ABSTRACT  Studies were conducted to assess proton leak kinetics (proton conductance) in breast muscle mitochondria isolated from broiler breeder males within a single genetic line exhibiting either high (HFE) or low (LFE) feed efficiency. Proton leak kinetics were determined by simultaneously measuring mitochondrial membrane potential and state 2 (resting) respiration rate in breast muscle mitochondria as succinate oxidation was progressively decreased by malonate. Control proton conductance was similar in HFE and LFE mitochondria and decreased to a similar extent in both groups in response to BSA. Although treatment of mitochondria with Glu or guanosine diphosphate had no effect, retinal increased and carboxyatractylate alone or in combination with Glu decreased proton conductance relative to control proton conductance in both HFE and LFE mitochondria. After treatment with either guanosine diphosphate or carboxyatractylate alone, proton conductance was lower in HFE compared with LFE mitochondria. With the exception of BSA, proton conductance in HFE mitochondria after the various chemical treatments was either less than or equal to, and never greater than, proton conductance in the LFE mitochondria. The results suggest that there are subtle differences in membrane characteristics (e.g., lipids, integral membrane proteins) that affect proton conductance in broiler muscle mitochondria that may in turn play a role in the phenotypic expression of feed efficiency.

Key words: broiler, feed efficiency, mitochondria, proton leak kinetics

INTRODUCTION

A link between mitochondrial function and feed efficiency (FE) in broilers within a single genetic line has been reported (Bottje et al., 2002). Over 90% of the adenosine triphosphate (ATP) produced in a cell comes from oxidative phosphorylation associated with the electron transport chain (ETC) of mitochondria. Proton motive force that develops from proton pumping across the inner mitochondrial membrane is used to drive ATP synthesis. However, protons may also flow back into the mitochondria at sites other than the F1F0-ATPase and effectively short circuit the coupling of ATP synthesis in a process called proton leak (Brand, 1995). Because proton leak represents up to 30% of oxygen consumption in isolated liver cells and up to 50% of oxygen use in a perfused muscle (Brand, 1990; Rolfe and Brand, 1996), proton leak could contribute as much as 25% of total basal metabolic rate of an animal (Rolfe and Brand, 1996, 1997; Rolfe et al., 1999). Thus, it is reasonable to hypothesize that proton leak could be involved in the phenotypic expression of FE in animals.

An overview of inherent (basal) and inducible proton conductance mechanisms has been provided (Parker et al., 2008). Basal proton conductance occurs partly by leakage across the phospholipid bilayer as membranes are intrinsically leaky to protons (Dilger et al., 1979), and partly through particular proteins (Brand et al., 2005), whereas inducible proton conductance is associated with intramembranous proteins such as anion carrier proteins [e.g., adenine nucleotide transporter (ANT), Glu transporter] and uncoupling proteins (UCP; Brown and Brand, 1991; Brookes et al., 1997; Rolfe and Brand, 1997). Over 50% of the basal proton conductance is due to ANT and continues even when ANT activity is inhibited by carboxyatractylate (CAT;
Brand et al., 2005). Inducible proton conductance is altered by various activators or inhibitors. Fatty acids increase proton conductance by stimulating the proton-translocating activities of ANT, UCP, and phosphate and Glu carrier proteins (Andreyev et al., 1988, 1989; Samartsev et al., 1997; Jaburek et al., 1999; Echtay et al., 2001). Bovine serum albumin has been shown to reduce proton conductance by removing fatty acids as well as reactive oxygen species (ROS) and products of lipid peroxidation such as 4-hydroxy 2-nonenal (Mellors et al., 1967; Echtay et al., 2002a, 2003; Parker et al., 2008). Uncoupling protein activity can also be stimulated by retinal (RET; Puigserver et al., 1996; Rial et al., 1999) and inhibited by both adenine and guanosine nucleoside di- and triphosphates (Cadenas et al., 2000).

Uncoupling represents a cellular inefficiency but also reduces oxidative stress by attenuating mitochondrial ROS production. The self-limiting feedback on mitochondrial ROS production by superoxide through mitochondrial uncoupling was shown by Skulachev (1996, 1997). Mitochondrial ROS production is enhanced when the mitochondrial membrane potential (MMP) is above 140 mV (Korshunov et al., 1997) and can be attenuated by uncoupling (Miwa et al., 2003). Echtay et al. (2002a,b) demonstrated that ROS-mediated uncoupling was due to increased UCP activity that could be inhibited by guanosine diphosphate (GDP). Uncoupling lowered ROS production in duodenal mitochondria obtained from broilers with low FE (LFE) but not in mitochondria obtained from broilers with high FE (HFE; Ojano-Dirain et al., 2007a). In that study, basal ROS production was significantly higher in LFE mitochondria and therefore uncoupling would be expected to have a greater effect on mitochondrial ROS production. Thus, it would appear that factors influencing MMP and proton conductance may contribute to higher mitochondrial ROS associated with the phenotypic expression of LFE in broilers (Bottje et al., 2002; Bottje and Carstens, 2009). Therefore, the present study was conducted to a) characterize proton conductance and b) determine what mechanisms may be responsible for any differences in proton conductance in muscle mitochondria obtained from broilers within a single genetic line that exhibit either LFE or HFE phenotypes.

**MATERIALS AND METHODS**

### Birds and Management

Feed efficiency was determined in broiler breeder male replacement stock (Cobb Vantress, Inc., Three Springs Farm, OK) that was handled and sampled as described previously (Bottje et al., 2002). A total of 18 birds identified as being either HFE (n = 9) or LFE (n = 9) phenotype were used in this study. Birds were killed with an overdose of sodium pentobarbital by i.v. injection into the wing vein and 7 to 10 g of breast muscle (pectoralis superficialis) obtained for mitochondrial studies. The animal handling and euthanasia for this project were approved by the Institutional Animal Care and Use Committee (protocol no. 02027).

### Mitochondrial Isolation

Breast muscle mitochondria were isolated according to Bhattacharya et al. (1991). The tissues were excised, finely minced in isolation medium A (100 mM sucrose, 10 mM EDTA, 100 mM Tris-HCl, 46 mM KCl, pH 7.4), and incubated at room temperature (25°C) in 14 mL of isolation medium A containing 20 mg of nagarse for 5 min. The minced tissue was homogenized in a Potter-Elvehjem vessel with a Teflon pestle of 0.16-mm clearance (Thomas Scientific, Swedesboro, NJ) and incubated for an additional 5 min on ice (4°C) with stirring. The homogenate was centrifuged (1,000 × g for 10 min) and resulting supernatant was centrifuged (10,000 × g for 15 min) to obtain the mitochondrial pellet that was resuspended and washed in 10 mL of isolation medium A plus 0.5% BSA (without nagarse). Mitochondria were pelleted by centrifugation (8,000 × g for 15 min) in incubation medium (230 mM mannitol, 70 mM sucrose, 20 mM Tris-HCl, 5 mM KH₂PO₄, pH 7.4). The resulting pellet was resuspended in 2 mL of incubation medium, placed on ice, and mitochondrial protein was determined spectrophotometrically.

### Mitochondrial Function

Mitochondrial function was determined according to Estabrook (1967). Oxygen consumption of mitochondria (expressed in nmol/min per milligram of protein) was measured polarographically with a Clark-type oxygen electrode in a thermostatically controlled reaction vessel equipped with magnetic stirring (Yellow Springs Instrument Co. Inc, Yellow Springs, OH). Muscle mitochondria (0.5 mg of protein per mL of final concentration) were added to the reaction vessel containing 1 mL of respiratory control ratio reaction buffer (220 mM d-mannitol, 70 mM sucrose, 2 mM HEPES, 3 mM KH₂PO₄, 5 μM rotenone, pH 7.0). After monitoring initial oxygen consumption (state 2 respiration) with succinate (10 mM) as the respiratory substrate, state 3 (active) respiration was initiated with 150 μM adenosine diphosphate (ADP; final concentration), followed by state 4 (resting) respiration when ADP levels became limiting. Also, uncoupled state 3 (State 3u) respiration rate was determined by adding 1 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone and state 4 respiration was determined after a 1-μg addition of oligomycin (state 4o respiration) to ensure complete inhibition of ATP synthase. Coupling of mitochondria was calculated by dividing active by resting respiration rates.

### Mitochondrial Proton Leak Kinetics

Proton leak kinetics were assessed as described previously (Brand, 1995). The respiration rate of mito-
Mitochondria in the presence of oligomycin to inhibit ATP synthesis is proportional to the rate that protons leak across the mitochondrial inner membrane. Consequently, the kinetic response of proton leak (proton conductance) to its driving force (proton motive force) can be measured by the relationship between respiration rate and MMP (in mV) when MMP is lowered by sequential additions of an ETC inhibitor such as malonate and ATP synthase is inhibited with oligomycin.

Respiration rate and MMP were measured simultaneously using electrodes sensitive to oxygen and to the potential-dependent probe triphenylmethylphosphonium (TPMP) ion (Brand, 1995). The measuring chamber contained proton leak medium (120 mM KCl, 5 mM KH₂PO₄, 3 mM HEPES, 1 mM ethylene glycol tetraacetic acid) with 5 µM rotenone (to inhibit electron flow through complex I), 1 µg/mL of oligomycin (to inhibit ATP synthase), and 65 ng/mL of nigericin (to serve as a ΔpH clamp such that all changes in proton motive force across the inner mitochondrial membrane would be expressed as MMP and reflected in TPMP ion distribution). Mitochondria were added to the proton leak media at a final concentration of 1 mg of mitochondrial protein/mL. After the TPMP-sensitive electrode was calibrated with sequential additions of 1 to 2 µM TPMP, succinate (4 mM) was added to initiate state 2 respiration. Respiration and MMP were progressively inhibited by sequential additions of malonate (an inhibitor of complex 3, 0.88 to 3.0 mM malonate per addition). The uncoupling agent carbonyl cyanide-p-trifluoromethoxyphenylhydrazone was added at the end of each run to fully dissipate the MMP and to release TPMP sequestered in the mitochondria back into the medium, allowing a baseline correction for any electrode drift. Mitochondrial membrane potential in millivolts (mV) was calculated based on the Nernst equation as:

\[
MMP = 61.5 \log ([TPMP] \text{ added} - \text{external} [TPMP])
\times \text{TPMP binding correction}/(0.001
\times \text{mg of protein/mL} \times \text{[TPMP]}),
\]

where the TPMP binding correction was 0.45 (µL/mg of mitochondrial protein)⁻¹ (Rolfe et al., 1994).

Proton leak kinetics were determined in mitochondria treated with proton leak medium alone (untreated control) or in the presence of several chemicals: 0.3% BSA, 1 mM GDP, 2 µM CAT, 12.5 µM RET, 10 mM Glu, 1 mM GDP in combination with 12 µM RET, and 1 mM GDP in combination with 10 mM Glu.

**Assessment of ANT Content by CAT Titer**

The ANT content of HFE and LFE mitochondria was determined using CAT titers (Brand et al., 2005). This method utilizes the chemical characteristic of CAT as a specific high-affinity inhibitor of ANT in which CAT molecules bind stoichiometrically to ANT until it is fully inhibited (Streicher-Scott et al., 1993). Consequently, ANT molar content in mitochondria is equal to the minimum amount of CAT needed to lower state 3 (active) respiration to state 4 (resting) respiration. Values are reported as nanomoles of CAT per milligram of protein.

**Statistical Analyses**

Data are presented as the mean ± SEM and means are separated by t-tests. A probability level of \(P \leq 0.05\) was considered statistically significant.

**RESULTS AND DISCUSSION**

Data used for phenotyping HFE and LFE broilers in this study are shown in Table 1. Similar to previous studies, HFE broilers had greater BW gain while consuming the same amount of feed as LFE broilers, resulting in better FE and feed conversion ratio. Six-week BW was lower in the HFE group, but there were no differences in 7-wk BW between groups. These results contrast with previous studies in which there were no differences in 6-wk BW, but HFE broilers weighed more at 7 wk (on the same amount of feed) compared with LFE broilers (Bottje et al., 2002). Also, FE in both groups was lower in the present study compared with previous studies (Bottje et al., 2002), probably due to the way in which FE was determined (i.e., the timing of feeding and feed withdrawal before and after the 1-wk time period for FE determination). Despite these differences in FE data, the relative differences between the HFE and LFE groups were identical (0.19) in this study and our first investigation of mitochondrial function and FE (Bottje et al., 2002). There were no differences in amount of protein in isolated mitochondria between HFE and LFE groups with values (±SE) of 1.35 ± 0.05 and 1.29 ± 0.04 mg of protein per milligram of breast muscle tissue, respectively, which is similar to previous studies.

Mitochondrial respiration rates and ETC coupling to ATP synthesis in isolated mitochondria in the presence (+) or absence (−) of BSA are shown in Table 2. Fatty acid free BSA sequesters fatty acids and lipid peroxides that can increase proton conductance (Figure 1) and resting respiration rate in isolated mitochondria (Table 2). It is apparent that state 2 and state 4 respiration rates (resting respiration before and after the addition and subsequent consumption of ADP) were decreased by BSA in both HFE and LFE mitochondria. Bovine serum albumin increased state 3 respiration in LFE mitochondria but had no significant effect on state 3 respiration in HFE mitochondria. There were no differences in active or resting respiration rates between LFE and HFE mitochondria with or without BSA in the medium. Bovine serum albumin treatment increased all ETC coupling determinations in both HFE and LFE groups (Table 2, bottom section). There were no differences in coupling between HFE and LFE mitochondria...
with or without BSA with the exception of higher state 3/state 2 values in LFE compared with HFE mitochondria treated with BSA. There were also no differences in respiration rate or coupling between HFE and LFE mitochondria provided with NADH-linked substrates (10 mM malate and 1 mM Glu) as the energy source (data not shown). The higher state 3/state 2 in LFE mitochondria treated with BSA (Table 1) is the first time higher ETC coupling values have been observed in LFE compared with HFE mitochondria in several tissues (breast and leg muscle, liver, duodenum) provided either flavin adenine dehydrogenase (FADH)-linked (succinate) or NADH-linked (Glu, malate, pyruvate either singly or in combination) energy substrates (Bot-tj et al., 2002; Ojano-Dirain et al., 2004; Iqbal et al., 2005) and may represent only statistical noise.

Initial MMP and state 2 respiration rates for breast muscle mitochondria from HFE and LFE groups are shown in Table 3. There were no differences in initial control and treatment MMP values between HFE and LFE groups or for any treatment compared with within-group control values with the exception of lower \((P < 0.05)\) MMP in HFE mitochondria receiving RET + GDP. Compared with control values, initial state 2 respiration was lowered by BSA and raised by RET treatments in both groups, raised by the RET + GDP combination in LFE mitochondria, and lowered in HFE mitochondria by CAT treatment. Finally, state 2 respiration was higher in LFE mitochondria compared with HFE mitochondria after RET + GDP and CAT treatments.

Control proton leak kinetics and after treatment of HFE and LFE mitochondria with BSA are shown in Figure 1. The nonlinear relationship of MMP to respiration is characteristic of proton leak kinetics reported in both birds and mammals (Brookes et al., 1998; Brand et al., 2003; Talbot et al., 2003; Cawthon et al., 2004). The BSA-mediated decrease of proton conductance was clearly similar in both LFE and HFE mitochondria and concurs with previous reports of BSA on proton conductance (Mellors et al., 1967; Echtay et al., 2002a, 2003; Parker et al., 2008). Thus, the presence

| Table 1. Growth performance data for broilers with low and high feed efficiency (FE)1 |
|-----------------|-----------------|-----------------|
| Variable        | High FE (n = 9) | Low FE (n = 9) |
| 6-wk BW (g)     | 2,490 ± 67      | 2,704 ± 28      | 0.009 |
| 7-wk BW (g)     | 3,132 ± 76      | 3,178 ± 30      | 0.580 |
| Gain (g)        | 642 ± 41        | 474 ± 36        | 0.041 |
| Feed (g)        | 989 ± 61        | 1,033 ± 85      | 0.354 |
| FE (g of gain/g of feed) | 0.65 ± 0.01 | 0.46 ± 0.01 | 0.0001 |
| FCR* (g of feed/g of gain) | 1.54 ± 0.02 | 2.19 ± 0.03 | 0.0001 |

1Values are mean ± SE of values shown in parentheses.

*Feed conversion ratio.

Table 2. Mitochondrial respiration rates and electron transport chain (ETC) coupling [ratio of active (state 3) divided by resting (state 4) respiration rates] in breast muscle mitochondria provided with succinate (10 mM) without BSA (−BSA) or with 0.3% BSA (+BSA) in the incubation medium obtained from broilers exhibiting high (HFE) and low (LFE) feed efficiency phenotypes1

<table>
<thead>
<tr>
<th>Item</th>
<th>HFE −BSA (n = 9)</th>
<th>HFE +BSA (n = 5)</th>
<th>P-value</th>
<th>LFE −BSA (n = 9)</th>
<th>LFE +BSA (n = 5)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration rate2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>State 2 respiration</td>
<td>31 ± 2</td>
<td>22 ± 2</td>
<td>0.018</td>
<td>35 ± 2</td>
<td>23 ± 1</td>
<td>0.001</td>
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<tr>
<td>State 3 respiration</td>
<td>138 ± 12</td>
<td>169 ± 14</td>
<td>NS</td>
<td>159 ± 11</td>
<td>193 ± 9</td>
<td>0.054</td>
</tr>
<tr>
<td>State 4 respiration</td>
<td>64 ± 3</td>
<td>48 ± 6</td>
<td>0.014</td>
<td>70 ± 5</td>
<td>47 ± 4</td>
<td>0.006</td>
</tr>
<tr>
<td>State 3u respiration*</td>
<td>283 ± 33</td>
<td>361 ± 20</td>
<td>NS</td>
<td>303 ± 27</td>
<td>381 ± 24</td>
<td>0.080</td>
</tr>
<tr>
<td>State 4o respiration*</td>
<td>35 ± 3</td>
<td>25 ± 3</td>
<td>0.055</td>
<td>40 ± 2</td>
<td>28 ± 4</td>
<td>0.002</td>
</tr>
</tbody>
</table>

ETC coupling

<table>
<thead>
<tr>
<th>Item</th>
<th>HFE −BSA (n = 9)</th>
<th>HFE +BSA (n = 5)</th>
<th>P-value</th>
<th>LFE −BSA (n = 9)</th>
<th>LFE +BSA (n = 5)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>State 3/State 2</td>
<td>4.4 ± 0.2</td>
<td>7.7 ± 0.3</td>
<td>&lt;0.001</td>
<td>4.6 ± 0.3</td>
<td>8.6 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>State 3u/State 2</td>
<td>9.1 ± 0.9</td>
<td>16.5 ± 0.9</td>
<td>&lt;0.001</td>
<td>8.8 ± 0.7</td>
<td>17.0 ± 0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>State 3/State 4</td>
<td>2.3 ± 0.1</td>
<td>4.3 ± 1.1</td>
<td>0.038</td>
<td>2.4 ± 0.2</td>
<td>4.4 ± 0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>State 3u/State 4</td>
<td>4.0 ± 0.1</td>
<td>7.5 ± 0.9</td>
<td>0.004</td>
<td>4.1 ± 0.2</td>
<td>7.4 ± 0.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>State 3u/State 4o</td>
<td>8.4 ± 0.7</td>
<td>16.1 ± 2.0</td>
<td>&lt;0.001</td>
<td>7.6 ± 0.5</td>
<td>14.5 ± 1.5</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

1LFE + BSA value is higher \((P = 0.01)\) than HFE + BSA value.

2The data represent the mean ± SE of n observations (in parentheses) for respiration rates and ETC coupling obtained without BSA (−BSA) or with 0.3% BSA (+BSA) in the incubation medium.

3Respiration rates (in nmol of oxygen consumed/min per milligram of mitochondrial protein) are shown for state 2 initial resting respiration [no adenosine diphosphate (ADP) present], state 3 active respiration (ADP in excess), and state 4 resting respiration (ADP limiting) as defined by Esta-brook (1967).

4Respiration rates in the presence of 1 µM of the uncoupling compound carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

5NS designates P-values >0.10.

*State 3u = uncoupled active respiration rate (ADP in excess) in the presence of 1 µM of the uncoupling compound carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

*State 4o = resting respiration rate (ADP depleted) in the presence of 1 µg/mL of the adenosine triphosphate synthase inhibitor, oligomycin.
of fatty acids or lipid peroxidation products, or both, under control conditions caused a similar degree of uncoupling in both HFE and LFE mitochondria.

The effects of CAT and Glu, either singly or in combination, on proton leak kinetics of HFE and LFE breast muscle mitochondria are shown in Figure 2. Carboxyatractylate is an inhibitor of ANT that, in addition to transporting nucleotides across the inner mitochondrial membrane, also acts as an uncoupling molecule for the mitochondrial inner membrane (Andreyev et al., 1988, 1989; Brand et al., 2005). The treatment of mitochondria with CAT clearly decreased proton leak (displacement of the proton kinetic curve down) in both HFE (Figure 2A) and LFE (Figure 2B) mitochondria, and the decrease in proton leak by CAT was greater in HFE than in LFE mitochondria (Figure 2C). However, ANT levels determined by CAT titer (Brand et al., 2005) in the present study revealed no difference between HFE and LFE mitochondria; ANT values were calculated to be 7.0 ± 0.4 (n = 4) and 6.9 ± 0.3 (n = 5) nmol of CAT/mg of mitochondrial protein, respectively. Although Ojano-Dirain et al. (2007b) reported higher mRNA expression of ANT in breast muscle of HFE compared with LFE broilers (Ojano-Dirain et al., 2007b), ANT protein expression was actually lower in HFE muscle compared with values in HFE muscle tissue (Iqbal et al., 2004). Although mRNA expression and protein expression do not always go hand in hand due to differences in mRNA stability and posttranslational modifications of proteins (Day and Tuite, 1998), a good correlation between ANT protein and mRNA expression was reported in penguin muscle (Talbot et al., 2004). Although the mechanism is not apparent, the results clearly show that CAT treatment (Figure 2A to C) resulted in lower proton conductance in HFE compared with LFE mitochondria.

Table 3. Initial mitochondrial membrane potential and state 2 respiration rates before proton leak assessment for breast muscle mitochondria that were untreated (control) or treated with BSA, guanosine diphosphate (GDP), retinal (RET), retinal + GDP (RET + GDP), carboxyatractylate (CAT), Glu, and Glu + CAT obtained from broilers with high (HFE) and low (LFE) feed efficiency.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HFE</th>
<th>LFE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP2</td>
<td>State 23</td>
<td>n</td>
</tr>
<tr>
<td>Control</td>
<td>200 ± 3</td>
<td>61 ± 4</td>
</tr>
<tr>
<td>+BSA</td>
<td>199 ± 3</td>
<td>28 ± 5a</td>
</tr>
<tr>
<td>+GDP</td>
<td>197 ± 3</td>
<td>64 ± 3</td>
</tr>
<tr>
<td>+RET</td>
<td>195 ± 4</td>
<td>72 ± 4a</td>
</tr>
<tr>
<td>+RET + GDP</td>
<td>187 ± 5a</td>
<td>60 ± 5a</td>
</tr>
<tr>
<td>+CAT</td>
<td>207 ± 5</td>
<td>47 ± 4a</td>
</tr>
<tr>
<td>+Glu</td>
<td>201 ± 6</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>+Glu + CAT</td>
<td>202 ± 7</td>
<td>55 ± 2</td>
</tr>
</tbody>
</table>

*Means within a column are different from control values (P < 0.05).

*HFE state 2 respiration mean values are lower than LFE mean values (P < 0.05).

1Values are mean ± SE of n observations shown for each.

2MMP = mitochondrial membrane potential (in mV).

3State 2 = state 2 respiration rate (in nmol of O/min per milligram of P).

Figure 1. The effect of 0.3% BSA on proton leak kinetics assessed by simultaneous measurements of mitochondrial inner membrane potential (mV) and respiration rate (nmol of O/min per milligram of protein) in breast muscle mitochondria obtained from broilers with high (HFE) and low (LFE) feed efficiency. Each data point represents the mean ± SE of observations shown in parentheses.
Figure 2. The effects of 2 µM carboxyatractylate (CAT; panels A to C), 10 mM Glu (panels D to F), and the combination of Glu and carboxyatractylate (Glu + CAT; panels G to I) on proton leak kinetics in breast muscle mitochondria obtained from broilers with high (HFE) and low (LFE) feed efficiency in: A) untreated HFE (HFE, Con, ◊) and CAT-treated (HFE + CAT, ▲) mitochondria, B) untreated LFE (LFE, Con, ■) and CAT-treated (LFE + CAT, Δ) mitochondria, C) HFE + CAT (▲) and LFE + CAT (Δ)-treated mitochondria, D) untreated control HFE (HFE, Con, ◊) vs. Glu-treated (HFE + Glu, ♦) mitochondria, E) untreated control LFE (LFE, Con, ■) and Glu-treated (LFE + Glu, ○) mitochondria, F) HFE + Glu (♦) and LFE + Glu (○)-treated mitochondria, G) untreated control HFE (HFE, Con, ◊) and Glu + CAT-treated (HFE + Glu + CAT, ●) mitochondria, H) untreated control LFE (LFE, Con, ■) and Glu + CAT-treated (LFE + Glu + CAT, Δ) mitochondria, I) HFE and LFE mitochondria received the Glu + CAT combination treatments, J) HFE control (Con, ◊) and HFE mitochondria receiving Glu + CAT combination (HFE + Glu + CAT, ●) and CAT treatment alone (HFE + CAT, ▲), K) LFE control (Con, ■) and HFE mitochondria receiving Glu + CAT combination (LFE + Glu + CAT, Δ) and CAT treatment alone (LFE + CAT, ○). Each data point represents the mean ± SE of observations shown in parentheses. MMP = mitochondrial membrane potential.
The substrates for the Glu-aspartate antiporter have been shown to inhibit mitochondrial uncoupling mediated by fatty acids (Samartsev et al., 2000). In the present study, Glu had no effect on proton conductance in either HFE (Figure 2D) or LFE (Figure 2E), and there was no difference in proton conductance between Glu-treated HFE and LFE mitochondria (Figure 2F). Similar to CAT treatment alone (Figure 2A and 2B), the CAT + Glu combination lowered proton conductance in both HFE (Figure 2G) and LFE (Figure 2H) mitochondria. Interestingly, although proton conductance was lower in HFE compared with LFE mitochondria following CAT alone (Figure 2C), the Glu + CAT treatment resulted in similar proton leak kinetics between HFE and LFE mitochondria (Figure 2I). A qualitative difference in the response of HFE and LFE mitochondria to the Glu + CAT combination vs. CAT alone can also be observed in Figure 2J and 2K; although Glu + CAT proton conductance is clearly intermediate between control and CAT conductance curves in HFE mitochondria (Figure 2J), in LFE mitochondria, the CAT proton conductance curve is intermediate to the control and Glu + CAT combination proton conductance curves (Figure 2K). Thus, in HFE mitochondria, Glu + CAT treatment appeared to attenuate the CAT-mediated decrease in proton conductance (Figure 2I), whereas the Glu treatment enhanced CAT-mediated proton conductance in LFE mitochondria (Figure 2K). The effects of BSA on respiration characteristics in HFE and LFE mitochondria presented in Table 1 indicate the possibility of greater sensitivity of LFE mitochondria to fatty acids with significant increases noted in state 3 respiration compared with HFE mitochondria. This also resulted in greater state 3/state 2 coupling in LFE compared with HFE mitochondria. With these observations, it is possible that Glu-mediated inhibition of fatty acid-mediated proton conductance was greater in LFE mitochondria accounting for the lack of difference in the Glu + CAT combination between HFE and LFE mitochondria (Figure 2I) as well as differences in proton conductance relationships for Glu + CAT and CAT alone for HFE (Figure 2J) and LFE (Figure 2K) mitochondria.

The addition of GDP had no effect on proton leak kinetics in HFE mitochondria (Figure 3A) and LFE (Figure 3B) breast muscle mitochondria. A slightly higher proton leak in LFE control mitochondria relative to HFE mitochondria (Figure 1) in combination with a slight increase in proton conductance by GDP (Figure 3B) resulted in greater proton conductance in LFE than in HFE mitochondria (Figure 3C).

Guanosine diphosphate inhibits proton conductance by blocking superoxide and 4-hydroxy 2-nonenal activation of UCP (Echtay et al., 2003; Talbot et al., 2003) and ANT (Parker et al., 2008). The lack of effect of GDP on proton conductance in HFE mitochondria (Figure 3A) could indicate that superoxide generation in HFE breast muscle mitochondria was negligible and would support findings of lower ROS generation (Bottje et al., 2002) and lower UCP expression (Ojano-Dirain et al., 2007b) in breast muscle of HFE compared with LFE broilers. These findings might be similar to reports in which GDP had no effect on proton conductance in breast muscle mitochondria from naive (i.e., not previously exposed to cold temperatures) penguins with low levels of UCP and that the ability of GDP to decrease proton conductance was directly related to UCP expression (Talbot et al., 2003, 2004). Rather than observ-

![Figure 3. The effect of 1 mM guanosine diphosphate (GDP) on proton leak kinetics in breast muscle mitochondria obtained from broilers with high (HFE) and low (LFE) feed efficiency in: A) untreated HFE (HFE, Con, ◊) and GDP-treated (HFE + GDP, ▲) mitochondria, B) untreated LFE mitochondria (LFE, Con, ■) and GDP-treated (LFE + GDP, ○) mitochondria, and C) showing differences in proton conductance between HFE + GDP (▲) and LFE + GDP (○)-treated mitochondria. Each data point represents the mean ± SE of observations shown in parentheses.](https://academic.oup.com/ps/article-abstract/88/8/1683/1537612)
ing a decrease in proton conductance, a slight increase in proton conductance was observed in GDP-treated LFE mitochondria (Figure 3B). We do know that avian UCP (avUCP) was higher ($P < 0.07$) in breast muscle from LFE broilers compared with HFE broilers (Ojano-Dirain et al., 2007b); therefore, if GDP was mediating an effect in the present study, a decrease in proton conductance should have been observed. Possibly, a different level of GDP was needed to block superoxide or lipid peroxide-mediated increases in proton conductance in broilers. What produced the slight increase in proton conductance in LFE mitochondria is not apparent, but it did result in greater proton conductance than in GDP-treated HFE mitochondria (Figure 3C). Further studies are needed to clearly define the role of GDP in proton leak kinetics in broilers.

It should be noted that 23 polymorphisms of the avUCP gene were reported in the same broiler line being investigated in the present study (Sharma et al., 2008). Of the 23 polymorphisms, 1 variant, the UCP-Val118Val allele, was associated with higher FE, whereas the UCPAla118Val was associated with lower FE. Also, a missense mutation was predicted in the transmembrane region of the UCP close to the mitochondrial inner membrane. Possibly, the atypical behavior of GDP on proton conductance in this study could be due to some structural differences in the avUCP in this broiler population.

The effect of RET alone and in combination with GDP is shown in Figure 4. Retinal is known to increase mitochondrial superoxide production (Klamt et al., 2005, 2008) and enhance UCP activity (Puigserver et al., 1996; Rial et al., 1999). In addition, Echtay et al. (2003) provided evidence that closely related chemical compounds, retinoic acid and trans-RET, could induce uncoupling of mitochondria directly and essentially function as a chemical analog of 4-hydroxy-trans-2-nonenal, which is a product of lipid peroxidation and capable of increasing proton conductance in mitochondria. In both HFE and LFE mitochondria, RET increased proton conductance (Figure 4A and C) that was unaffected by the presence of GDP (Figure 4B and D). Slightly greater proton conductance was observed in LFE mitochondria in response to RET compared with HFE mitochondria (Figure 4E). The inability of GDP to prevent or attenuate RET-induced increases in proton conductance in broilers would suggest atypical behavior of GDP on proton conductance in this study could be due to some structural differences in the avUCP in this broiler population.

**Figure 4.** The effect of 12 µM retinal (RET) and retinal in combination with 1 mM guanosine diphosphate (GDP) on proton leak kinetics in breast muscle mitochondria obtained from broilers with high (HFE) and low (LFE) feed efficiency in: A) untreated control HFE mitochondria (HFE, Con, ◊) and RET-treated mitochondria (HFE + RET, --○--); B) HFE mitochondria that were untreated (HFE, Con, ◊), RET-treated mitochondria (HFE + RET, --○--), and HFE mitochondria treated with RET in combination with GDP (HFE + RET + GDP, ●); C) untreated control LFE mitochondria (LFE, Con, ■) and RET-treated mitochondria (LFE + RET, □); D) untreated control LFE mitochondria (LFE, Con, ■), RET-treated mitochondria (LFE + RET, □), and mitochondria treated with RET in combination with GDP (LFE + RET + GDP, ●); and E) showing proton conductance in HFE + RET and LFE + RET-treated mitochondria. Each data point represents the mean ± SE of observations shown in parentheses. MMP = mitochondrial membrane potential.
in proton conductance is once again incongruent with findings by Echtay et al. (2003) but is consistent with the lack of effect of GDP on proton conductance described above. It is possible that the increase in proton conductance in response to RET may have been mediated via modifications of ANT as was demonstrated by Echtay et al. (2003).

A summary is provided in Table 4 to show differences in proton conductance compared with control values (column A) and relative differences of proton conductance curves for each treatment between HFE and LFE mitochondria (column B). It is apparent that there are both differences and similarities in proton leak kinetics between HFE and LFE mitochondria. No treatment resulted in a proton conductance in HFE mitochondria that was higher than in LFE mitochondria. Second, sequestration of fatty acids and lipid oxidation products by BSA produced the largest decrease in proton conductance in both groups, resulting in virtually identical proton conductance curves. Third, the CAT treatment reduced proton conductance in both groups, but the reduction was clearly greater in HFE mitochondria and the combination of Glu + CAT abrogated the difference in proton conductance between groups observed with CAT treatment alone. Although it is tempting to suggest that the results from the CAT treatment indicate the presence of greater amounts of ANT in HFE mitochondria, the fact that amounts of ANT determined by CAT titer were nearly identical in both groups would indicate that one or more other mechanisms produced the differences in HFE and LFE mitochondria response to CAT. What this mechanism might be is not apparent, yet could be very significant with regard to regulation of proton leak in HFE muscle mitochondria. Finally, RET moderately raised proton conductance in both groups compared with control values, with proton conductance being slightly less in the HFE group. The RET + GDP combination eliminated differences between groups observed with RET treatment alone.

The results of this study indicate that subtle differences in proton leak kinetics exist in mitochondria obtained from broilers exhibiting either a HFE or LFE phenotype in this genetic line. The differences could be a consequence of critical mitochondrial components such as proteins and lipids integral to the mitochondrial membrane. As with all studies of this kind, the present study provides a snapshot of proton conductance in tissue at a specific age. It is reasonable to hypothesize that even a small difference in proton conductance between the HFE and LFE groups could be very significant energetically to the animal over long time periods (e.g., several weeks). Although mechanisms responsible for these differences are not clear from this study, it does provide additional insight into relationships of mitochondrial function with phenotypic expression of FE in broilers. Further studies in this area could provide insight into the relationship of proton leak mechanisms and higher mitochondrial ROS production that have been previously reported (Bottje et al., 2002; Bottje and Carstens, 2009).

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respiration rate in working skeletal muscle and liver and to SMR. 