Lactate Dehydrogenase and Citrate Synthase activity in cardiac and skeletal muscle of lowland and highland tinamous

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Tinamous (Tinamidae) have the smallest heart in relation to body mass compared to any other flying bird today (Bishop 1997). This means that heart size is likely to restrict aerobic metabolism. Tinamous inhabit areas from sea level to 4800 m a.s.l., which means that the high altitude living species, *Nothoprocta ornata* (NO), is exposed to hypoxia. In this study the activity of the two metabolic enzymes Lactate Dehydrogenase (LDH) and Citrate Synthase (CS) was measured and the ratio between the enzyme activities calculated to examine if the small heart of the tinamous affects their aerobic/anaerobic metabolism. The activity of the two enzymes was measured in the heart and the gastrocnemius muscle in the three species *Nothoprocta ornata* (NO), *Nothoprocta perdicaria* (NP) and *Gallus gallus* (GG). CS activity was significantly higher in the heart compared to the skeletal muscle and LDH activity was significant higher in the skeletal muscle than in the heart in all three species. The LDH/CS ratio was significantly higher in NO’s skeletal muscle than in chickens but there was no significant difference between species in the heart. The higher ratio in NO’s muscle could be a sign of a higher anaerobic metabolism that is used in the muscles to compensate for the small heart NO have. In conclusion, the Tinamous’ small heart can result in changed activities of LDH and CS in the heart and gastrocnemius muscle.

Tinamidae, Lactate Dehydrogenase, Citrate Synthase, cardiac and skeletal muscle and high altitude
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1 Abstract

Tinamous (*Tinamida*) have the smallest heart in relation to body mass compared to any other flying bird today (Bishop 1997). This means that heart size is likely to restrict aerobic metabolism. Tinamous inhabit areas from sea level to 4800 m a.s.l., which means that the high altitude living species, *Nothoprocta ornata* (NO), is exposed to hypoxia. In this study the activity of the two metabolic enzymes Lactate Dehydrogenase (LDH) and Citrate Synthase (CS) was measured and the ratio between the enzyme activities calculated to examine if the small heart of the tinamous affects their aerobic/anaerobic metabolism. The activity of the two enzymes was measured in the heart and the gastrocnemius muscle in the three species *Nothoprocta ornata* (NO), *Nothoprocta perdicaria* (NP) and *Gallus gallus* (GG). CS activity was significantly higher in the heart compared to the skeletal muscle and LDH activity was significant higher in the skeletal muscle than in the heart in all three species. The LDH/CS ratio was significantly higher in NO’s skeletal muscle than in chickens but there was no significant difference between species in the heart. The higher ratio in NO’s muscle could be a sign of a higher anaerobic metabolism that is used in the muscles to compensate for the small heart NO have. In conclusion, the Tinamous’ small heart can result in changed activities of LDH and CS in the heart and gastrocnemius muscle.

2 Introduction

The family Tinamidae is found only in the Neotropics and its 45 species are distributed at high and low altitudes (Cabot 1992, Davies 2002). The Ornate Tinamou (*Nothoprocta ornata*; NO) is found at high altitudes between 2500 to 4800 m a.s.l. (meters above sea level), and the Chilean Tinamou (*N. perdicaria*; NP) inhabits sea level to 2000 m a.s.l. habitats (Cabot 1992, Davies 2002). Tinamidae belongs to the Palaeognathae which is an ancient bird group that includes the Ratites (Ostriches, Kiwis, Emus, Rheas, Cassowaries and the extinct elephant bird) (Philips 2010). Tinamous have the smallest heart compared to body mass than any other flying bird today (Bishop 1997), that means that heart size is likely to restrict aerobic metabolism, particularly upon exercise or stress (Altimiras et al. in preparation). An important question is if the tinamou’s aerobic/anaerobic metabolism varies in comparison to the metabolism of species of similar size and ecological habits but with a “normal” avian-sized heart as the chicken (*Gallus gallus*; GG). The comparison between two tinamou species of the same genus, but with a different altitudinal distribution allows us too to evaluate the adaptive role of enzymatic activity in this ancient group of birds.
To see if the small heart of the tinamou has an impact on their aerobic/anaerobic metabolism the ratio between the activities of the two enzymes Lactate Dehydrogenase (LDH) and Citrate Synthase (CS) will be measured. A lower ratio of LDH/CS implies a higher oxidative metabolism (Esteva et al. 2009). LDH catalyze the production of ATP by the conversion of pyruvate to lactate and vice versa and convert NADH to NAD⁺. LDH is thus related to the anaerobic metabolism (He et al. 2013). CS is catalyzing the first reaction in the citric acid cycle, the reaction between coenzyme A (acetyl CoA) and oxaloacetic acid to form citric acid and is related to the aerobic metabolism (Hochachka et al. 1977).

My hypothesis is that the tinamous would have a higher anaerobic metabolism than the chickens, due to the size of their heart and between the two tinamou species the Ornate Tinamou would have a higher anaerobic metabolism because there is already evidence that animals which are exposure to high altitude hypoxia tend to use more anaerobic metabolism than animals which inhabits at low altitude (Wang et al. 2008).

3 Materials and methods

3.1 Sampling and homogenization
In this study samples from eight Ornate Tinamous, seven domestic chickens and eight Chilean Tinamous were obtained. The left ventricle of the heart and the left gastrocnemius muscle were removed and immediately frozen in a cryoshipper (-80°C) until the samples could be permanently placed in a -80°C freezer to be preserved. Homogenization of the samples took place within 4 weeks after freezing. Then, 100 mg tissue was homogenized on ice with a Teflon-to-glass tissue homogenizer in 1.9 ml of homogenization buffer (175 mM KCl, 2 mM EDTA, 10 mM Tris, pH 7.0).

3.1.1 Enzymatic activity
Citrate Synthase and Lactate Dehydrogenase activities were determined using standard procedures (Sheafor 2003).

The final dilution of the gastrocnemius muscle was 1:2000 for both enzyme trials. The heart homogenate had a final dilution of 1:1000 in the LDH measurements and 1:2000 in the CS measurements. All the measurements were done in a spectrophotometer (Quimis, Q798U) at room temperature (20-22°C). The reaction solutions were mixed in a 1 ml cuvette. The final composition for LDH measurements was 0.25 mM
NADH, 10mM pyruvate and 100 mM TRIS-HCl buffer pH 7. As a control the sample was measured in the spectrophotometer (340 nm) before adding pyruvate as a substrate for the reaction. After 2 min pyruvate was added and the measurements continued for 6 min. The final composition for CS measurements was 0.2 mM AcetylCoA, 0.1 mM DTNB, 0.5 mM oxaloacetate in 100 mM TRIS-HCl buffer at pH 8.0 in a 1 ml cuvette. Before adding the oxaloacetate the reaction buffer was measured in a spectrophotometer at 412 nm to get a control value. After 2 min the reaction was initiated by oxaloacetate and the measurements continued for 6 more min.

3.2 Statistical analysis

Enzymatic activity was measured at least twice in all samples from each of the two tissues from the three species. The mean value from the different runs from each sample was used for statistical analysis using a one way analysis of variance (ANOVA) and then continued by Tukey’s post hoc test. All statistic analyses were done with the program Minitab 16. A statistical significance was accepted at p < 0.05.

4 Results

The results show a significantly higher CS activity in the heart from _Nortoprocta ornata_ (NO) compared to _Gallus gallus_ (GG) (F_{2,16} = 6.01 ; p=0.011) without any significant difference between _N. perdicaria_ (NP) and NO or NP and GG (Fig. 1). The CS activity in the gastrocnemius muscle was significantly higher in GG compared to both NO and NP (F_{2,20} = 40.756 ; p < 0.001) but there were no significant higher CS activity in NP compared to NO (Fig. 1).
Figure 1. CS activity in the heart and gastrocnemius muscle of the three different species Notoprocta ornata (NO), Notoprocta perdicaria (NP) and Gallus gallus (GG). Means+−s.d. eller s.d. Dissimilar letter indicate significant differences between groups.

The LDH activity in heart was significant higher in NO than in NP (F_{2,17}=3.82; p=0.043) but there were no significant difference in the gastrocnemius muscle between the three species (F_{2,20}=0.46; p=0.639) (Table 2). There was also a significantly higher activity in the gastrocnemius muscle compared to the activity in the heart in all three species (F_{2,37}=27.854; p < 0.001) (Fig. 2).

Figure 2. LDH activity in the heart and gastrocnemius muscle of the three species Notoprocta ornata (NO), Notoprocta perdicaria (NP) and Gallus gallus (GG). Dissimilar letter indicate significant differences between groups.

The LDH/CS ratio in the heart and in the gastrocnemius muscle in NO, NP and GG are shown in Table 1. The ratio in the gastrocnemius muscle in the Ornate tinamou was significant higher compared to the chicken. There were not found any significant difference in the LDH/CS ratio between the tree species in the hearts.

Table 1. The ratio between the two enzymes LDH and CS in all three species and the two different tissues (heart and gastrocnemius muscle). No significant difference between the species in heart but No had a significant higher ratio than GG. Dissimilar letter indicate significant differences between groups. The values are means ± SD.
5 Discussion

I was unable to do the enzymatic measurements at temperatures close to normal body temperatures and instead, all the measurements were done at room temperature (20-22 °C). Other researchers have performed their enzymatic measurements in 30-40°C to mimic the body temperature as much as possible (Fernández et al. 2011, Letout et al. 2005, Schippers et al. 2012 and Sheafor 2003). In order to compare my data with other studies I used the concept of Q_{10} to estimate enzymatic activities at temperatures close to body temperature. I thus assume that my reactions follow the ordinary Q_{10} concept, i.e. for each ten degrees the speed of the chemical reaction doubles. So to be able to compare my results with other studies my results are multiplied by four (Table 2 and 3).

The CS activity measured in the heart in NO, NP and GG matched the results from previous studies made on mammals and coots (Table 2). There were larger differences in the gastrocnemius muscle between my results and previous studies. Coots and pikas had much higher CS activity in the muscles compared to NO and NP but quite similar to GG (Table 2).

The LDH activity from my results in heart was very low for all three species compared to both mice and coots. The LDH activity from gastrocnemius muscle was more similar to the mouse than to coot which had approximately three times higher LDH activity in their muscularis digitorum longus (EDL) than the tinamous and kickens. The Japanese quail also had a very high LDH activity in the muscle, approximately nine times higher than my results.

Table 2. CS activity in heart and gastrocnemius muscle (micromol min^{-1} mg^{-1} wet tissue) in the present study (Ornate tinamou and Chilean tinamou) compared with literature data.

<table>
<thead>
<tr>
<th>Ratio LDH/CS</th>
<th>Heart</th>
<th>Gastrocnemius muscle</th>
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<tbody>
<tr>
<td>NO</td>
<td>0.61 ± 0.25^a</td>
<td>52.2 ± 35.9^b</td>
</tr>
<tr>
<td>NP</td>
<td>0.40 ± 0.17^a</td>
<td>38.3 ± 28.6^bc</td>
</tr>
<tr>
<td>GG</td>
<td>0.84 ± 0.39^a</td>
<td>12.0 ± 8.36^c</td>
</tr>
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</table>
Table 3. LDH activity in heart and gastrocnemius muscle (micromol min\(^{-1}\) mg\(^{-1}\) wet tissue) in the present study (Ornate tinamou and Chilean tinamou) compared with literature data.

<table>
<thead>
<tr>
<th></th>
<th>Heart</th>
<th>Gastrocnemius muscle</th>
<th>EDL</th>
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<tbody>
<tr>
<td>Ornate tinamou*</td>
<td>278 ± 64</td>
<td>22.4 ± 9.8</td>
<td></td>
</tr>
<tr>
<td>Chilean tinamou</td>
<td>263 ± 27</td>
<td>19.8 ± 8.4</td>
<td></td>
</tr>
<tr>
<td>Chicken*</td>
<td>222 ± 72</td>
<td>75.1 ± 20</td>
<td></td>
</tr>
<tr>
<td>Coot(^{a})</td>
<td>203 ± 25</td>
<td>65.2 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>Coot(^{a})</td>
<td>203 ± 28</td>
<td></td>
<td>59.0 ± 4.7</td>
</tr>
<tr>
<td>Mouse(^{b})</td>
<td>320 ± 13</td>
<td>35.9 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>Mouse(^{b})</td>
<td>273 ± 7</td>
<td>38.5 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>Pika(^{c})</td>
<td>304 ± 9.2</td>
<td>52.2 ± 4.6</td>
<td></td>
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<tr>
<td>Pika(^{c})</td>
<td>262 ± 13</td>
<td>46.7 ± 2.3</td>
<td></td>
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Values are means ± SD, my values are multiplied by four in this table

*High altitude species or populations

\(^{a}\) León-Velarde et al. 1993 (U · g prot \(^{-1}\) on a wet tissue basis) (at 25 °C)

\(^{b}\) Schippers et al. 2012 (at 37 °C)

\(^{c}\) Sheafor 2003 (at 40 °C)

Chickens living at high altitude had a significant higher CS activity in the gastrocnemius muscle than both tinamous living at either high or low altitude, which indicates that the chicken has a higher potential for aerobic metabolism in the gastrocnemius muscles compared to the tinamous. The CS activity in the heart also differed between the species. Ornate tinamou had a significant higher CS activity in heart tissue compared to chickens but not in comparisons to Chilean tinamou, which indicates that NO has a higher potential for aerobic metabolism in the
heart compared to the chickens. This has also been seen in mice (Phyllotis), pikas (Ochotona). Studies made by Schippers et al. (2012) and Sheafor (2003) showed that Phyllotis and Ochotona species from high altitude had a higher CS activity in the cardiac muscle compared to Phyllotis and Ochotona species from low altitude. Zhang et al. 2008 did not look at CS activity but instead they turned to Succinate Dehydrogenase (SDH), which also is an indicator of aerobic metabolism (He et al. 2013). Zhang et al. 2008 found a significant higher SDH activity in heart from Tibetan chickens (native at high altitude) compared to Shouguan and Dwarf recessive white chickens (low land species). The results also show that the CS activity was higher in the heart compared to the gastrocnemius muscle in all three species which indicates that the heart uses more aerobe metabolism than the gastrocnemius muscle. The results from the LDH runs also show that the heart uses more aerobe metabolism than the gastrocnemius muscle. The heart had a significant lower LDH activity compared to the gastrocnemius muscle in all three species. León-Velarde et al. 1993 also saw a lower LDH activity in the heart compared to muscles. The high difference between the LDH and CS activity in the heart and gastrocnemius muscle could be explained by the knowledge that the heart is supporting the whole body with oxygen and the function of the heart is crucial, especially in hypoxia (Schippers et al. 2012).

In other studies it has been seen that lizard populations grown up at high altitude, have a lower LDH activity in heart than populations grown up at low altitude (He et al. 2013). A lower LDH activity was also found in the heart from rats exposed to hypoxia by Esteva et al. 2009 but Zhang et al. (2008) did not find any significant difference in the LDH activity in hearts from Tibetan chickens (native at high altitude) compared to Shouguan and Dwarf recessive white chickens (lowland species). I could see a significant higher LDH activity in heart from the high altitude living species NO. Maybe NO is compensating for the small heart they have (which do not have enough capacity to support the whole body with sufficient oxygen during exercise or stress) by not having a lower LDH activity in heart compared to NP even if NO is exposed to hypoxia. There was no significant difference in LDH activity between the species in the gastrocnemius muscle. This result supports work by Schippers et al. (2012) and Esteva et al. (2009), who did not find any difference in LDH activity in mice or rats skeletal muscle before and after exposure to hypoxia. Neither did León-Velarde et al. (1993) in Andean coots. León-Velarde et al. (1993) also concluded that the LDH activity does not have any significant participation in the adaptation to high altitude.
The LDH/CS ratio which is an indicator of the amount of anaerobic/aerobic metabolism (Hochachka et al. 1983) was significantly higher in NO in the gastrocnemius muscle than in GG. This suggests that the small heart of NO have made the bird use more anaerobic metabolism i.e. they have higher LDH/CS ratio than the chickens with “normal” sized avian heart. Chilean tinamou had a relatively high LDH/CS ratio but it was not significantly higher than the chickens. Even if this species is not living at high altitude it also has a small heart which makes it more important for it to be able to use anaerobic metabolism. This could explain the relative high LDH/CS ratio in the gastrocnemius muscle for NP. Tanaka et al. (1997) showed that the LDH/CS ratio decreased in rats skeletal muscles after they were exposed to hypoxia. My results for chickens agrees with Tanaka et al. (1997), who suggested that chronic exposure to hypoxia induces oxidative enzyme activity in skeletal muscles which can explain the low LDH/CS ratio I got in chickens. But on the other hand Esteva et al. 2009 did not see any significant change in LDH/CS ratio in skeletal muscles after the rats were exposed to hypoxia. This also supports the idea that the higher ratio in NO’s muscle could be to compensate for the small heart. The LDH/CS ratio did not show any significant difference in the heart between the species which could be explained by the fact that the heart should always be supported with oxygen and thereby do not need that much anaerobe enzyme activity even if the heart is small or exposed to hypoxia.

5.1 Conclusion

To conclude, the Ornate tinamou is probably compensating for the small size of their heart by increasing the potential for anaerobic metabolism in the gastrocnemius muscle compared to chickens. NO also has a higher potential for aerobic metabolism in the heart compared to chickens. Both NO and NP had a lower CS activity in the muscles than chickens, which could be due to the higher reliance NO and NP have on the anaerobic activity in the gastrocnemius muscles, especially during stress or exercise. I did not find any significant differences between the two tinamous, except for the LDH activity in heart, where NO had a significantly higher activity. It would be interesting to do further studies and see if the enzymatic activities in Ornate tinamou would change if the bird was brought down to sea level.
6 Acknowledgements

I would like to thank my supervisor Dr. Jordi Altimiras for supporting me in my work and giving me the chance to go to Bolivia to make my studies there. I would also like to thank Dr. Alvaro Garitano, Dr. Isabel Morales Belpaire and all lovely people in the labs for all the practical help at UMSA.

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