYSKYLDRRAQDFVQWLMT	29
Duck glucagon	HSQGTFTSDYSKYLDRRAQDFVQWLMT	29
Turkey glucagon	HSQGTFTSDYSKYLDRRAQDFVQWLMT	29
	*****:*****:*****	

Fig. 1. Alignment of avian and alligator insulin and glucagon amino acid sequences obtained from the National Center for Biotechnology Information (NCBI) as described in Tables 2 and 3. Alignments performed using the online program ClustalW2. Small hydrophobic residues (AVFPMILW) shown in red, acidic residues (DE) in blue, basic residues (RK) in magenta and hydroxyl, sulfhydryl as well as amine residues (STYHCNGQ) appear green.

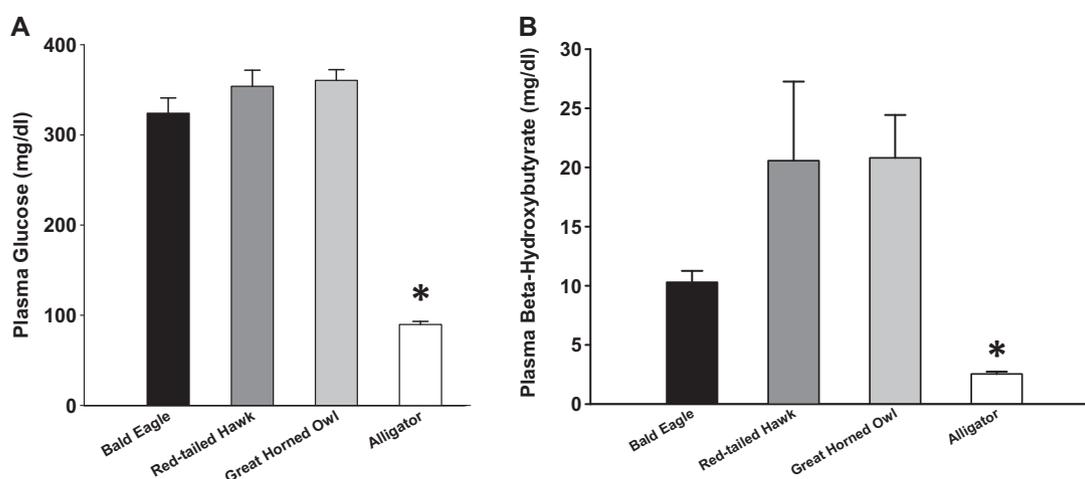


Fig. 2. Comparison of metabolic substrate concentrations in birds and alligators. (A) Fasting plasma glucose levels. Alligators have significantly lower concentrations than birds. Ranges (in mM/l glucose) are as follows: 15.7–20.1 bald eagles, 17.9–22.2 great horned owls, 17.2–22.1 red-tailed hawks, 2.89–4.83 American alligators. (B) Fasting plasma β -hydroxybutyrate (BHO) concentrations. Levels are significantly lower in alligators compared to birds. Eagles, hawks and owls have significantly higher BHO concentrations compared to alligators. Data expressed as means \pm SEM; * $p \leq 0.05$ versus all birds; $n = 4$ bald eagles, 6 great horned owls, 5 red-tailed hawks, and 8 American alligators.

alligators (4.98 ± 0.19 mM/l; Fig. 2A). Similarly, plasma triglyceride concentrations were comparable ($H = 4.92$, $df = 2$, $p = 0.085$) for all birds examined (owls: 72.5 ± 2.5 ; eagles: 78.5 ± 9.2 ; hawks: 61.8 ± 2.6 mg/dl), whereas triglyceride concentrations for alligators were below the detection limit (50 mg/dl). Fig. 2B shows significantly greater plasma β -hydroxybutyrate concentrations ($H = 17.72$, $df = 3$, $p < 0.001$) in birds compared to alligators (2.54 ± 0.2 mg/dl), with the highest concentrations observed in hawks (20.6 ± 6.7 mg/dl) and owls (20.8 ± 3.6 mg/dl). β -hydroxybutyrate concentrations in eagles were 10.3 ± 0.96 mg/dl.

3.2. Measurement of plasma glucoregulatory hormones

Plasma glucagon concentrations were similar between the birds examined (owls: 64.8 ± 4.00 pg/ml; eagles: 74.8 ± 24.4 pg/ml; hawks: 44.2 ± 8.5 pg/ml). Plasma glucagon concentrations were significantly greater in owls as compared with alligators where plasma glucagon was 33.3 ± 1.72 pg/ml ($H = 8.93$, $df = 3$, $p = 0.03$). Plasma insulin concentrations were greater in eagles (1090.8 ± 255.0 pg/ml) and hawks (1194.8 ± 253.2 pg/ml) compared to owls (175.7 ± 84.0 pg/ml), which had the lowest concentrations (Table 4; $F = 5.90$, $df = 3$, $p = 0.005$). Plasma insulin concentrations in alligators (846.2 ± 160.9 pg/ml) were not significantly different from any avian species examined in this study (Table 4).

Plasma insulin and glucose levels were significantly positively correlated in bald eagles as measured by regression analyses ($F = 42.99$, $df = 1$, $p = 0.022$). There were no significant relationships between substrate and hormone concentrations in any other species examined.

Table 4
Plasma insulin and glucagon concentrations.

Animal	Insulin (pg/ml)	Glucagon (pg/ml)	Approximate ratio
Red-tailed hawk	1194.8 ± 253.2	44.2 ± 8.48	27:1
Great horned owl	175.7 ± 84.01^a	64.8 ± 4.00	2.7:1
Bald eagle	1090.8 ± 255.0	74.7 ± 24.40	14.5:1
Alligator	846.2 ± 160.9	33.3 ± 1.72	25:1

Data expressed as mean \pm SEM.

^a $p \leq 0.05$ versus eagles, hawks; $n = 4$ bald eagles, 6 great horned owls, 5 red-tailed hawks, and 8 American alligators.

3.3. Alignment of published insulin and glucagon sequences

Insulin (90–94%) and glucagon (96–100%) are highly conserved between birds and alligators (Tables 2 and 3; Fig. 1).

4. Discussion

The major findings of this study are: (i) insulin and glucagon are highly conserved between alligators and birds; (ii) red-tailed hawks, great horned owls and bald eagles had higher plasma glucose, triglyceride and β -hydroxybutyrate concentrations than alligators; (iii) plasma glucagon concentrations were similar for all birds examined and great horned owls had significantly higher plasma glucagon concentrations than alligators; (iv) plasma insulin was highest in eagles and hawks whereas owls had the lowest concentrations; and (v) alligator insulin concentrations were not different from those of any avian species examined in this study.

Data from the present study indicate that carnivorous birds have plasma glucose concentrations that are 3.6–4 times higher than that of alligators (Fig. 1). Moreover, glucose concentrations for all carnivorous birds examined in this study were higher than that of domestic chickens (12.1 mM/l; O'Donnell et al., 1978) but are comparable to data from other carnivorous avian species (Table 1). As carnivorous birds rely more heavily on gluconeogenesis than chickens, this supports our original hypothesis (Migliorini et al., 1973; Veiga et al., 1978). The plasma glucose concentrations quantified for alligators in the present study (4.98 mM/l; Fig. 2) are similar to those of other reptiles. In a large data set, Dessauer (1970) reported mean plasma glucose concentrations of 4.48 ± 3.0 mM/l (1.39–10.67 mM/l) for 55 species of reptiles from four families. In contrast, glucose is maintained at consistently higher concentrations across avian species (Braun and Sweazea, 2008). For example, blood sugar concentrations of black vultures (*Coragyps atratus*) are remarkably stable during a prolonged fasting period of 3 days, which is attributed to increased gluconeogenesis (Migliorini et al., 1973).

The respiratory quotient (RQ) of humans and rats running at 75% of their maximum rate of oxygen uptake is ≥ 0.90 , indicative of the use of primarily carbohydrates. In contrast, birds rely primarily on fatty acids to fuel the high metabolic demands of flight as evidenced by an RQ of 0.73 for pigeons in flight (Rothe et al., 1987; Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002; Kuzmiak et al., 2012). Moreover, isolated mitochondria from birds

have been shown to preferentially oxidize fatty acids (Kuzmiak et al., 2012). Although alligators similarly rely on fatty acids for metabolism (Black et al., 1963), their comparably lower metabolic rate may explain why birds have relatively higher concentrations of plasma ketones (β -hydroxybutyrate) and triglycerides compared with alligators (Section 3.2; Fig. 2B). In fact, the highest levels of β -hydroxybutyrate were observed in hawks and owls (Fig. 2B).

Chicken plasma and endocrine pancreatic glucagon concentrations are reportedly 5–10 times those typically found in mammals (Hazelwood, 1973). In addition, pancreatic glucagon concentrations are 20-fold and 100–200-fold higher in chickens and domestic pigeons (*Columba livia*), respectively, as compared to humans (Pollock, 2006). Similar observations have been made for bald eagles and red-tailed hawks that have insulin to glucagon ratios of 0.18 and 0.44, respectively, indicative of higher pancreatic glucagon as compared to insulin concentrations (Minick and Duke, 1991; Minick et al., 1996). Further, the α - to β -cell ratio in the pancreas of spotted owlets (*Athene brama*) is 2.4:1, indicative of a greater capacity to produce glucagon than insulin (Guha and Ghosh, 1978). Thus, glucagon appears to be a prominent gluco-regulatory hormone in many avian species. For mammals, high concentrations of the catabolic hormone glucagon are seen in diabetes, starvation and exercise (Hazelwood, 1984). These findings support prior claims that birds are normally in a catabolic state (Hazelwood, 1984). Moreover, studies have demonstrated that birds are highly sensitive to glucagon as administration of this hormone results in hyperglycemia as well as increases in plasma free fatty acids and triglycerides (Hazelwood, 1973). Similar to birds, Indian sand lizards (*Uromastix hardwickii*) are resistant to the glucose-lowering effects of insulin, which has been attributed to their large number of pancreatic α -cells (Kumar and Khanna, 1977). Bullfrogs also have large numbers of pancreatic α -cells as compared to β -cells (α/β ratio: 7.38) (Tomita and Pollock, 1981). In contrast, Lance et al. (1984) found that the alligator pancreas contains 54 mg/kg insulin and 26.5 mg/kg glucagon, an α/β ratio of 0.49. However, a major criticism of analyzing pancreatic gluco-regulatory hormones is the potential for misleading results as regional heterogeneity in the expression of α - and β -cells often exists in the pancreas of reptiles and birds (Bonner-Weir and Weir, 1979). Moreover, studies have shown that insulin and glucagon expression are not limited to the endocrine tissue, but can also be found in the exocrine tissue of the alligator pancreas (Buchan et al., 1982).

In the present study, there were no significant differences between glucagon concentrations in the carnivorous species examined, despite significant differences in plasma insulin (Table 4). In addition, insulin concentrations for all species examined were higher than glucagon concentrations (Table 4) as well as values measured for eagles and hawks in previous studies (Minick and Duke, 1991; Minick et al., 1996). The high concentrations of insulin reported in the current study may be attributed to the use of a radioimmunoassay specific for avian (chicken) insulin whereas prior studies used radiolabeled mammalian insulin, which may be less specific. The data suggest that glucagon may be more important in the regulation of substrate metabolism in granivorous species, such as chickens. Considering the role of insulin in promoting elevated free fatty acid concentrations in fasted birds (Hazelwood, 1973), it is not surprising that triglyceride and β -hydroxybutyrate concentrations were also high in the birds examined in the present study (see Section 3.2; Fig. 2B).

Insulin was derived early in evolution and comparisons of porcine and primitive Atlantic hagfish (*Myxine glutinosa*) insulin demonstrate that this hormone has been well-conserved across vertebrate evolution (Peterson et al., 1975; Muggeo et al., 1979). Moreover, due to their common ancestry, reptilian insulin and glucagon are more related to avian homologs than those of mammals (Lance et al., 1984) and show a high degree of conservation

(Fig. 1). For invertebrates, insulin participates predominantly in tissue growth and development, whereas insulin-like growth factors assume this function in vertebrates with insulin taking on the role of a gluco-regulatory hormone (Chan and Steiner, 2000). In the present study, plasma insulin concentrations were highest in eagles and hawks, whereas owls had the lowest concentrations (Table 4). These findings are similar to those of studies by Minick and colleagues, which showed lower plasma insulin concentrations for great horned owls (Minick, 1986) as compared to bald eagles (Minick and Duke, 1991) or red-tailed hawks (Minick et al., 1996). Unlike these prior studies, however, all species examined in the present study had higher fasting insulin to glucagon ratios (Table 4), which in mammals is suggestive of diminished insulin sensitivity. These findings are also in contrast to granivorous species that have insulin to glucagon ratios of 0.5 or less, indicative of higher glucagon expression patterns and the need to mobilize sufficient energy to maintain high metabolic rates (Pollock, 2006). However, the findings for glucagon should be interpreted with caution as glucagon degradation by plasma proteases, as seen in human samples, cannot be ruled out (Harris et al., 1978).

The high concentrations of both glucose and insulin observed in the present study are consistent with prior studies which concluded that birds are remarkably resistant to the glucose-lowering effects of insulin (Chen et al., 1945). Pancreatectomy of ducks, pigeons, and chickens results in transient hyperglycemia without the development of glycosuria (Nelson et al., 1942). These findings led to the hypothesis that an extra-pancreatic source of insulin may exist in some birds. However, this potential source has yet to be identified (Colca and Hazelwood, 1976; Sitbon et al., 1980). Likewise, partial pancreatectomy of Indian sand lizards (*U. hardwickii*) results in transient hypoglycemia with levels returning to normal within one week (Khanna and Kumar, 1975). Further studies of turtles (*Chrysemys dorsignii*) show that administration of the pancreatic β -cell toxin, streptozotocin, results in hyperglycemia in only 14% of the animals (Muniz and Marques, 1989) indicating that some reptiles may also be insulin resistant.

Previous studies have shown that alligators are similarly glucose intolerant as demonstrated by prolonged elevations in plasma glucose concentrations after a glucose tolerance test (Coulson and Hernandez, 1983). Moreover, juvenile alligators similar in size to the ones used in the present study have demonstrated sensitivity to the glucose-lowering effects of bovine, but not alligator, insulin (Lance et al., 1993). De-pancreatized alligators develop prolonged hyperglycemia ultimately ensuing in death from ketosis 2–4 months post-operation if insulin injections are not administered to normalize blood sugar (Penhos et al., 1967). Similarly, carnivorous birds such as hawks, falcons, buzzards, and ravens develop glycosuria following pancreatectomy (Nelson et al., 1942). Moreover, studies have demonstrated that great horned owls are as sensitive to insulin as mammals in that pancreatectomy results in marked hyperglycemia and death that is preventable with insulin treatment (Nelson et al., 1942). However, others have shown that great horned owls are resistant to hypoglycemic convulsions associated with supraphysiologic doses of insulin (Scott and Chen, 1946).

In conclusion, plasma glucose, triglyceride and β -hydroxybutyrate concentrations for American alligators are relatively low as the animals have very low metabolic rates (Stevenson et al., 1957). Carnivorous birds develop high insulin, glucose, triglyceride, and β -hydroxybutyrate concentrations in response to fasting. Owls in particular maintain the lowest fasting insulin in combination with the highest plasma glucose concentrations (Table 4 and Fig. 2). Together, these findings suggest that owls may be less sensitive to the glucose-lowering effects of insulin as compared to the other species examined. Alternatively, the findings may point to higher rates of gluconeogenesis in

owls as compared to the Falconiformes examined in this study. Studies have shown that plasma glucose concentrations in barn owls remain elevated 3.5 h after an intravenous glucose challenge whereas levels in chickens return to normal within 30 min, suggestive of relative glucose intolerance in barn owls (Myers and Klasing, 1999). For owls, this glucose intolerance has been attributed to low hepatic glucokinase activity in combination with low suppression of gluconeogenesis in the presence of exogenous glucose (Myers and Klasing, 1999). The ability to maintain high plasma glucose concentrations through endogenous production likely protects the animals during periods of scarce food resources (Brand-Miller and Colagiuri, 1999). Insulin resistance in humans is thought to have evolved as a result of high-protein, low-carbohydrate diets as well as during periods of fasting. It has additionally been hypothesized that insulin resistance was considered the normal genotype for humans near the end of the last Ice Age (Brand-Miller and Colagiuri, 1999). Insulin resistance is thought to have similarly evolved in carnivores to shuttle glucose away from skeletal muscles to ensure glucose availability for the brain and other vital organs (Brand-Miller and Colagiuri, 1999). High-protein diets promote gluconeogenesis and glycogenolysis, both of which may contribute to the comparatively high plasma glucose concentrations measured for the raptors examined in this study as compared to non-carnivorous species of birds that have been examined by others (Brand-Miller and Colagiuri, 1999).

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