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

3
4 **Running Title:** *HCD content of freshwater turtles.*

5
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21
22 **Highlights:**

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

27 **Abstract:**

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

40

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

42

43 INTRODUCTION

44 The ability to maintain acid-base balance within homeostatic limits is essential to maintain cellular
45 function (1). Acid-base homeostasis is constantly challenged by various internal and external factors.
46 For example, anaerobic metabolism is required for continued ATP regeneration when turnover
47 exceeds the oxidative capacity of the cell. This results in hydrogen cation (H^+) accumulation and
48 increased metabolic acidosis (2), which has adverse consequences for numerous cellular processes,
49 including reduced glycolytic enzyme activity, inhibition of oxidative phosphorylation and impaired
50 phosphorylcreatine (PCr) resynthesis (3–5). To avoid these adverse consequences, living organisms
51 have evolved a diverse range of pH regulatory strategies. Intracellular buffers, such as bicarbonate,
52 phosphates, proteins and histidine containing dipeptides (HCDs), provide an important “first line of
53 defence” against intracellular pH perturbations. Simultaneously, “dynamic buffering” is the process
54 by which excess H^+ is removed from the cell via Na/H^+ exchangers and monocarboxylate transporters
55 (6, 7).

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

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

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

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

95

96 *Animals:*

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

111

112 *Tissue sampling and preparation*

113 Turtles were euthanized by rapid decapitation. After the plastron was removed with a bone saw,
114 samples of ventricle, liver and pectoralis muscle were removed and immediately flash-frozen with
115 freeze clamps pre-chilled in liquid nitrogen, and then stored at -80°C until analysed. All samples were
116 subsequently lyophilised and powdered before extraction was performed using perchloric acid
117 [HClO₄], EDTA and potassium bicarbonate [KHCO₃] (26). The neutralised supernatant was collected
118 using a centrifugal filter (0.2 µm), checked to ensure a pH close to 7 and then stored at -80°C until
119 high-pressure liquid chromatographic (HPLC) analysis.

120

121 *Chromatographic determination of histidine-containing dipeptides*

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

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

139

140 *Data analysis*

141 Data were analysed using the SAS statistical package (SAS[®] University Edition, SAS Institute Inc., USA),
142 and are presented as mean \pm 1SD. Carnosine content ($\text{mmol}\cdot\text{kg DM}^{-1}$) was analysed using mixed model
143 analysis with animals assumed as a random factor and tissue (3 levels; liver/ventricle/*m. pectoralis*)
144 and environment (2 levels; 3 or 20°C) assumed as fixed factors. Tukey–Kramer adjustments were
145 performed when a significant F value was obtained. Results were interpreted according to the
146 statistical probabilities of rejecting the null hypothesis (H_0) and in the following categories: $p > 0.1$: no
147 evidence against H_0 ; $0.05 < p < 0.1$: weak evidence against H_0 ; $0.01 < p < 0.05$: some evidence against
148 H_0 ; $0.001 < p < 0.01$: strong evidence against H_0 ; $p < 0.001$: very strong evidence against H_0 (29).
149 Effect sizes (ES) were calculated as the mean difference between the two groups of turtles, divided by
150 the pooled standard deviation and are reported alongside their 95% confidence interval. The
151 theoretical buffering contribution of the observed HCD content for a reduction of 0.6 pH units was
152 calculated using a derivation of the Henderson Hasselbalch equation (14) namely: $\beta_{\text{HCD}} = \{[\text{HCD}]/(1 +$
153 $10^{(\text{pHi} - \text{pKa})})\} - \{[\text{HCD}]/(1 + 10^{(\text{pHi} - \text{pKa})})\}$. For this calculation we assumed the physiologically relevant
154 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
155 and 7.209 at 20° and 3°C (32).

156

157 **RESULTS**

158 *HCD Content*

159 HCDs were detected in all examined tissues (Figure 1, Panel A). Carnosine was found in the *m.*
160 *pectoralis* ($16.09 \pm 7.00 \text{ mmol}\cdot\text{kgDM}^{-1}$) and balenine in the liver, whilst both balenine and carnosine
161 ($6.08 \pm 2.95 \text{ mmol}\cdot\text{kgDM}^{-1}$) were found in the ventricle. Visual inspection of the chromatograms
162 suggested that very small amounts of balenine were present in two of the *m. pectoralis* samples and
163 very small amounts of carnosine in two of the liver samples, although these were below the limits of
164 interpolation and quantification of the detection software. There was very strong evidence of an effect
165 of tissue on MCarn content ($p < 0.0001$), but no effect of environment ($p = 0.17$). There was strong
166 evidence of a tissue x environment interaction ($p = 0.007$), with post-hoc analysis indicating that the
167 turtles acclimated to 3°C had a higher total *m. pectoralis* HCD content ($\text{mmol}\cdot\text{kg DM}^{-1}$) than those kept
168 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
169 were no differences in MCarn content between environments for the other tissues (both $p = 0.99$).
170 Skeletal muscle carnosine content equated to a buffering contribution of 6.83 ± 1.47 and 4.10 ± 1.93
171 $\text{mmol}\cdot\text{kg DM}^{-1} (0.6 \text{ pH unit})^{-1}$ for 3 (pHi: 7.4 – 6.8) and 20°C (pHi: 7.1 – 6.5; $p = 0.04$; ES: 1.59).

172

173

174 **DISCUSSION**

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

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

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

211 Despite these turtles' relatively low HCD content when considered relative to other species, the cold-
212 acclimated turtles did have a higher content than their counterparts who were maintained at 20°C,
213 which implies a regulatory role for the HCDs in adapting to this stressor. Skeletal muscle forms the
214 largest mass of unmineralized tissue in the turtle, so higher muscle carnosine content could have
215 quantitatively important consequences for acid-base regulation, assuming that it is the predominant
216 histidylated peptide contained therein. Turtles have long been known to be alpha-stat regulators,
217 which means they decrease their PCO₂ in order to increase their relative alkalinity for the purpose of
218 defending the dissociation fraction (alpha) of the imidazole functional groups in histidine residues
219 within their proteome, thereby preserving their charge and conformational states (24). Based on

220 Reeves (24), the small change in MCarn observed in the present study would have minimal impacts
221 on intracellular pH or Pco₂. Thus, the most important effect will be on the buffering power of the
222 muscle. The pKa of carnosine is increased in colder conditions (7.209 at 3°C vs 6.702 at 20°C), which
223 would contribute to the maintenance of the increased alkalinity, given that each temperature-specific
224 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
225 the theoretical buffering contribution of the observed MCarn content indicated a higher buffering
226 capacity in the cold-acclimated turtles, again suggesting that this dipeptide may play an important role
227 in acid-base regulation at this temperature. Increases of the magnitude observed herein are roughly
228 comparable to those observed in humans in response to commonly used BA dosing protocols (38),
229 and would necessitate either a large increase in carnosine synthesis, a large reduction in carnosine
230 degradation, or perhaps a combination of both (39). Given that no external BA source was available
231 to these turtles, increased synthesis could only have occurred via increased endogenous production,
232 alongside an increase in carnosine synthase activity. This seems unlikely to be the only factor,
233 considering the time-periods that were investigated, and so a reduced degradation rate may have
234 contributed (at least in part) to the observed increases. This is, of course, speculative and further
235 research is required both to confirm our findings, and to explore the biokinetics of cold-induced MCarn
236 increases in these ectothermic vertebrates. It is also important to highlight that the number of turtles
237 in each group was small (n = 5), and our estimates are necessarily based on between group
238 comparisons, which does increase the risk of sampling error. As such, caution must be applied when
239 interpreting the magnitude of increase.

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

250 An interesting finding was the distinct HCD profile within the different tissues. It was unsurprising that
251 skeletal muscle had the largest HCD content, as it has previously been estimated that approximately
252 99% of the total carnosine is located in this tissue (11). In studies of this kind, three HCD forms are
253 commonly investigated, namely carnosine, and its methylated analogues balenine and anserine. Most
254 species contain at least two HCD forms and this varies largely between phylogenetically distinct
255 species, but is similar within the same, or congeneric, species. Primarily carnosine, and very small
256 quantities of balenine, were identified in these painted turtles, with the latter being absent in skeletal
257 muscle. However the uniformity of HCD distribution of different types in different tissues does imply
258 that they may exert type- or tissue-specific effects. Some biological differences have been reported
259 between the HCD analogues, *e.g.*, each HCD has a subtly different pKa (estimated to be 7.03, 7.04 and
260 6.83 for anserine, balenine and carnosine at mammalian temperatures, *i.e.*, ~37°C), while distinct anti-
261 radical effects of the different HCD analogues have also been suggested (40, 41). The functional
262 relevance of these subtle physiological differences is unknown and represents an interesting avenue
263 for future research. Interestingly, evidence is emerging that other HCD forms are also endogenously
264 produced (*e.g.*, oxidized HCDs), and that these may also exert distinct, physiological, roles (42). Our
265 method was not developed to detect these molecules, but their consideration in future studies would
266 represent an interesting opportunity to further advance understanding of this topic.

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

275 **Figure Legends:**

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

280

281 **Figure 2:** Skeletal muscle HCD content of various species

282 Note: The contents shown are indicative, and likely to vary based upon species sub-type and on the
283 muscle type. More detailed overviews of HCD variation in different species are provided elsewhere
284 (11–13).

285

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292 declare.

293

294

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