A Comparative Study of Hummingbirds and Chickens Provides Mechanistic Insight on the Histidine Containing Dipeptide Role in Skeletal Muscle Metabolism

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Abstract
Histidine containing dipeptides (HCDs) have numerous ergogenic and therapeutic properties, but their primary role in skeletal muscle remains unclear. Potential functions include pH regulation, protection against reactive oxygen/nitrogen species, or Ca2+regulation. In recognition of the challenge of isolating physiological processes in-vivo, we employed a comparative physiology approach to investigate the primary mechanism of HCD action in skeletal muscle. We selected two avian species (i.e., hummingbirds and chickens), who represented the extremes of the physiological processes in which HCDs are likely to function. Our findings indicate that HCDs are non-essential to the development of highly oxidative and contractile muscle, given their very low content in hummingbird skeletal tissue. In contrast, their abundance in the glycolytic chicken muscle, indicate that they are important in anaerobic bioenergetics as pH regulators. This evidence provides new insights on the HCD role in skeletal muscle, which could inform widespread interventions, from health to elite performance.

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A Comparative Study of Hummingbirds and Chickens Provides Mechanistic Insight on the Histidine Containing Dipeptide Role in Skeletal Muscle Metabolism

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Histidine containing dipeptides (HCDs) have numerous ergogenic and therapeutic properties, but their primary role in skeletal muscle remains unclear. Potential functions include pH regulation, protection against reactive oxygen/nitrogen species, or Ca²⁺ regulation. In recognition of the challenge of isolating physiological processes in-vivo, we employed a comparative physiology approach to investigate the primary mechanism of HCD action in skeletal muscle. We selected two avian species (i.e., hummingbirds and chickens), who represented the extremes of the physiological processes in which HCDs are likely to function. Our findings indicate that HCDs are non-essential to the development of highly oxidative and contractile muscle, given their very low content in hummingbird skeletal tissue. In contrast, their abundance in the glycolytic chicken muscle, indicate that they are important in anaerobic bioenergetics as pH regulators. This evidence provides new insights on the HCD role in skeletal muscle, which could inform widespread interventions, from health to elite performance.

The histidine containing dipeptide (HCD) carnosine, and its related methylated analogues (anserine and baleine), are formed by β-alanine and L-histidine, and are purported to have pleiotropic ergogenic and therapeutic effects. These include the enhancement of high-intensity exercise performance1, protection against the effects of senescence2, neuro-protection3,4 and tumour inhibition5. These influences may occur due to a number of mechanisms, namely intracellular proton buffering, protection against reactive species and/or the regulation of Ca²⁺ transients and sensitivity6,7. Their primary role, however, is unknown. This renders targeted intervention difficult, meaning that the full therapeutic and ergogenic potential of these dipeptides remains unexploited. The abundance of HCDs (carnosine) in human skeletal muscle (approximately 20–30 mmol.kgDM⁻¹ in m. vastus lateralis8), along with evidence of a positive influence on exercise performance1, implies an important role in skeletal muscle metabolism. Elucidation of their primary role in skeletal muscle is challenging, however, as all potential processes to which they may contribute up-regulate concurrently and cooperatively in response to high-intensity activity. Similarly, many of the conditions in which HCDs have been reported to convey a therapeutic benefit are multi-factorial, therefore potentially benefitting from most, or all, of the HCDs purported biological functions.

In recognition of the challenge of isolating biological processes in-vivo, we employed a comparative physiology approach to provide new insight into the primary mechanism of HCD action in skeletal muscle metabolism. More specifically, we selected two physiologically distinct avian species (namely hummingbirds and chickens), whose unique skeletal muscle types represented the extremes of biological processes in which HCDs are most likely to exert their primary influence. An overview of the contrasting characteristics of the hummingbird and chicken m. pectoralis are described in Fig. 1. Of particular relevance to this investigation, is the hummingbird's
remarkable skeletal muscle characteristics, which gives rise to its unique locomotive ability. Hummingbirds have an outstanding capacity to accelerate and to alter flight speed, trajectory and body orientation\textsuperscript{9,10}. They are also capable of beating their wings with sufficient frequency to hover. This characteristic is unique among avian species, and occurs due to their extraordinarily high wing-beat frequency, which is the fastest of all vertebrates\textsuperscript{11,12}. This is achieved through highly developed contractile properties, and requires optimised regulation of Ca\textsuperscript{2+} transients and sensitivity\textsuperscript{13}. Hummingbirds also have the highest mass-specific metabolic rate of all vertebrates\textsuperscript{14}, achieved through an outstanding capacity to deliver, uptake and utilize oxygen\textsuperscript{9}, allowing a constant supply of aerobically generated energy to the working muscles\textsuperscript{15,16}. This extremely advanced oxidative system, must be accompanied by an equally well-developed system to neutralise the metabolic by-products of oxidative phosphorylation, namely reactive species\textsuperscript{17}. Consequently, if the main role of HCDs in skeletal muscle metabolism is to act either in the primary protection against reactive species or in the regulation of calcium transients and sensitivity, then they would be abundantly expressed in the hummingbird flight musculature. In contrast to their outstanding oxidative and contractile properties, hummingbirds have limited capacity for anaerobic metabolism\textsuperscript{18,19}, most likely because their aerobic capacity renders anaerobic bioenergetics largely unnecessary. Indeed the phosphofructokinase:lactate dehydrogenase enzyme activity ratio in hummingbirds is far higher than in other vertebrates\textsuperscript{19}, indicating that the glycolytic conversion of glucose to pyruvate is designed for complete oxidation through the krebs cycle and electron transport chain, and not for conversion of pyruvate to lactate, as occurs when insufficient oxygen is available for complete oxidative metabolism in the mitochondria. Given that intramuscular acidosis occurs as a result of hydrogen ion accumulation during anaerobic metabolism, the highly advanced aerobic capacities of the hummingbird, and thus, reduced reliance on anaerobic metabolism mean that their flight musculature is not routinely exposed to high acid loads, and therefore has limited requirement for a highly adapted intracellular physicochemical buffering system. Therefore if the primary role of carnosine is to act as an intracellular buffer, high levels would not be required in the highly aerobic hummingbird tissue. The HCD content of the hummingbird flight muscle is, however, unknown.

Figure 1. Characteristics of the Hummingbird & Chicken M. Pectoralis\textsuperscript{6,9,11,19,20,22,37,38,56–60}.
Conversely, the chicken flight musculature has evolved as a largely vestigial tissue, and is primarily composed of glycolytic muscle fibres, rendering these muscles capable of short bursts of powerful contraction when required (e.g., to escape imminent danger). They have limited capacity for oxidative metabolism, or muscle contraction and, therefore, they cannot undertake sustained flight. Accordingly, chicken flight is largely dependent on anaerobic energy metabolism. Chickens have previously been reported to have high skeletal muscle HCD content\(^6,21,22\), and this has been interpreted as implying a primary role for HCDs as intracellular proton buffers\(^23\). The legs are the primary locomotive unit of this land-based bird, and are known to have a greater oxidative capacity, but lower HCD content than the predominantly glycolytic \(m.\) pectoralis\(^{22}\). As such, the chicken \(m.\) vastus lateralis would represent an “intermediate” muscle type between the highly oxidative hummingbird, and highly glycolytic chicken \(m.\) pectoralis.

The primary aim of this study was to measure the HCD content of the flight muscle (\(m.\) pectoralis) in both species, along with the chicken \(m.\) vastus lateralis. Cytochrome C oxidase, subunit IV (COX IV) and lactate dehydrogenase (LDH) content, superoxide dismutase (SOD) activity and \textit{in vitro} muscle buffering capacity (\(\beta_m\)), were also measured, and used to provide an indication of the aerobic and anaerobic capacities of the muscle types under investigation, thus offering further mechanistic insight into the role of HCDs in skeletal muscle metabolism.

**Results**

**HCD content.** Total HCD content (i.e., carnosine + anserine) was significantly different between species (\(p < 0.001\) for all between-muscle sample comparisons; Fig. 2), with the HCD content of hummingbird \(m.\) pectoralis (7.46 ± 2.6 mmol.kgDM\(^{-1}\)) being substantially lower than both the chicken \(m.\) vastus lateralis (91.18 ± 9.10 mmol.kgDM\(^{-1}\)) and \(m.\) pectoralis (206.69 ± 17.76 mmol.kgDM\(^{-1}\)). Consistently higher levels of anserine compared to carnosine were recorded in all tissues (\(p < 0.001\) for all within sample comparisons).

**Muscle characterisation.** Data on COX IV and LDH content, SOD activity and \(\beta_m\) are presented in Fig. 3. Hummingbird \(m.\) pectoralis had substantially higher COX IV content and SOD activity than either of the two chicken muscle samples (\(p < 0.001\) for all between-species comparisons). The difference in COX IV and SOD activity between chicken \(m.\) vastus lateralis and \(m.\) pectoralis were non-significant (\(p = 0.949\) and 0.058). Hummingbird \(m.\) pectoralis had substantially lower LDH content than either chicken muscle type (\(p < 0.01\) for all between species comparisons), while chicken \(m.\) vastus lateralis had more LDH than \(m.\) pectoralis (\(p < 0.01\)). \(\beta_m\) of the chicken \(m.\) pectoralis was higher than \(m.\) vastus lateralis (\(p < 0.001\)), while both chicken samples had a higher \(\beta_m\) than the hummingbird \(m.\) pectoralis (both \(p < 0.01\); Fig. 3, Panel D). Calculation of the non-HCD buffering capacity using the Henderson-Hasselbalch equation indicated that the HCD buffering contribution (\(\beta_{m_{\text{HCD}}}\)) was responsible for all of the reported variation in \(\beta_m\) between the three muscle samples (\(\beta_{m_{\text{non-HCD}}} = 79.2 ± 9.9\) mmol.kgDM\(^{-1}\); \(p > 0.05\) for all comparisons) (Fig. 3, Panel D).

**Prediction analysis.** Pearson’s bivariate correlation analysis showed that total HCD content was strongly and significantly correlated with COX IV (\(R = −0.777\); \(p < 0.001\)), SOD activity (\(R = −0.889\); \(p < 0.001\)) and \(\beta_m\) (\(R = −0.931\); \(p < 0.001\)) but not with LDH (\(r = 0.327\); \(p = 0.217\)). Multiple linear regression was used to identify the predictive value of these independent variables on the primary outcome (total HCD content). Only those variables that had a statistically significant bivariate correlation with total HCD were included in the model (namely COX IV, SOD activity and \(\beta_m\)). Variables were entered using the stepwise method. COX IV did not statistically contribute to the model and was excluded. Both \(\beta_m\) and SOD activity significantly contributed to the prediction of total HCD content (\(F(2, 14) = 118.3, p < 0.001, R^2 = 0.944\)). The linearity of the correlation between \(\beta_m\) and total HCD (Fig. 4, Panel B) confirms that \(\beta_{m_{\text{non-HCD}}}\) is essentially the same in all three muscles, which, from the intersect with the y-axis, is approximately 80 mmol.kgDM\(^{-1}\).
Discussion

The renowned physiologist August Krogh famously stated "For a large number of problems, there will be an animal of choice, or a few such animals on which it can be most conveniently studied"\(^24,25\). In recognition of the difficulty of studying isolated mechanistic pathways in in-vivo models, we employed a comparative physiology approach to examine the primary HCD role in skeletal muscle metabolism. The very low HCD content in the hummingbird tissue, which has remarkable oxidative and contractile properties, indicates that HCDs are not essential to these processes, and that their primary physiological function is unlikely to involve the primary reduction of reactive species, nor the regulation of Ca\(^{2+}\) transients or sensitivity. In contrast, HCDs were abundant in chicken \textit{m. pectoralis} and \textit{m. vastus lateralis}. These tissues (particularly the chicken \textit{m. pectoralis}) have limited oxidative capacity and are, therefore, largely reliant upon anaerobic forms of energy metabolism that subsequently challenges pH homeostasis. Collectively, these findings indicate that the primary physiological function of HCDs, within skeletal muscle metabolism, is to act as intracellular physicochemical buffers.

Our assertion that the primary physiological role of the HCDs is to buffer H\(^+\) across the physiological pH range, is supported by the strong and positive linear relationship reported between total HCD content and \(\beta m\).
The ratio of carnosine: anserine in these birds shown herein is interesting. Anserine is a carnosine analogue; the primary difference between these two molecules being that anserine has 1-methyl histidine in place of L-histidine, which is found in carnosine. The physiological relevance of the methylated versus the non-methylated forms of these HCDs is not clear. Anserine was consistently higher than carnosine in all of the muscle samples investigated in the current study (see Fig. 2). Conversely, carnosine is believed to be the only HCD in human skeletal muscle (~20–30 mmol.kgDM⁻¹), although anserine is expressed in other human tissues (e.g., the kidney). In vitro evidence indicates that carnosine has the capacity to influence muscle contractility by enhancing the sensitivity of the sarcoplasmic reticulum Ca²⁺ release channels. This is often proposed as a potential mechanism that may underpin the effect of carnosine on high-intensity exercise performance, which is characterised by high contractile activity. Hummingbirds have the highest wingbeat frequency recorded for any avian species, along with remarkable dexterity. Indeed, it has been proposed that the m. pectoralis of the hummingbird has two primary functions, namely locomotion and thermogenesis, both of which rely on calcium release and re-uptake from the sarcoplasmic reticulum Ca²⁺ pumps. Given the low HCD levels in the hummingbird flight musculature, accompanied by their high reliance on Ca²⁺ release for locomotion and thermogenesis, it is unlikely that HCDs are primarily involved in the development of a highly functional muscle. Increased intramuscular acidity has been reported to influence sarcoplasmic reticulum Ca²⁺ release and, therefore, a role for HCDs in maintaining the pH of the intracellular environment may indirectly act to regulate muscle contractility during intense exercise.

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Some functional differences between carnosine and anserine have been reported, including distinct pK\textsubscript{a}s and protective properties against reactive species\cite{49,50}. The distinct physiological role of these dipeptides, and the unique contribution of anserine versus carnosine to skeletal muscle metabolism, along with tissue-specific HCD effects, represent exciting research opportunities that warrant further investigation.

**Conclusion**

In conclusion, our findings indicate that HCDs are non-essential to the development of a highly oxidative and contractile muscle, given their very low content in hummingbird *m. pectoralis*, indicating that their main physiological role is unlikely to involve protection against primary reactive species or the regulation of Ca\textsuperscript{2+}. In contrast, HCD content was highest in chicken *m. pectoralis*, a tissue that has adapted to primarily metabolise substrates via anaerobic bioenergetic pathways, experiencing regular challenges to pH homeostasis as a result. Collectively, our results indicate that the primary mechanistic role of HCDs in skeletal muscle metabolism is to enhance anaerobic bioenergetics by acting as an intracellular physicochemical buffer.

**Methods**

**Sample collection.** Samples were collected from wild hummingbirds (amazilia fimbriata, n = 5) and free-living domestic chickens (gallus domesticus, n = 6). Wild-type and free-living birds were selected to replicate natural living conditions and behaviours as closely as possible. All hummingbirds were captured in the state of Sao Paulo Brazil, using mist nets (Ecotone\textsuperscript{®} M-14/2). Permission to capture wild hummingbirds was obtained from the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA no: 49347-2). Experimental procedures for this study were approved by the Bioscience Institute of the University of Sao Paulo Bioethics Committee (CEUA protocol number 222/2015), and all experiments were performed in accordance with all institutional and governmental guidelines and regulations. Hummingbirds were sacrificed using anaesthesia on the day of capture. Chicken samples were obtained from birds raised on a farm in the state of Sao Paulo. All chickens were sacrificed on-site by cervical dislocation. Following muscle dissection, all samples were flash frozen in liquid nitrogen, then stored at −80 °C until further analysis.

**Analytical procedures. HCD content.** Total HCD content was determined using high performance liquid chromatography (HPLC) with UV detection (Hitachi Ltd., Tokyo, Japan), according to previously described methods\cite{51}. Deproteinised muscle extracts were obtained from 3–5 mg of lyophilised samples\cite{52}. All chromatography was carried out at room temperature. Deproteinized extracts (20 µL) were injected via an auto sampler using a cut injection method, and chromatographic separation was achieved using an Atlantis HILIC silica column (4.6 × 150 mm, 3 μm; Waters, Massachusetts, USA) attached to an Atlantis Silica column guard (4.6 × 20 mm, 3 μm). The method used two mobile phases: Mobile phase A (0.65 mM ammonium acetate, in water/acetonitrile (25:75) (v/v)), and mobile phase B (4.55 mM ammonium acetate, in water/acetonitrile (70:30)), with both solutions adjusted to pH 5.5 and filtered using a 0.2 μm filter membrane. The separation condition comprised a linear gradient from 0 to 100% of solvent B for 13 min at a flow rate of 1.4 mL·min\textsuperscript{−1}. Separation was monitored using an ultraviolet detector at a wavelength of 214 nm. Standard curves for carnosine and anserine were performed prior to analysis using concentrations of 0.1, 0.25, 0.5, 1, 2.5, and 5 mM, and regression equations generated using the area under the curve (AUC) of generated peaks (R\textsuperscript{2} = 0.999 and >0.999 for carnosine and anserine). Carnosine content was quantified by plotting the AUC of each sample against standard curve data, and reported as mmol·kg\textsuperscript{DM}·\textsuperscript{−1}.

**Aerobic and anaerobic enzyme content.** The abundance of COX IV (a subunit of cytochrome C oxidase, and the terminal enzyme in the respiratory chain) and LDH (which catalyzes the inter-conversion of pyruvate and NADH to lactate and NAD\textsuperscript{+}) were analysed using the Western Blot technique. Lysis buffer (RIPA, Sigma-Aldrich Ltd), with added phenylmethylsulfonyl fluoride (PMSF) (1 mM) and protease and phosphatase inhibitors were added to the muscle samples (10 µL of buffer·mg\textsuperscript{−1} of wet muscle). The muscle was homogenized using a bench-top motor driven homogenizer, with intermittent vortexing. The homogenate was then centrifuged at 12,902 relative centrifugal force (RCF), and the supernatant extracted. Total protein content was assessed using the Bradford technique, and the results used to calculate the quantity of Laemmli loading buffer required (1.5 μL·µg). Samples were separated by SDS-PAGE in pre-cast polyacrylamide gels (4–20%, Bio Rad Laboratories Inc), with equal loading in each well (30 µg of protein). A molecular mass marker (Precision Plus TM Dual Color Standards, Bio Rad Laboratories Inc) was used to locate the proteins of interest (COX IV: 17 kDa; LDH: 37 kDa). Proteins were transferred to a nitrocellulose membrane using gel electrophoresis (75 minutes at 100 V, 4 °C). Total protein transfer was visualised using Ponceau staining (Supplementary File 1), and the membranes were then washed for 3 × 10 minutes in TBS-T, followed by a 10 min wash in TBS. The membranes were blocked in a 5% blocking solution (TBS-T with non-fat milk powder) for 2 hours then the wash procedure was repeated. Membranes were then incubated with the primary antibody for 12–14 hours at 4 °C (Cell Signalling Technology® #2012 (LDHA) & 4850 (COX IV; 3E11)). Following the primary antibody incubation, membranes were washed as described above, then incubated in an anti-rabbit, horseradish peroxidise (HRP) linked secondary antibody (Cell Signalling Technology® # 7074S). Excess secondary antibody was washed, and the membranes were exposed to an enhancing solution at room temperature, then visualised by chemiluminescence using the Image Quant LAS 4000 (GE Healthcare®). Protein content was quantified using densitometry (Scion Image\textsuperscript{®}), and all bands were normalised to a positive control comprising human *m. vastus lateralis*, with 40 mg of protein.

**Superoxide dismutase (SOD) activity.** Superoxide dismutase (SOD) activity was measured based on the inhibition of xanthine/xanthine-oxidase-driven cytochrome C reduction by the activity level of SOD\cite{53,54}. Approximately 25–35 mg of wet muscle was homogenized in phosphate buffer (50 mM KH\textsubscript{2}PO\textsubscript{4}, 50 mM K\textsubscript{2}HPO\textsubscript{4}, pH 7.8, protease inhibitors; 1:4 mg·mL\textsuperscript{−1}), using a bench-top motor driven homogenizer. The homogenate was centrifuged...
at 12,902 RCF for 15 min, and the supernatant used to measure SOD activity. Protein content was measured using the Bradford technique. Initially, the rate of cytochrome C reduction was measured in the absence of sample for 5 minutes at an absorbance of 550 nm, through mixing a solution containing EDTA (0.1 mM), xanthine (50μM) and cytochrome C (20μM) and xanthine oxidase (diluted in the phosphate buffer (50 mM), with another solution containing phosphate buffer (50 mM) with added EDTA (0.1 mM) and xanthine oxidase (0.2 U·mL−1). Subsequently, the cytochrome C reduction rate was measured in the presence of the sample homogenate, containing 20μg of protein. SOD activity was calculated according to the difference in the two reduction rates, and measured within the linear range of the assay. SOD activity is expressed in units (U), whereby 50% inhibition of the cytochrome C reduction rate was considered to represent 1U of SOD activity.

Muscle buffering capacity (βm). The non-bicarbonate skeletal muscle buffering capacity (βm) was assessed using the homogenate titration technique, as previously described. Whole muscle samples were lyophilised, then extracts of powdered tissue (approximately 2.5–3.5 mg) were homogenized by intermittent vortexing (5 x 20s passes, interspersed with 30 s periods on ice) in a non-buffered NaF solution (10 mM; 30 mgDM·ml−1). Samples were subsequently equilibrated for 5 minutes at 37°C and with constant motion, using a thermorimer. The pH was measured using a micro-electrode (InLab Micro; Mettler Toledo) connected to a pH meter. Prior to starting the experiment, the pH of the muscle homogenates was adjusted to 7.1, using an NaOH solution (0.02 M). Homogenates were subsequently titrated to pH 6.5 through the repeated addition of 2 μL of a HCl solution (10 mM). The total amount (moles) required to change the pH from 7.1 to 6.5 was recorded, and the value normalised to the starting weight (kg) of dry tissue used in each experiment (mmol.kgDM−1). The HCD contribution to total muscle buffering capacity was calculated using a derivation of the Henderson-Hasselbalch equation, as previously described according to the calculation:

\[
\beta_{HCD} = \left(\frac{[HCD]}{(1 + 10^{(6.5-pK_a)})} \right) - \left(\frac{[HCD]}{(1 + 10^{(7.1-pK_a)})} \right)
\]

where [HCD] is the content of either carnosine and anserine in mmol.kgDM−1, and assuming a pKa of 6.83 and 7.04 for carnosine and anserine.

For each sample, the buffering contributed specifically by carnosine and anserine between pH 7.1 and 6.5 was calculated using the above equation, and the sum of the HCD contribution to βm was subtracted from the total muscle buffering capacity to provide a measure of the non-HCD buffering capacity, i.e. \(\beta_{non-HCD} = \beta_m - \beta_{HCD}\).

Statistical Analysis. Data were analysed using the Statistical Package for Social Sciences (SPSS version 17.0). One way ANOVA, with tukey post hoc adjustment, was used to locate differences between the three muscle types (hummingbird m. pectoralis, chicken m. vastus lateralis, chicken m. pectoralis) for all outcome measures. Bivariate correlation analysis between all independent variables (COX IV, LDH, SOD activity and βm) and the dependent variable (total HCD content) was conducted using Pearson’s correlation coefficient. Multiple linear regression was used to identify the predictive contribution of these independent variables on the primary outcome of interest, namely total HCD content. Variables were entered using the stepwise function, which functions by building a predictive model through adding or removing variables based on the t-statistics of their estimated coefficients. Statistical significance was accepted at the level of p < 0.05. All outcomes are reported as mean ± 1SD.

Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author Contributions
B.G., E.D., C.S., G.G.A., R.C., J.E.P.W.B. and R.H. participated in the design and/or the interpretation of the reported results. E.D., B.S., W.S.D. and I.H. participated in the acquisition and/or the analysis of data. E.D. wrote the manuscript, and all authors reviewed and edited this manuscript, before agreeing the final version.

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