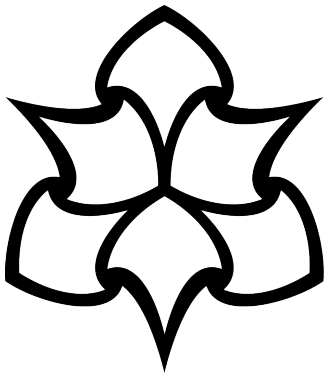

Skeletal Muscle Histidine Containing Dipeptide Contents are Increased in Freshwater Turtles (*Chrysemys picta bellii*) with Cold-Acclimation

Running Title: *HCD content of freshwater turtles.*

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Highlights:

- pH regulation is a major challenge for overwintering freshwater turtles.
- Histidine containing dipeptides are important intracellular buffers.
- Turtles acclimated to 3°C had higher HCD content than those at 20°C.
- HCDs may be important pH regulators in cold-acclimated turtles.

Abstract:

Freshwater turtles found in higher latitudes can experience extreme challenges to acid-base homeostasis while overwintering, due to a combination of cold temperatures along with the potential for environmental hypoxia. Histidine containing dipeptides (HCDs; carnosine, anserine and balenine) may facilitate pH regulation in response to these challenges, through their role as pH buffers. We measured the HCD content of three tissues (liver, cardiac and skeletal muscle) from the anoxia-tolerant painted turtle (*Chrysemys picta bellii*) acclimated to either 3 or 20°C. HCDs were detected in all tissues, with the highest content shown in the skeletal muscle. Turtles acclimated to 3°C had more HCD in their skeletal muscle than those acclimated to 20°C (carnosine = 20.8±4.5 vs 12.5±5.9 mmol·kg DM^{-1}; ES = 1.59 (95%CI: 0.16 – 3.00), P = 0.013). The higher HCD content shown in the skeletal muscle of the cold-acclimated turtles suggests a role in acid-base regulation in response to physiological challenges associated with living in the cold, with the increase possibly related to the temperature sensitivity of carnosine's dissociation constant.

Key-Words: alphastat; pH; acid-base; carnosine; buffering; hypoxia; hibernation.

INTRODUCTION

The ability to maintain acid-base balance within homeostatic limits is essential to maintain cellular function (1). Acid-base homeostasis is constantly challenged by various internal and external factors. For example, anaerobic metabolism is required for continued ATP regeneration when turnover exceeds the oxidative capacity of the cell. This results in hydrogen cation (H^+) accumulation and increased metabolic acidosis (2), which has adverse consequences for numerous cellular processes, including reduced glycolytic enzyme activity, inhibition of oxidative phosphorylation and impaired phosphorylcreatine (PCr) resynthesis (3–5). To avoid these adverse consequences, living organisms have evolved a diverse range of pH regulatory strategies. Intracellular buffers, such as bicarbonate, phosphates, proteins and histidine containing dipeptides (HCDs), provide an important "first line of defence" against intracellular pH perturbations. Simultaneously, "dynamic buffering" is the process by which excess H^+ is removed from the cell via Na/H^+ exchangers and monocarboxylate transporters (6, 7).

In vertebrates, the HCD carnosine (beta-alanyl-L-histidine), along with its methylated analogues balenine (beta-alanyl-1-methyl-L-histidine) and anserine (beta-alanyl-3-methyl-L-histidine), are considered to be important intracellular buffers with acid dissociation constants (pKa's) at physiological temperatures (*i.e.,* 36°C) that render them ideally placed to buffer across physiological pH ranges (8) - for skeletal muscle this is approximately 7.1 – 6.5 (9, 10). Previous studies indicate that these dipeptides are abundant in the skeletal muscle of species with a large capacity for anaerobic energy metabolism, and that have adapted to tolerate high acid loads (11–13). These species include sprinters, such as thoroughbred racehorses and greyhounds (14); avian species with a limited ability for aerobically fueled flight, such as chickens (15, 16); and aquatic mammals that undergo prolonged periods of hypoxia while diving, such as blue or fin whales (17).

North American pond turtles have a remarkable tolerance to hypoxia (to the point of anoxia), during which they experience major challenges to pH homeostasis (18). As such, they represent a fascinating model to investigate pH buffering. During winter, many of these turtles, especially those found in higher latitudes, are forced to overwinter in anoxic water at the bottom of small ponds and swamps. The western painted turtle (*Chrysemys picta bellii)* can survive anoxia at 3°C for more than 170 days, despite oxygen levels being undetectable (18, 19). This remarkable ability to withstand anoxia results from three main evolutionary adaptations: extreme metabolic suppression, large tissue glycogen stores, and a marked capacity to withstand metabolic acidosis (20, 21). Painted turtles can tolerate very high circulating lactate, with plasma levels of up to 200 mM recorded (21), while blood pH falls to ~7.2 from normal pH of 8.1 at 3°C (18), representing a remarkable capacity for buffering and pH regulation. To put this into context, humans undertaking exhaustive exercise experience plasma lactate increases of ~14-18 mM, concomitant to a large export of H^+ out of the muscle, which generally leads to a reduction in pH from approximately 7.4 to 7.1. Turtles' buffering ability is largely achieved via the shell (22), which buffers pH by releasing calcium and magnesium carbonates, and via the direct uptake of lactate and H^+ (23). Less certain in these species is the contribution of intracellular physicochemical buffers in unmineralized tissues, such as the HCDs, which have previously been reported to be abundant in species who experience large challenges to acid-base regulation (13).

In addition to the challenges that extreme hypoxia poses, these ectothermic vertebrates also hibernate in near freezing conditions, which has implications for pH regulation, particularly with regards to the charge state of histidyl residues within proteins. Reeve's alpha-stat hypothesis states that ectotherms shift intracellular and extracellular pH according to temperature in order to maintain constant imidazole ionization, also called alpha (24, 25). HCDs comprise the majority of these intracellular imidazole compounds (11) and as such are likely to contribute toward maintenance of

alpha. Therefore, painted turtles are exposed to two major acid-base stressors while hibernating during winter – anoxia and low temperatures. Theoretically, HCD content may contribute to defending against both these stressors, although little is currently known about the HCD content of these ectothermic vertebrates, nor whether these contents are affected by temperature. The aim of this exploratory study, therefore, was to determine the skeletal muscle, liver and heart HCD content of freshwater western painted turtles who were acclimated to either 3°C or 20°C.

Animals:

Ten adult painted turtles (*Chrysemys picta bellii*; Niles Biological, Sacramento, CA, USA) of both sexes were acclimated to either 3°C (n = 5) or 20° C (n = 5). Prior to temperature acclimation they were maintained for 1-3 months in large tanks filled with partially dechlorinated St. Louis municipal tap water under natural Minnesota photoperiod. The turtles had access to a drying platform, an incandescent light bulb for basking, and a 10W UVB light for nutritional purposes. Air and water temperatures were maintained between 18-22°C. During this time, turtles were fed commercial turtle pellets *ad libitum* three times per week. For the temperature acclimation, the 20°C turtles (n = 5) were transferred to an ~80-liter temperature-controlled aquarium with water temperature thermostatted to 20°C (YSI Model 72) and were held there, without food, for 36-48 hours. The 3°C turtles (n=5) were placed into an ~80-liter aquarium with water temperature initially thermostatted to 20°C. The set temperature was lowered by 2°C per day for just over 8 days until it reached 3°C, and then held there for an additional two weeks. The turtles were not fed during the acclimation period. All procedures were approved by the Saint Louis University Institutional Animal Care and Use Committee (IACUC protocol 2198).

Tissue sampling and preparation

Turtles were euthanized by rapid decapitation. After the plastron was removed with a bone saw, samples of ventricle, liver and pectoralis muscle were removed and immediately flash-frozen with freeze clamps pre-chilled in liquid nitrogen, and then stored at -80°C until analysed. All samples were subsequently lyophilised and powdered before extraction was performed using perchloric acid [$HClO_4$], EDTA and potassium bicarbonate [$KHCO_3$] (26). The neutralised supernatant was collected using a centrifugal filter (0.2 µm), checked to ensure a pH close to 7 and then stored at -80°C until high-pressure liquid chromatographic (HPLC) analysis.

Chromatographic determination of histidine-containing dipeptides

The HCD content was determined by HPLC (Hitachi, Hitachi Ltd., Tokyo, Japan), as per Mora et al. (27) using an Atlantis HILIC silica column (4.6×150 mm, 3 µm; (Waters, Massachusetts, USA). All chromatography was conducted at room temperature. Samples were analysed in duplicate and injected via an autosampler using a loop injection method. Two mobile phases were used. Mobile phase A: 0.65 mM ammonium acetate, in water/acetonitrile (25:75) (v/v). Mobile phase B: 4.55 mM ammonium acetate, in water/acetonitrile (70:30). The pH of both solutions was adjusted to 5.5 using hydrochloric acid and thereafter filtered under vacuum through a 0.2 µm filter membrane. The separation condition comprised a linear gradient from 0 to 100% of solvent B in 13 min at a flow rate of 1.4 $mL \cdot min^{-1}$. Separation was monitored using an ultraviolet detector at a wavelength of 214 nm. The area under the curve (AUC) for carnosine was obtained and used to estimate the content by

comparison to standards of 100, 250, 500 and 1000 µM. The in-house variability of the extraction and analysis methods is 4.0 and 2.5% (28). Another peak in close proximity to carnosine was detected in several samples, which, based on chromatograms from the original article describing our procedure could only be balenine or anserine (27). The retention times of samples spiked with carnosine and anserine led us to conclude the peak could only correspond to balenine. Balenine data are reported as AUC, which were used to compare contents between the animals acclimated to the different temperatures.

Data analysis

Data were analysed using the SAS statistical package (SAS® University Edition, SAS Institute Inc., USA), and are presented as mean±1SD. Carnosine content (mmol·kg DM^{-1}) was analysed using mixed model analysis with animals assumed as a random factor and tissue (3 levels; liver/ventricle/*m. pectoralis*) and environment (2 levels; 3 or 20°C) assumed as fixed factors. Tukey–Kramer adjustments were performed when a significant F value was obtained. Results were interpreted according to the statistical probabilities of rejecting the null hypothesis (H0) and in the following categories: $p > 0.1$: no evidence against H0; $0.05 < p < 0.1$: weak evidence against H0; $0.01 < p < 0.05$: some evidence against H0; $0.001 < p < 0.01$: strong evidence against H0; $p < 0.001$: very strong evidence against H0 (29). Effect sizes (ES) were calculated as the mean difference between the two groups of turtles, divided by the pooled standard deviation and are reported alongside their 95% confidence interval. The theoretical buffering contribution of the observed HCD content for a reduction of 0.6 pH units was calculated using a derivation of the Henderson Hasselbalch equation (14) namely: β_{HCD} ={[HCD]/(1 + 10^(pHi – pKa))} – {[HCD]/(1 + 10^(pHi – pKa))}. For this calculation we assumed the physiologically relevant pHi range was 7.1 down to 6.5 at 20°C (30) and 7.4 to 6.8 at 3° (31) and pKa's for carnosine of 6.702 and 7.209 at 20° and 3°C (32).

RESULTS

HCD Content

HCDs were detected in all examined tissues (Figure 1, Panel A). Carnosine was found in the *m. pectoralis* (16.09 ± 7.00 mmol·kgDM^{-1}) and balenine in the liver, whilst both balenine and carnosine (6.08 ± 2.95 mmol·kgDM^{-1}) were found in the ventricle. Visual inspection of the chromatograms suggested that very small amounts of balenine were present in two of the *m. pectoralis* samples and very small amounts of carnosine in two of the liver samples, although these were below the limits of interpolation and quantification of the detection software. There was very strong evidence of an effect of tissue on MCarn content ($p<0.0001$), but no effect of environment ($p=0.17$). There was strong evidence of a tissue x environment interaction ($p=0.007$), with post-hoc analysis indicating that the turtles acclimated to 3°C had a higher total *m. pectoralis* HCD content (mmol·kg DM^{-1}) than those kept at 20°C (20.8 ± 4.5 Vs 12.5 ± 5.9; ES = 1.59 (95% CI: 0.16 – 3.00; $p = 0.013$; Figure 1, Panel B). There were no differences in MCarn content between environments for the other tissues (both $p=0.99$). Skeletal muscle carnosine content equated to a buffering contribution of 6.83 ± 1.47 and 4.10 ± 1.93 mmol·kg DM^{-1} $(0.6\ pH\ unit)^{-1}$ for 3 (pHi: 7.4 – 6.8) and 20°C (pHi: 7.1 – 6.5; $p = 0.04$; ES: 1.59).

DISCUSSION

The purpose of this study was to determine the intracellular HCD content of anoxia-tolerant painted turtles acclimated to either 3 or 20°C. Overall, the observed HCD contents were unremarkable, when considered relative to those reported in other species. The turtles acclimated to 3°C had a higher *m.pectoralis* carnosine content than those maintained at 20°C, while liver and cardiac HCD contents were not different. This indicates that intramuscular carnosine content may be instrumental in the adaptive response of these ectotherms to a cold environment.

HCD content varies widely between species and is abundant in the skeletal muscle of those with highly evolved capacities to withstand exercise-induced or environmental hypoxia (11, 13). As such, it may have been expected to observe very high HCD levels in painted turtles, given their remarkable capacity to withstand extreme hypoxia, during which they accumulate blood lactate to levels approaching 200 mM (21). This was not, however, the case and the painted turtles investigated herein had low HCD contents when considered within the context of other species (see figure 2). Observed contents were also similar to those reported in other turtle species, including those who do not experience similar challenges to acid-base balance, *e.g.,* the green sea turtle (*Chelonia mydas;* Suborder Cryptodira*)* and eastern long-necked turtle (*Chelodina longicollis;* Suborder Pleurodira) (33, 34), and other species that also experience seasonal variations in water temperature, *i.e.,* Chinese soft-shell turtle(35). It must be highlighted, however, that turtle HCD content has not been comprehensively characterized, with some of these previous studies based on single animals and using a variety of measurement techniques. As such, these comparisons should be interpreted with caution.

The reason for the relatively low intramuscular carnosine content observed in painted turtles (see Figure 2), despite the extreme challenges to acid-base regulation that they face, is unclear, but it could relate to the large availability of other mineralized buffers, along with the length of time across which acidosis occurs in this species. Turtles, and painted turtles in particular, can use the calcium and magnesium carbonate stored in their mineralized tissues (*i.e.,* shell and skeleton) to buffer the acidosis that slowly accumulates over months (23). In contrast, the acidosis incurred by the sprint or diving animals previously reported to be abundant in HCDs (12, 13) is more acute, and occurs when intramuscular H^+ generation is in excess of that which can be actively transported out of the cell. As such, intracellular buffering agents, including tissue HCD contents, may have a greater physiological importance for these animals as opposed to turtles, who experience a more gradual and prolonged acid base stressor while hibernating. In support of this assertion are data suggesting relatively low non-bicarbonate buffering capacities in turtles (34, 36) compared to cetaceans, such as whales (37), who experience the dual challenges of locomotion and hypoxia while diving. In contrast, turtles remain largely stationary when hibernating in anoxic conditions and appear to have solved the buffering problem by exporting H^+ to the circulation, where it is subsequently buffered by calcium and magnesium carbonates released by the shell, or taken up directly (as lactate and H^+) and buffered by the shell (23).

Despite these turtles' relatively low HCD content when considered relative to other species, the cold-acclimated turtles did have a higher content than their counterparts who were maintained at 20°C, which implies a regulatory role for the HCDs in adapting to this stressor. Skeletal muscle forms the largest mass of unmineralized tissue in the turtle, so higher muscle carnosine content could have quantitatively important consequences for acid-base regulation, assuming that it is the predominant histidylated peptide contained therein. Turtles have long been known to be alpha-stat regulators, which means they decrease their PCO_2 in order to increase their relative alkalinity for the purpose of defending the dissociation fraction (alpha) of the imidazole functional groups in histidine residues within their proteome, thereby preserving their charge and conformational states (24). Based on

Reeves (24), the small change in MCarn observed in the present study would have minimal impacts on intracellular pH or Pco_2. Thus, the most important effect will be on the buffering power of the muscle. The pKa of carnosine is increased in colder conditions (7.209 at 3°C vs 6.702 at 20°C), which would contribute to the maintenance of the increased alkalinity, given that each temperature-specific pKa is within the mid-range of the assumed pHi at 3°C (7.4 – 6.8) and 20°C (7.1 – 6.5°C). Estimation of the theoretical buffering contribution of the observed MCarn content indicated a higher buffering capacity in the cold-acclimated turtles, again suggesting that this dipeptide may play an important role in acid-base regulation at this temperature. Increases of the magnitude observed herein are roughly comparable to those observed in humans in response to commonly used BA dosing protocols (38), and would necessitate either a large increase in carnosine synthesis, a large reduction in carnosine degradation, or perhaps a combination of both (39). Given that no external BA source was available to these turtles, increased synthesis could only have occurred via increased endogenous production, alongside an increase in carnosine synthase activity. This seems unlikely to be the only factor, considering the time-periods that were investigated, and so a reduced degradation rate may have contributed (at least in part) to the observed increases. This is, of course, speculative and further research is required both to confirm our findings, and to explore the biokinetics of cold-induced MCarn increases in these ectothermic vertebrates. It is also important to highlight that the number of turtles in each group was small (n = 5), and our estimates are necessarily based on between group comparisons, which does increase the risk of sampling error. As such, caution must be applied when interpreting the magnitude of increase.

Although the importance of the HCDs to intracellular acid-base regulation is well-recognised, these dipeptides are also thought to contribute toward a number of other biological processes that may be relevant to the dual challenges of anoxia and cold temperatures. Carnosine may play a role in metal ion chelation, antioxidant activity, protein carbonylation and glycoxidation, nonpolysomal proteolysis, and nitric oxide metabolism (18). Of these, carnosine's antioxidant activity may be most relevant to severely hypoxic and anoxic tissues like those in overwintering turtles, which, theoretically, experience an increase in ROS from xanthine oxidase activity with the reperfusion of oxygen during spring emergence (39). The higher carnosine content, as observed in the cold-acclimated turtles, could, potentially, increase the antioxidant activity of skeletal muscle and reduce any ROS-mediated injury that might occur during tissue reperfusion.

An interesting finding was the distinct HCD profile within the different tissues. It was unsurprising that skeletal muscle had the largest HCD content, as it has previously been estimated that approximately 99% of the total carnosine is located in this tissue (11). In studies of this kind, three HCD forms are commonly investigated, namely carnosine, and its methylated analogues balenine and anserine. Most species contain at least two HCD forms and this varies largely between phylogenetically distinct species, but is similar within the same, or congeneric, species. Primarily carnosine, and very small quantities of balenine, were identified in these painted turtles, with the latter being absent in skeletal muscle. However the uniformity of HCD distribution of different types in different tissues does imply that they may exert type- or tissue-specific effects. Some biological differences have been reported between the HCD analogues, *e.g.,* each HCD has a subtly different pKa (estimated to be 7.03, 7.04 and 6.83 for anserine, balenine and carnosine at mammalian temperatures, *i.e.,* ~37°C), while distinct anti-radical effects of the different HCD analogues have also been suggested (40, 41). The functional relevance of these subtle physiological differences is unknown and represents an interesting avenue for future research. Interestingly, evidence is emerging that other HCD forms are also endogenously produced (*e.g.,* oxidized HCDs), and that these may also exert distinct, physiological, roles (42). Our method was not developed to detect these molecules, but their consideration in future studies would represent an interesting opportunity to further advance understanding of this topic.

In conclusion, we measured HCDs in the ventricle, liver and *m.pectoralis* of the freshwater turtle, with balenine predominating in the liver, carnosine in the skeletal muscle and a mixture of the two in cardiac tissue. Tissue HCD content was relatively low compared to other species with large buffering requirements, implying that overall intracellular buffering may not make a substantial contribution to these turtles' remarkable capacity to withstand oxygen deprivation. Nonetheless, the HCD content of the *m. pectoralis* was higher in those turtles that were acclimated to 3°C, as opposed to 20°C, which implies a regulatory role for the HCDs in responding to challenges to acid-base homeostasis that occur at colder temperature.

Figure Legends:

Figure 1: **Panel A:** Individual area under the curve (AUC) HCD content of the turtles maintained at 3 and 20°C. L = liver; V = ventricle and P = Pectoralis. **Panel B:** Mean area under the curve (AUC) HCD content of turtles maintained at 3 or 20°C. Muscle carnosine content in the different tissues and environments. *p=0.013 Pectoral at 20 °C compared to 3°C.

Figure 2: Skeletal muscle HCD content of various species

Note: The contents shown are indicative, and likely to vary based upon species sub-type and on the muscle type. More detailed overviews of HCD variation in different species are provided elsewhere (11–13).

Declarations:

The is work was partially funded by a National Science Foundation CAREER grant (1253939) awarded to Daniel Warren. Eimear Dolan (2019/05616-6 and 2019/26899-6), Bryan Saunders (2016/50438-0) and Bruno Gualano (2017/13552-2) are financially supported by the *Fundação de Ampara a Pesquisa do Estado do São Paulo* (FAPESP). Bryan Saunders has received a grant from Faculdade de Medicina da Universidade de São Paulo (2020.1.362.5.2). None of the authors have any conflict of interest to declare.

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