CHAPTER 5: SEPSIS AND UCP3

5.1 Introduction

The mitochondrial uncoupling protein 3 (UCP3) belongs to a family of mitochondrial transporter proteins found predominantly in skeletal muscle and heart (Boss, Samec et al. 1997). Despite its name and high sequence homology with UCP1, the exact function of UCP3 remains controversial (Bézaire, Seifert et al. 2007). Increased expression of UCP3 has been reported in many pathological states as well as starvation and sepsis (Yu, Barger et al. 2000; Dulloo, Samec et al. 2001; Sun, Wray et al. 2003). Whilst some evidence points towards a protective function of UCP3 in reducing mitochondrial ROS production by mild uncoupling of oxidative phosphorylation, the energetic implications are still poorly understood (Brand and Esteves 2005; Costford, Seifert et al. 2007).

Mice lacking UCP3 show little change in body weight regulation, exercise tolerance, fatty acid metabolism or cold-induced thermogenesis compared to their wild-type counterparts (Vidal-Puig, Grujic et al. 2000). Studies using *Ucp3*$^{(-/-)}$ mice have shown little in the way of phenotypic difference to wild-type mice (Bézaire, Seifert et al. 2007). However, it is likely that UCP3 function is highly regulated and its activation requires stimulation by fatty acids, ROS and ROS by-products (Brand and Esteves 2005). Upregulation of compensatory proteins may explain the lack of difference in these knockout mice, though this was not reported in the original description (Vidal-Puig, Grujic et al. 2000).

In the previous chapter, I found an unchanged proton leak in mitochondria isolated from skeletal muscle of starved and septic mice, while ROS production was increased. Up-regulation of UCP3 shown in previous studies (Yu, Barger et al. 2000; Sun, Wray et al. 2003) could be a counter-mechanism to limit this increased ROS production and its damaging effect on the contractile apparatus of the muscle cell. In this chapter I explore the importance of UCP3 on metabolic changes, muscle dysfunction and survival from sepsis by comparing wild-type and *Ucp3*$^{(-/-)}$ mice.

5.2 Methods

5.2.1 Breeding of *Ucp3*$^{(-/-)}$ mice

Ucp3$^{(+/-)}$ heterozygote mice were kindly provided by Prof Martin Brand (Dunn Institute, Cambridge, UK). They were originally created by Gong *et al* (Gong, Monemdjou et al. 2000) and already backcrossed 10 generations onto C57Bl/6 mice. Male and female heterozygote mice were bred to produce homozygote offspring. Ear punches were performed to mark the mice and to obtain tissue for *Ucp3* genotyping. Homozygote mice were used to form multiple *Ucp3*$^{(-/-)}$ breeding triplets (2 females, one male). C57Bl/6 wild-type mice were bought from Charles River (Margate, UK)

5.2.1.1 DNA extraction from ear punches and tail snips

Ear punches and mouse tail tips (3mm) were snipped after cold spray anaesthesia. These were digested overnight in 500µl of digestion buffer (100 mM Tris HCl (pH 8), 5 mM EDTA, 200 mM NaCl, 0.2% Sodium Dodecyl Sulfate-SDS) with proteinase K (100 µg/ml- Sigma-Aldrich P8044) at 55°C with occasional shaking.

Phenol/Chloroform/isoamyl alcohol (25:24:1) 0.5 ml was used to extract the DNA with gentle inversion followed by centrifugation (13,000 rpm in microfuge for 5 mins). Chloroform 0.5 ml was then added to the supernatant and gently inverted and centrifuged again for 5 mins. Sodium acetate (3 M pH 5.2) was added at 1/10 volume of the supernatant with 2 volumes of 100% ethanol. The resulting mixture was mixed by gentle inversion for 1 minute to precipitate the DNA which was removed using a pipette and transferred to a new tube. This was washed with 0.5ml of 70% ethanol and allowed to air dry for 5 mins before dissolution in 100 µl of Tris-acetate EDTA (TEA) buffer (40 mM Tris acetate, 1 mM EDTA). All reagents were purchased from Sigma-Aldrich.

The concentration of DNA in each sample was measured using a Thermo Scientific Nanodrop^{TM} Spectrophotometer (Wilmington, USA) and samples used to perform polymerase chain reaction (PCR).

5.2.1.2 PCR for UCP3 genotyping of mice

PCR primers were obtained from Sigma-Aldrich with the following base sequences:

- P1 = UCP3KO F1.2 (5'-GCAAAACCAAATTAAGGG-3')
- P2 = UCP3KO F2 (5'-CATAGGCAGCAAAGGAAC-3')
- P3 = UCP3KO R1.2 (5'-CTTTATGGTTTACACAGC-3')

PCR reactions were performed with 75 ng of DNA in 25 µl of reaction buffer containing:

- Taq buffer (10x) 2.5 µl (final conc. 1x)
- Nucleotides (dNTP)10mM (0.5 µl - final conc. 0.2 mM)
- PCR primers mixture 0.75 µl (final conc. 3 ng/µl)
- Taq DNA polymerase 0.25 µl (final amount ~1.25 U)
- Double distilled water 20 µl

PCR program (Eppendorf Mastercycler® machine, Cambridge, UK):

- 1 cycle 95°C 5 mins
- 35 cycles of 95°C 30 s, 58°C 30 s, 72°C 30 s
- 1 cycle 72°C 5 mins

Samples were run on a 1% agarose/TAE gel to resolve the 400 base pair (wild type) and 490 base pair bands (knockout).

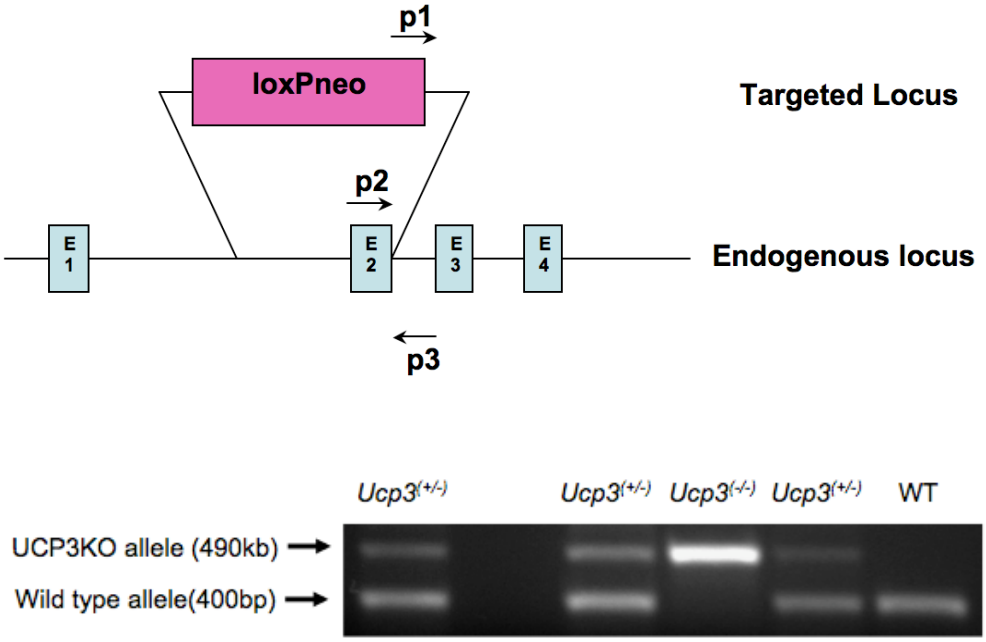

Figure 5.1 PCR products of tail/ear snips taken from different mice during the breeding phase to produce UCP3 knockout mice from heterozygote pairs. The product of the knockout allele with an inserted cassette was ~490 kilo-base pairs while the wild-type allele is ~400 kilo-base pairs.

5.2.2 Western blotting for UCP3 protein

The effect of sepsis on UCP3 protein abundance was measured at 10h and 20h following i/p faecal slurry. Wild-type fed sham and septic mice (see Chapter 2 for set-up) were culled at specified time-points with tissues snap-frozen in liquid N_2 and stored at -80°C. Approximately 100 mg of heart and skeletal muscle were homogenised with an overhead stirrer (Stuart Scientific,) in 900µl of ice-cold homogenisation buffer (sucrose 320 mM, EDTA 1 mM, Tris HCl 10 mM at pH 7.4).

SDS (2% final concentration) was used for protein precipitation with proteinase inhibitors (S8820-Sigma-Aldrich) added to stop protein degradation. Following mixing for 1 minute, samples were centrifuged at 12,000 rpm for 10 mins. The supernatant was transferred to a new ice-cold tube for protein estimation using BCA protein assay kit (Thermo Fischer Scientific) and read at 560nm in a multi-plate reader with appropriate BSA standards (Biotek Instruments).

To detect UCP3 protein, 10 µg of protein was separated on a 12% SDS polyacrylamide gel and transferred to nitrocellulose. Membranes were blocked for 1 hour at room temperature in Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) Marvel, then probed overnight at 4˚C with the primary antibody (Rabbit anti-UCP3: ABR) at 1:1000 dilutions in blocking buffer. Each sample was loaded twice in the same gel, and each gel repeated once.

Subsequently, the membranes were incubated for 1h at room temperature with peroxidase-conjugated 2˚ goat-anti-rabbit antibody (DAKO, USA) diluted to 0.1µg/mL in blocking buffer, then cross-reacted proteins were visualised by enhanced chemiluminescence (Amersham Biosciences) on photographic film (3M, Diegem, Belgium). Densitometry measurements were made using Image J image processing and National Institute of Health analysis software after scanning the films. A single control sample was used in each gel and all results normalized to this control allowing comparison between samples in different gels.

Alpha-tubulin (1:5000 dilution, Abcam 7291, Cambridge) was used to ensure equal loading of gels and transfer of proteins from gel to

membrane. Beta-actin was also used, but produced very variable results. Each membrane was also Coomassie stained at the end to ensure equal loading of the gels and transfer to membranes.

5.2.3 *In vivo* experiments with *Ucp3*$^{(-/-)}$ and wild-type mice

5.2.3.1 Mouse model of sepsis

Section 2.2.1.3 in Chapter 2 provides a fuller description of the model and the methods for the various *in vivo* experiments. In short, age-matched male wild-type and *Ucp3*$^{(-/-)}$ mice received i/p faecal slurry (or saline in sham groups) at time 0h. They were housed at room temperature with 12h light-dark cycles and fluid resuscitated at 0, 6, 18, 30, 42h time-points with s/c dextrose-saline. A validated clinical scoring system was used to assess the severity of sepsis at 24h in each mouse (see Chapter 2).

5.2.3.2 Survival study

Survival from sepsis in *Ucp3*$^{(-/-)}$ mice was compared with age-matched (20 week old) wild-type mice. Experiments were performed on three separate weeks. Each week, 7 genotyped *Ucp3*$^{(-/-)}$ mice and 7 wild-type mice were block-randomized to sham or septic. The same numbers of wild-type and *Ucp3*$^{(-/-)}$ animals were used per week to limit bias of survival results due to any variation of potency of the faecal slurry.

5.2.3.3 Metabolic and cardiac output measurements

Echocardiograms were performed at 0, 6 and 24h as described in section 2.2.3. Ascending aorta peak flow velocity, velocity time integral (VTI) and heart rate were measured by pulsed wave Doppler. These measurements were used to measure stroke volume and cardiac output Metabolic rate was measured continuously with mice housed in metabolic boxes. This was started 4 hours prior to i/p injection of faecal slurry or saline and continued for 24 hours following (described in Section 2.2.4). Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured and respiratory exchange ratio (RER) calculated.

5.2.3.4 Isometric force and cyclical power measurements

Age-matched *Ucp3*$^{(-/-)}$ and wild-type mice were split into fed sham, starved sham and septic groups. At 24 hours post-injection of slurry or saline, diaphragm muscle strips were dissected. Maximal isometric force, maximal power generation and repetitive cyclical stimulation with power measurement were performed as described in Chapter 3.

5.2.3.5 Measurement of diaphragm mitochondrial transmembrane potential in *Ucp3*$^{(-/-)}$ mice

Mitochondrial transmembrane potential ($\Delta\psi_m$) was measured in diaphragm muscle strips of fed sham and septic *Ucp3*$^{(-/-)}$ mice using tetramethylrhodamine methyl ester (TMRM) as described in chapter 4

(section 4.2.2). Identical dye concentration, incubation time, imaging conditions were for the *Ucp3*$^{(-/-)}$ mice.

5.3 Results

5.3.1 UCP3 protein abundance in septic wild-type mice

UCP3 protein abundance was measured in hearts and skeletal muscles of 20-week old septic and fed sham wild-type mice. Measurements were made at 10h (6 mice) and 24h (8 mice) in the severe septic group, and only at the 24h time-point in the sham group (6 mice). The specificity of the UCP3 antibody was evaluated using tissue taken from UCP3 knockout mice (fig. 5.2).

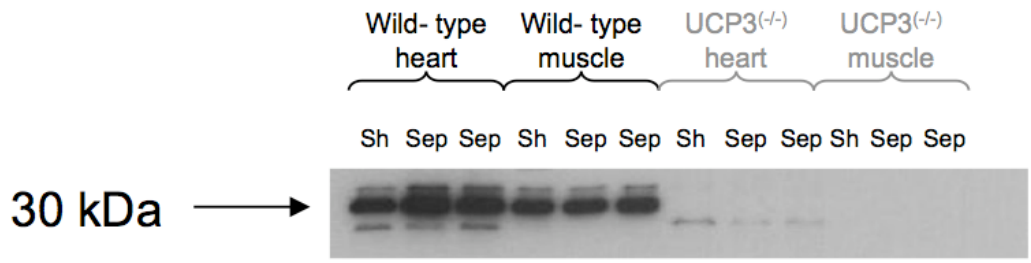

Figure 5.2 Western blots showing representative bands of UCP3 protein in skeletal muscle and heart homogenates from wild-type mice (left 6 lanes). These bands are missing in homogenates from skeletal muscle and hearts of *Ucp3*$^{(-/-)}$ mice. Lower molecular weight ghost bands appear in the heart samples. These were also present in samples from *Ucp3*$^{(-/-)}$ mouse heart but absent in skeletal muscle. Sh=Sham, Sep=Septic.

5.3.1.1 Cardiac muscle UCP3 protein is not elevated in severely septic mice at 10hrs or 24 hrs.

There were no statistical differences in the Coomassie-normalized UCP3 protein densitometry values between sham 24h, septic 10h and septic 24h heart homogenates (fig.5.4). However, there was a statistically significant 18% reduction in the abundance of α-tubulin at 10h and 24h in the septic groups when compared to the sham 24h ($p<0.05$, t-test). This was despite equal loading seen on the Coomassie stain (fig. 5.3). The ratio of UCP3/α-tubulin protein level showed a 50% rise at 10h (non-significant), which returned to normal at 24h in the septic group.

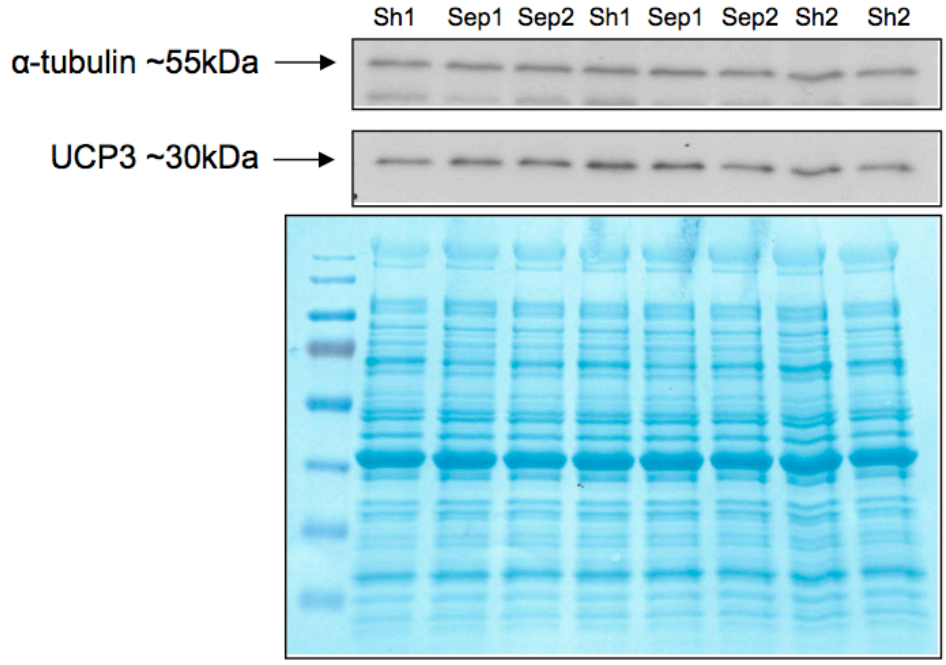

Figure 5.3 Representative Western blots of heart homogenates for α-tubulin (top), UCP3 (middle) and Coomassie stain (bottom). Two sham samples (Sh1, Sh2), a 10h septic (Sep1) and a 24h septic sample (Sep2) are shown. The Coomassie stain shows all the protein bands on the membrane in the different lanes, and confirms equal loading and transfer.

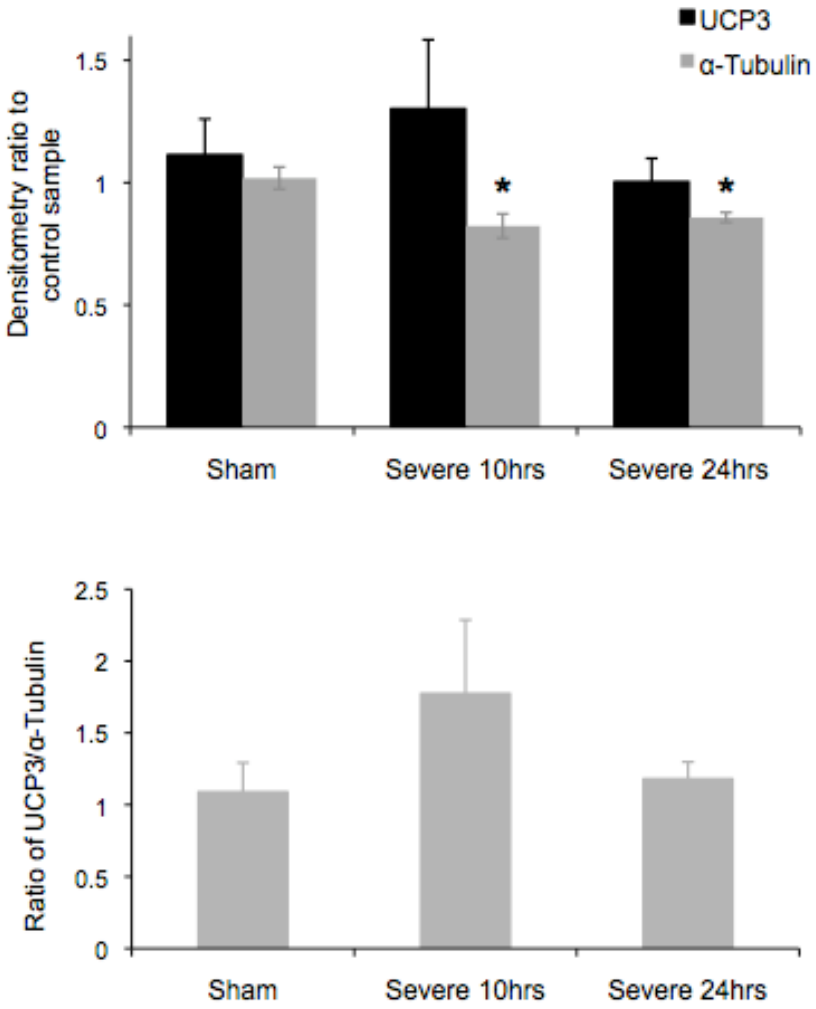

Figure 5.4 Densitometry measurements (normalized to control) of heart homogenates for UCP3 and α-tubulin (top panel). UCP3/α-tubulin ratio (bottom panel) shows a non-significant increase at 10 h, returning to sham levels at 24 h. Results are means ± SEM. (* $p<0.05$, ANOVA)

5.3.1.2 Diaphragm muscle UCP3 protein abundance is increased in severe septic mice at 24h.

Western blotting for UCP3 and α-tubulin were performed in diaphragm muscle homogenates isolated from fed sham and septic mice (fig. 5.5). Similar to heart, the normalized α-tubulin densitometry was

reduced in septic mice (22% reduction in 10h septic, and 12% in the 24h severe septic groups), although statistically significant only at 10h (Fig 5.6B). There was a 28% increase in UCP3 protein densitometry in severe sepsis mice at 24h (fig. 5.6A). When normalized to α-tubulin densitometry, this increase was 81% (median of sham ratio 0.69 vs. 24h severe septic ratio 1.24; $p<0.016$ Mann-Whitney U test) [fig. 5.6C]. Coomassie staining confirmed equal loading.

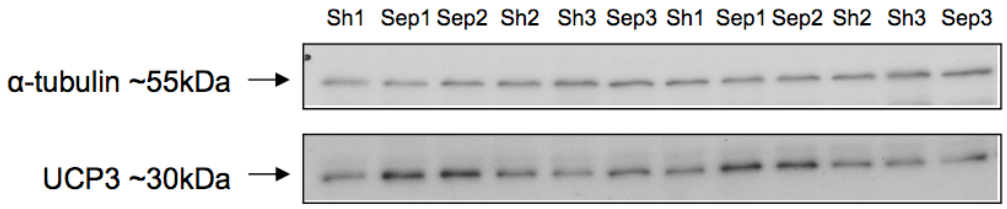

Figure 5.5 Representative Western blot of α-tubulin and UCP3 of skeletal muscle homogenates isolated from 24h Sham mice (Sh1, Sh2, Sh3), 10h severely septic mice (Sep3), and 24h severely septic mice (Sep2, Sep3).

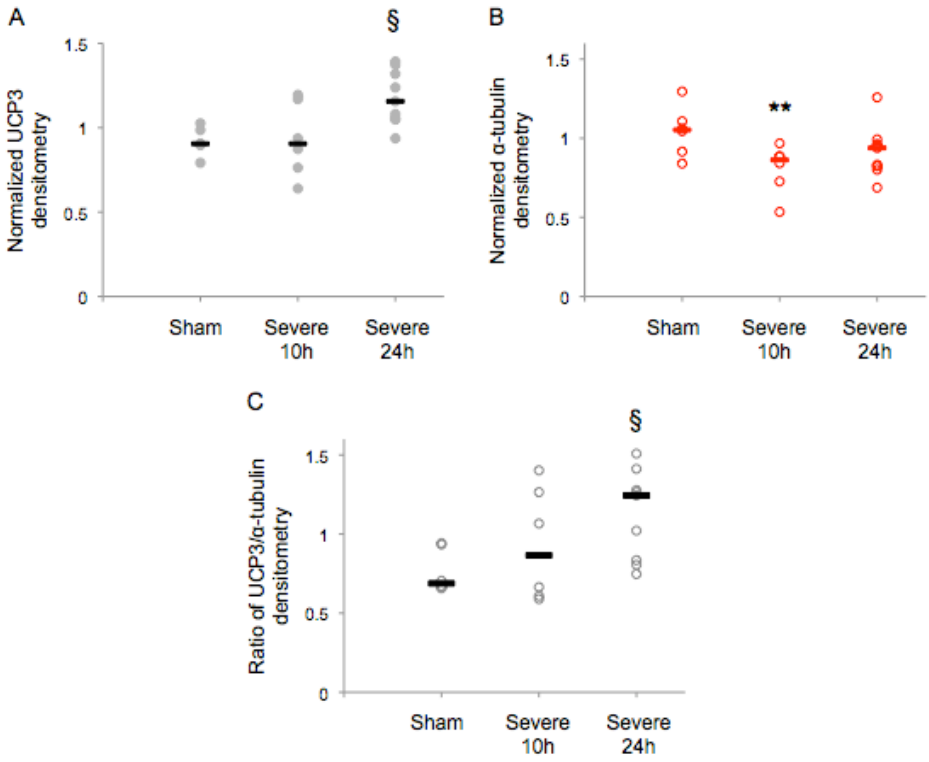

Figure 5.6 Plots of normalized densitometry results of UCP3 protein (A) and α-tubulin (B) as a ratio of absolute densitometry value to internal control of skeletal muscle. The ratio of UCP3 densitometry to α-tubulin is shown in the bottom panel (C). Samples were from 24h fed sham mice (n=6), 10h severe septic (n=6) and 24h severe septic mice (n=8). Median values are depicted by the horizontal bar. Mann-Whitney U test was used to test for statistical significance. § $p<0.016$ compared to both 10h septic and sham groups. **$p<0.016$ when the results compared to sham only.

5.3.2 Sepsis survival similar in wild-type and *Ucp3*$^{(-/-)}$ mice

Twenty-one wild-type and 21 *Ucp3*$^{(-/-)}$ mice were used for these experiments in 3 consecutive weeks. Figure 5.7 shows survival rates

with no mortality in the sham mice. Mortality at 72h was 75% in both the septic wild-type and *Ucp3*$^{(-/-)}$ mice.

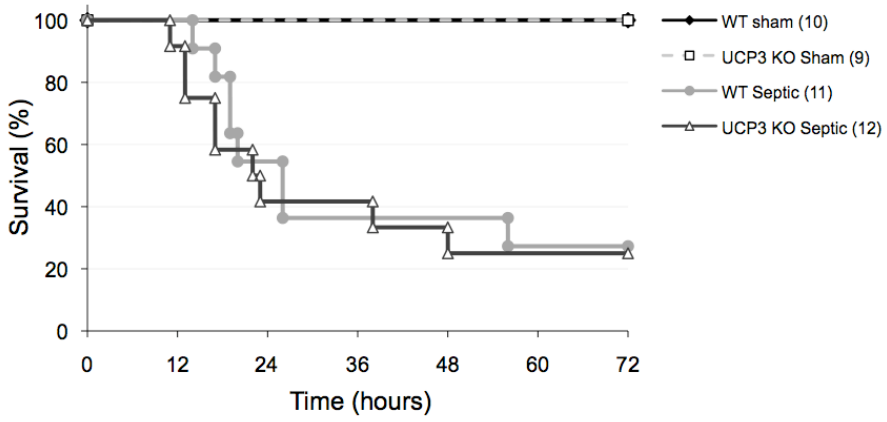

Figure 5.7 Kaplan-Meier plot of survival of 20 week old septic *Ucp3*$^{(-/-)}$ mice and age- and gender-matched wild-type C57bl/6 mice.

5.3.3 Metabolic parameters in response to sepsis are similar in wild-type and *Ucp3*$^{(-/-)}$ mice.

Rectal temperature was measured at 0, 6, 18 and 24h in wild-type and *Ucp3*$^{(-/-)}$ fed sham and septic mice (9-14 per group). No difference was noted in between the septic groups. Temperatures in wild-type fed sham mice were marginally but statistically significantly higher than fed sham knockouts ($p<0.05$) [fig. 5.8]. There were no differences in starved sham mice (data not shown).

As shown in Chapter 2, starved sham mice lost significant amounts of weight over 24h, whereas septic mice gained weight (fig. 5.9). No differences in weight were found between the *Ucp3*$^{(-/-)}$ and wild-type mice ($p>0.34$ independent sample t-test).

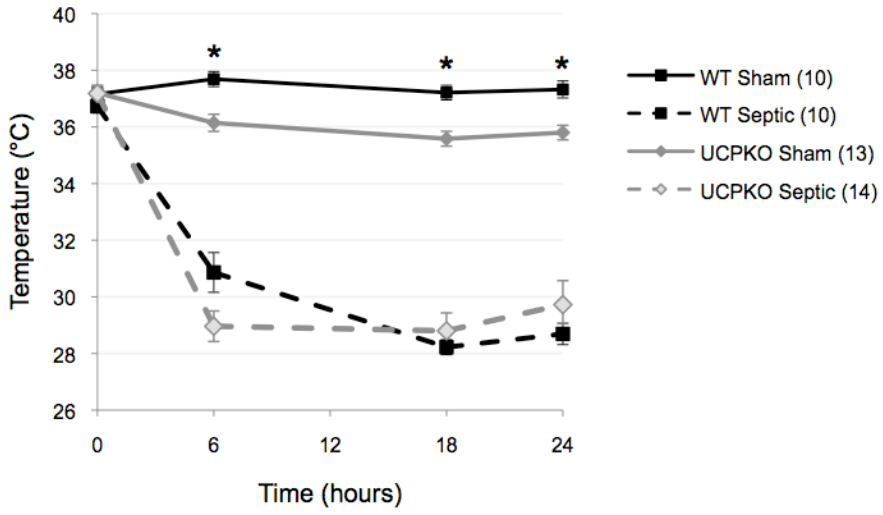

Figure 5.8 Rectal temperature of fed sham and septic wild-type and *Ucp3*$^{(-/-)}$ mice. The number in each group is shown in brackets. Results are mean ± SEM. *p<0.05 comparing fed sham wild-type and *Ucp3*$^{(-/-)}$ mice (repeated measures t-test).

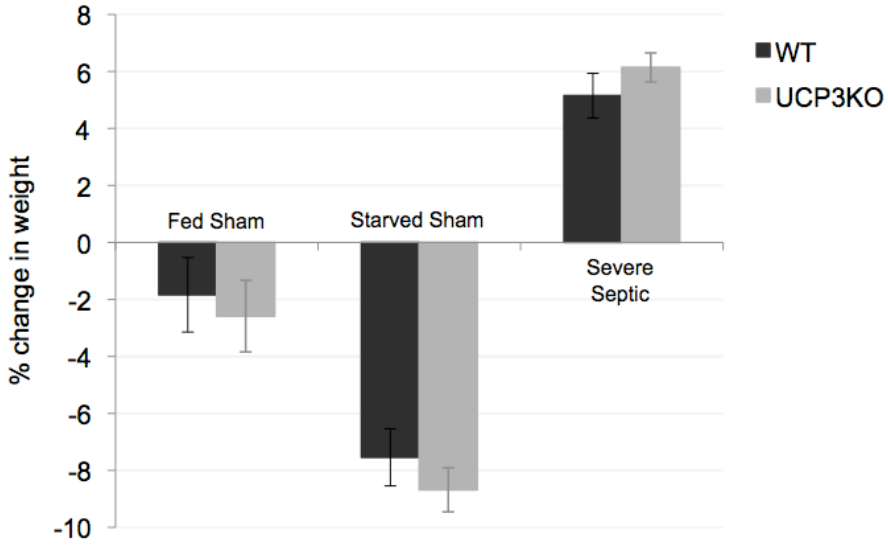

Figure 5.9 Percentage body weight change at 24 h in wild-type and *Ucp3*$^{(-/-)}$ septic and sham mice. Starvation was started after i/p injection of faecal slurry. All mice received s/c fluid resuscitation at 0, 6, and 18h.

Total body oxygen consumption (VO_2) was measured in age-matched starved sham and septic *Ucp3*$^{(-/-)}$ mice and compared to wild-type mouse data shown in Chapter 2. Starvation was started immediately after i/p injection of faecal slurry or saline. No differences in VO_2 response to starvation alone, or to sepsis, were noted between the two genotypes (Fig 5.10). VO_2 was already significantly reduced 1h post injection of slurry (time 0h in the VO_2 graph of fig 5.10). A plot of temperature vs. VO_2 can be seen in figure 5.11.

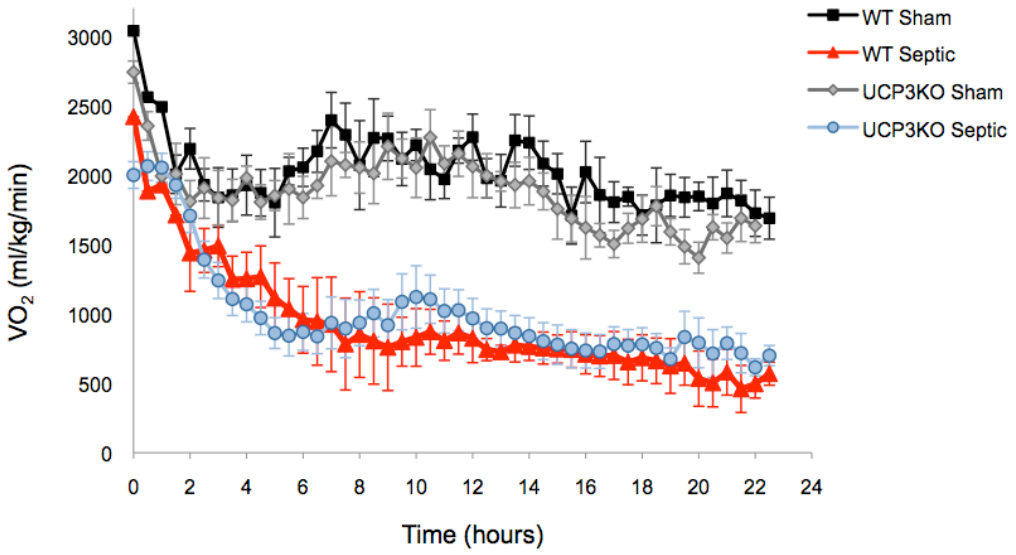

Figure 5.10 Total body oxygen consumption (VO_2) of starved sham and septic wild-type and *Ucp3*$^{(-/-)}$ mice. In the *Ucp3*$^{(-/-)}$ group, there were 7 sham mice and 9 septic. There were 6 sham and 7 septic mice in the wild-type group. No statistical difference was noted between the groups.

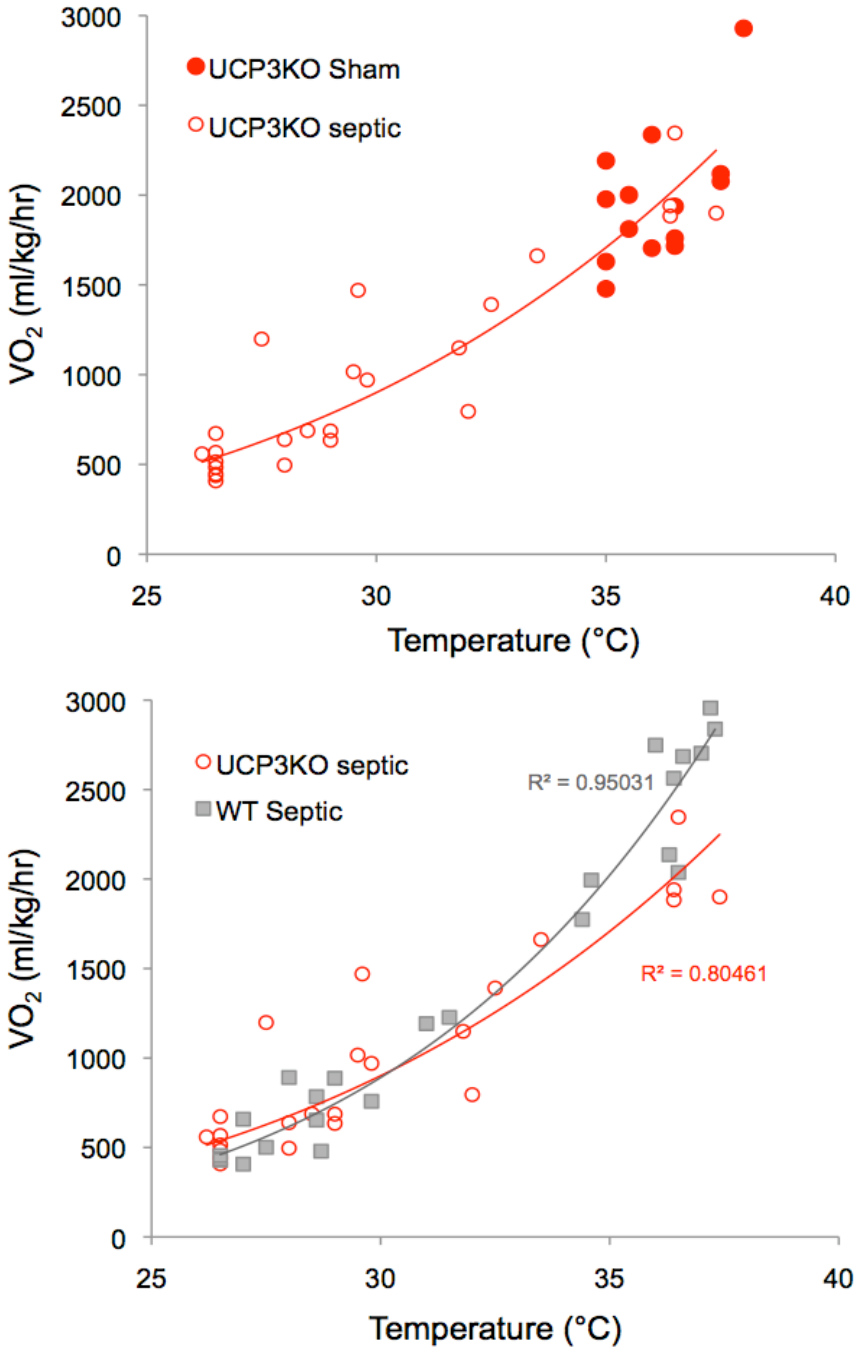

Figure 5.11 Plot showing the relationship between temperature and VO_2 of sham and septic *Ucp3*$^{(-/-)}$ mice (top panel). Comparison between wild-type and *Ucp3*$^{(-/-)}$ septic groups is shown in the lower panel. No statistical differences were found between the two genotypes.

5.3.4 Skeletal muscle mitochondrial transmembrane potential ($\Delta\psi_m$) in severe septic *Ucp3*$^{(-/-)}$ mice

Diaphragm muscle strips from 22 ± 3 weeks old *Ucp3*$^{(-/-)}$ fed sham and severe septic mice were loaded with fluorescent dye (TMRM 100 nM) to measure $\Delta\psi_m$. The baseline resting $\Delta\psi_m$ in fed sham *Ucp3*$^{(-/-)}$ mouse diaphragms were similar to the WT fed sham values presented in Chapter 4 (p=0.64). However, unlike the septic WT $\Delta\psi_m$ which was significantly lower than WT sham at baseline, $\Delta\psi_m$ of the septic *Ucp3*$^{(-/-)}$ mouse diaphragms were equal to sham mouse values (fig. 5.12).

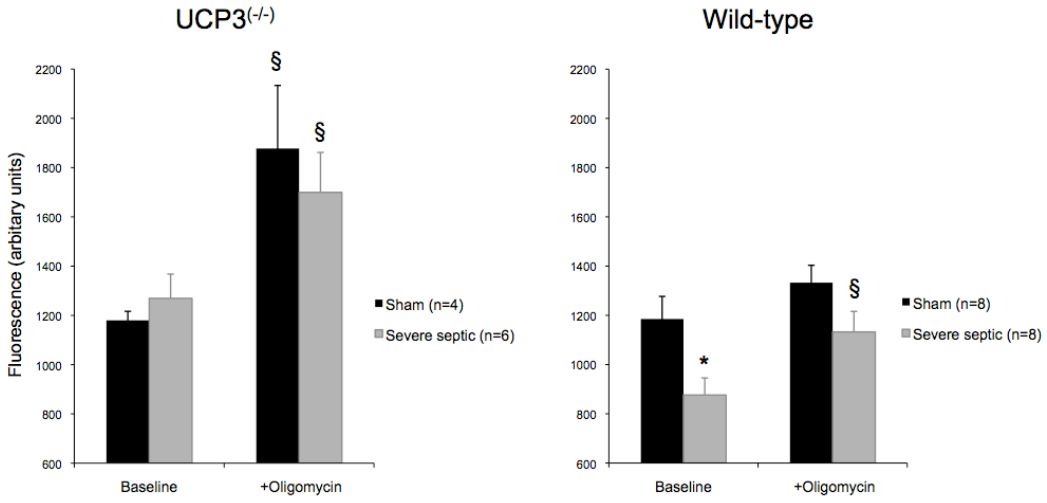

Figure 5.12 TMRM fluorescence of resting diaphragm muscle strips from *Ucp3*$^{(-/-)}$ and wild-type fed sham and septic mice before and after addition of oligomycin. *p<0.05 WT sham vs. septic baseline fluorescence (independent sample t-test); § p<0.05 comparing fluorescence after addition of oligomycin to the baseline value (repeated measure t-test).

Thus, mitochondrial transmembrane potential was reduced in WT septic mice but unaffected in *Ucp3*$^{(-/-)}$ septic mice. Addition of oligomycin to block ATP synthase led to an increase in $\Delta\psi_m$ in both sham (58 ± 19%) and septic groups (34 ± 6%) [$p<0.05$, repeated measures t-test]. This shows that $\Delta\psi_m$ is not being maintained by the reverse function of ATP synthase. The increase in $\Delta\psi_m$ after oligomycin was significantly higher in *Ucp3*$^{(-/-)}$ mice compared to wild-type mice.

5.3.5 Severely septic *Ucp3*$^{(-/-)}$ mice exhibit the same degree of cardiovascular changes as wild-type mice

The cardiovascular response of *Ucp3*$^{(-/-)}$ mice to severe sepsis was evaluated and compared to age-matched (20 week old) wild-type mice (fig. 5.13). All mice were fluid resuscitated at 0, 6 and 18h. Severity of illness was scored at 24h. Nine sham and 14 septic *Ucp3*$^{(-/-)}$ mice were set up. Three of the septic group developed only mild sepsis, and 4 died before 24h. Only sham and severe septic mouse results were analyzed. These were compared to the results of 15 wild-type sham and 13 severe septic mice described in Chapter 2. Isoflurane anaesthesia was kept to a minimum and titrated to loss of withdrawal reflex. Observed MAC values were significantly reduced in the severe septic mice (1.5-2% in sham/naïve mice, and 0.5-1% in severe septic mice; $p<0.05$ t-test).

Similar to the wild-type mice, *Ucp3*$^{(-/-)}$ exhibited a reduction in heart rate and cardiac output over the 24h time-period. Apart from a marginally higher peak velocity in the *Ucp3*$^{(-/-)}$ sham mice ($p<0.05$), no other differences were found between the two genotypes.

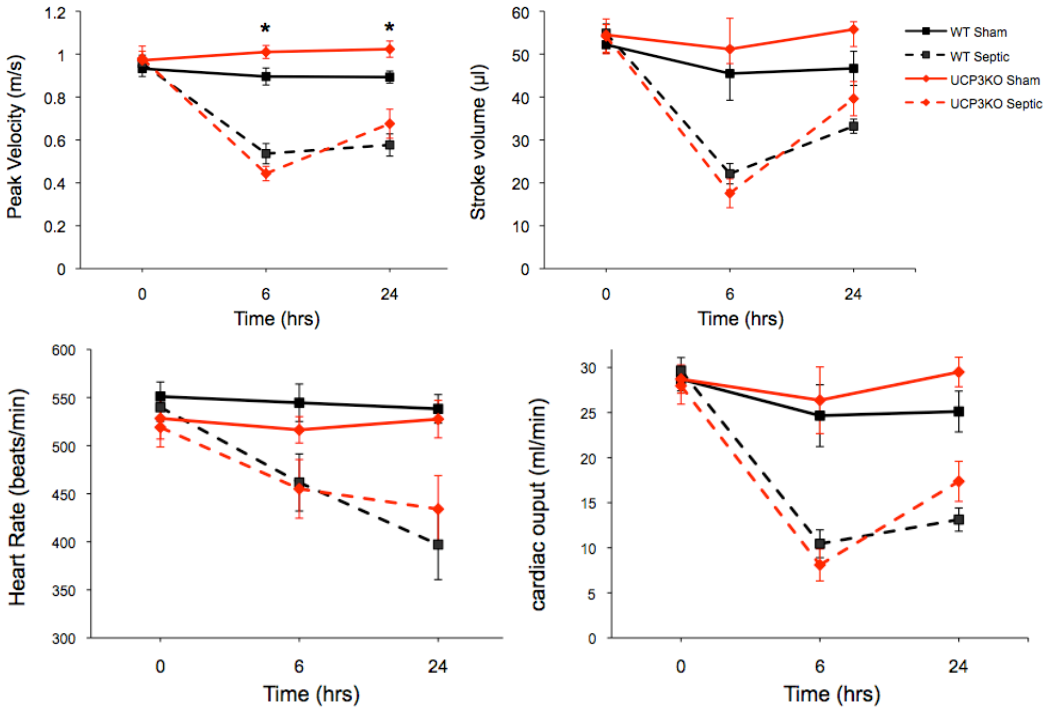

Figure 5.13 Echocardiography results of wild-type and *Ucp3*$^{(-/-)}$ mice following i/p injection of faecal slurry or saline. All septic mice were in the severe category according to the clinical scoring scale. Results are mean ± SEM. *p<0.02 comparing the sham values between the two genotypes.

5.3.6 Septic skeletal muscle dysfunction and fatigue unaltered by absence of UCP3 protein

Muscle function was measured in diaphragm muscle strips isolated from fed sham, starved sham and starved septic *Ucp3*$^{(-/-)}$ mice. The demographics of the wild-type and *Ucp3*$^{(-/-)}$ mice were similar (table 5.1).

Maximal tetanic isometric force (350ms stimulation at 100Hz) was equal in all the three groups of *Ucp3*$^{(-/-)}$ mice (fig. 5.14A; p=0.5). However, cyclical power generated by starved sham and septic *Ucp3*$^{(-/-)}$ mice was significantly lower than that seen in *Ucp3*$^{(-/-)}$ fed sham mice (median

values of starved sham and starved septic were 26% and 34% lower, respectively, than that of fed sham mice; p=0.02, Mann-Whitney U test) [fig. 5.14B]. The pattern of power reduction and fatigue with repeated cyclical power loops were also similar between the three *Ucp3*$^{(-/-)}$ groups (fig. 5.15). No differences were noted in either maximal isometric force, power generation or fatigue between wild type and knockouts.

	Wild-type mice			***Ucp3*$^{(-/-)}$ mice**		
	Fed sham	**Starved Sham**	**Starved Septic**	**Fed sham**	**Starved Sham**	**Starved Septic**
Number	9	8	9	8	8	10
Age (wks)	36±1	34±1	34±1	35±2	35±1	35±2
Wt (g)	31.6±1.4	31.2±1.2	33.5±1.5	32.9±1.2	35.3±1.1	34.6±1.1

Table 5.1 Demographics of wild-type and *Ucp3*$^{(-/-)}$ mice used for muscle function studies.

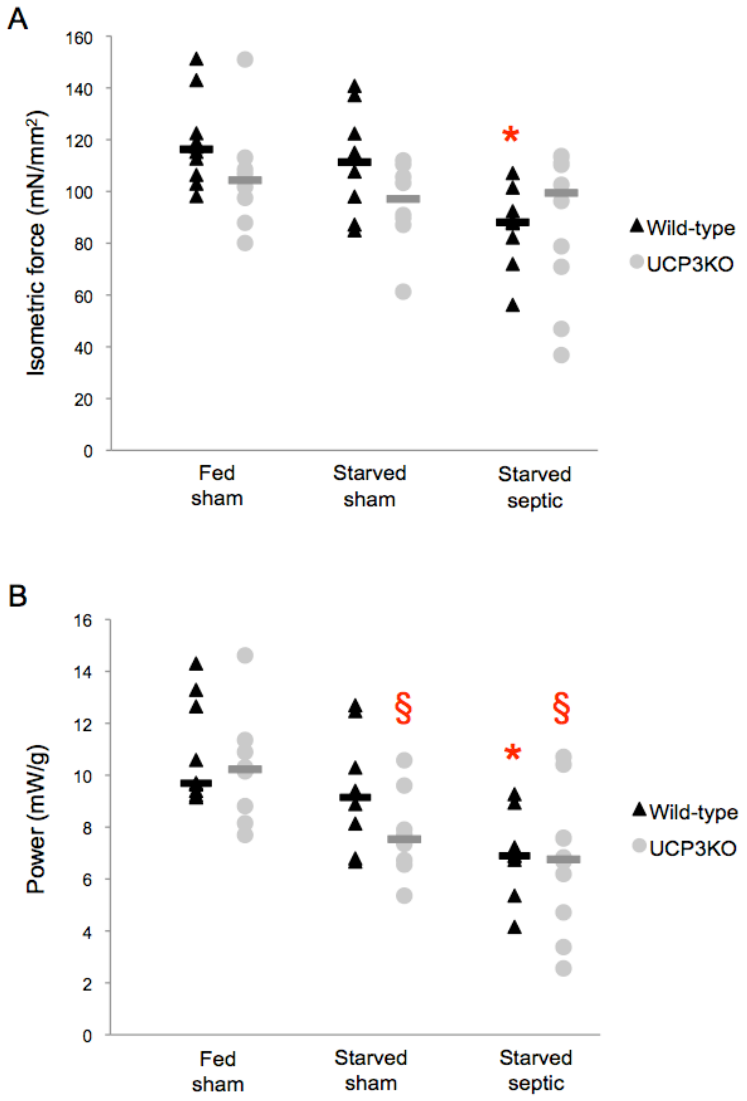

Figure 5.14 (A) Diaphragm strip maximal isometric force generated by 350ms tetanic stimulation in wild-type and *Ucp3*$^{(-/-)}$ mice and normalized to cross-sectional area. **(B)** Power generated by the muscle strips in a work-loop cycle with phasic tetanic stimulation and sinusoidal cyclical changes in length (4Hz; ±0.4mm) simulating *in vivo* muscle function. The triangles and circles represent individual values for wild-type and *Ucp3*$^{(-/-)}$ mice respectively. The horizontal lines represent the median value.

(*p<0.05 WT septic vs. WT fed and WT starved sham. § p<0.05 *Ucp3*$^{(-/-)}$ starved sham and septic vs. *Ucp3*$^{(-/-)}$ fed sham. One-way ANOVA)

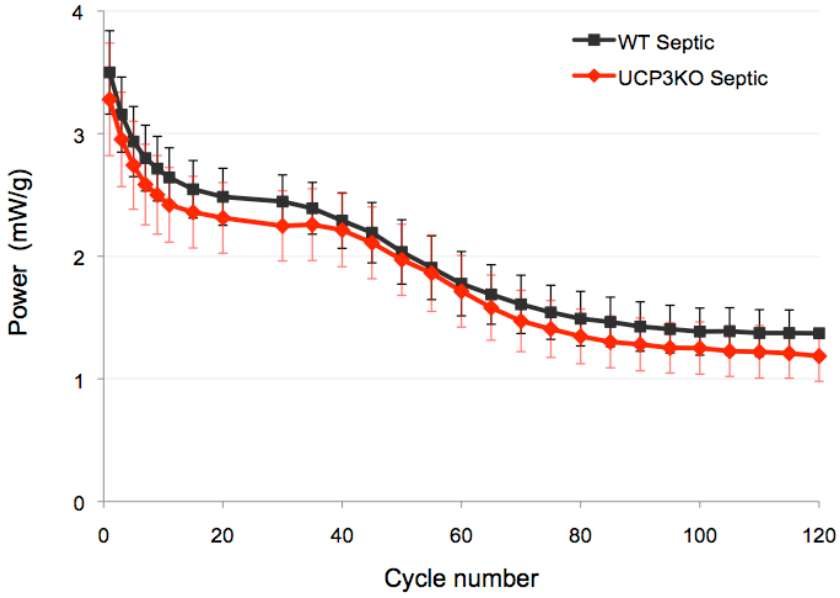

Figure 5.15 Graph of power output (normalized to wet weight) of the starved septic diaphragm muscle strips from wild-type and *Ucp3*$^{(-/-)}$ mice during repeated work-loop cycles over a one-minute time period (2Hz). Characteristic triphasic graph shows a rapid reduction in the power output at the beginning of the protocol, followed by a plateau where mitochondrial stimulation counteracts further power loss. However, build-up of phosphate and reduction in ATP eventually leads to a further reduction of power output.

5.4 Discussion

5.4.1 UCP3 protein abundance in septic wild-type mice

The results presented here confirm previous findings that skeletal muscle UCP3 is up-regulated in sepsis (Yu, Barger et al. 2000; Sun, Wray et al. 2003). There was a 28% increase in UCP3 protein abundance (81% when normalized to α-tubulin) in diaphragms at 24h. This was despite a 10-20% reduction in structural protein (α-tubulin) abundance, a finding likely related to increased protein degradation and reduced synthesis in sepsis (Hasselgren 1999).

The increased UCP3 protein abundance seen here is, however, more modest than previous reports and discordant with observations in cardiac muscle. Yu *et al* (Yu, Barger et al. 2000) reported a 5-fold increase in UCP3 mRNA abundance at 6h, and 2-fold at 24h, in mice treated with LPS. Similarly, Sun *et al* (Sun, Wray et al. 2003) reported a 2 to 3-fold increase in UCP3 mRNA and protein levels of skeletal muscle isolated from rats 16h after caecal ligation and puncture. They also found a larger increase in white, fast-twitch muscle (extensor digitorum longus) than in red, slow-twitch muscle (soleus).

Starvation alone leads to increased UCP3 mRNA and protein expression, likely mediated through increased plasma free fatty acid levels (Boss, Samec et al. 1997; Cadenas, Buckingham et al. 1999). The function of this paradoxical rise is not fully understood but is the basis of another suggested function of UCP3, namely switching from carbohydrate to lipid metabolism (Dulloo, Samec et al. 2001; Nedergaard

and Cannon 2003). This offers an alternative explanation for the increased UCP3 protein abundance observed in this and previous studies of sepsis. I found that mice stop eating following induction of intraperitoneal sepsis and switch to lipid metabolism (see Section 2.3.5). This may explain the rise in UCP3 abundance as metabolism shifts from predominantly carbohydrate to lipid utilisation. It would be interesting to investigate the effect of force-feeding or continuous i/v glucose infusions on metabolic pattern and UCP3 protein abundance during sepsis.

5.4.2 Sepsis mortality in *Ucp3*$^{(-/-)}$ mice

According to current knowledge, the absence of UCP3 should lead to greater ROS production by skeletal muscle mitochondria. This may result in greater ROS-mediated contractile dysfunction compared to WT mice (Callahan, Stofan et al. 2001). On the other hand, a lack of UCP3 may have a beneficial effect with higher coupling of mitochondrial respiration and efficiency (Vidal-Puig, Grujic et al. 2000; Cline, Vidal-Puig et al. 2001), during a time of cellular stress. However, the observed mortality in my model of sepsis was unchanged in *Ucp3*$^{(-/-)}$ mice compared to age-matched wild-type mice.

The lack of difference in mortality between wild-type and *Ucp3*$^{(-/-)}$ mice may be due to the high mortality rate in this model of sepsis (60% by 24h). This might have masked more subtle differences between the two genotypes. UCP3 may have a more significant role in milder forms of sepsis although this was not investigated in this study.

5.4.3 Sepsis related metabolic changes in *Ucp3*$^{(-/-)}$ mice

No differences in the hypothermic and hypometabolic response to sepsis or starvation were found between wild-type and *Ucp3*$^{(-/-)}$ mice. These results confirm recent data that have questioned the role of UCP3 in thermogenesis and basal metabolic rate. Barger *et al* (Barger, Barnes et al. 2006) found no significant role for UCP3 in non-shivering thermogenesis in arctic ground squirrels, even at an ambient temperature of -10°C. Similar conclusions were reached by Gong *et al* (Gong, Monemdjou et al. 2000) who found no difference in metabolic and body temperature responses following stimulation of wild-type and *Ucp3*$^{(-/-)}$ mice with tri-iodothyronine, a β_3- agonist or LPS. Also, no difference in heat production was found in skeletal muscle of *Ucp3*$^{(-/-)}$ and UCP3 over-expressed mice (Barclay, Woledge et al. 2009). However, supraphysiological stimulation using 3,4-methylenedioxymethamphetamine (MDMA) produced significant rises in temperature and death in wild-type but not *Ucp3*$^{(-/-)}$ mice (Mills, Banks et al. 2003; Mills, Rusyniak et al. 2004). These results imply that UCP3 may have the capacity for heat generation, but not under physiological conditions (Bezaire, Spriet et al. 2005).

Results from metabolic studies in transgenic mice over-expressing UCP3 protein or *Ucp3*$^{(-/-)}$ mice have produced varying results (Bezaire, Spriet et al. 2005). While mice over-expressing UCP3 were hyperphagic and lean (Clapham, Arch et al. 2000), *Ucp3*$^{(-/-)}$ mice showed a very similar metabolic phenotype to wild-type equivalents (Vidal-Puig, Grujic et al. 2000). My results reflect these findings with identical weight loss and VO_2

changes following starvation and sepsis in wild-type and *Ucp3*$^{(-/-)}$ mice. Yu *et al* (Yu, Barger et al. 2000) also found the same hypothermic and hypometabolic response following injection of LPS in wild-type mice, despite a rise in UCP3 expression.

Data from the previous chapter showed no differences in proton leak kinetics in 24h wild-type fed sham and septic mice despite a 28% absolute rise in skeletal muscle UCP3 protein level. Increased UCP3 protein abundance is also seen in starvation, but once again I failed to see any increase in proton leak in the starved sham group. Similar results were shown by Cadenas *et al* (Cadenas, Buckingham et al. 1999) and Bezaire *et al* (Bézaire, Hofmann et al. 2001) who reported unchanged skeletal muscle mitochondrial proton leak in wild-type and *Ucp3*$^{(-/-)}$ mice, even when increased UCP3 protein levels were observed following 24h of starvation in the wild-type mice. These results therefore argue against UCP3 having a significant role in the bioenergetic changes occurring in starvation and sepsis in mice.

5.4.4 Sepsis and $\Delta\psi_m$ in *Ucp3*$^{(-/-)}$ mice

In Chapter 4, I showed that $\Delta\psi_m$ of mitochondria in diaphragms of septic wild-type mice was significantly reduced compared to sham mice. However, as shown here, $\Delta\psi_m$ of septic *Ucp3*$^{(-/-)}$ mice was equal to both *Ucp3*$^{(-/-)}$ sham and wild-type sham mice. This signifies that diaphragm $\Delta\psi_m$ of *Ucp3*$^{(-/-)}$ mice is unaffected by sepsis. Interestingly, addition of

oligomycin led to a more pronounced rise in $\Delta\psi_m$ when compared to the wild-type diaphragms, but the significance of this is unclear.

These findings support the uncoupling role of UCP3, so that when its expression is increased, transmembrane potential is low (as seen with lower $\Delta\psi_m$ in wild-type septic muscle). When the protein was absent, transmembrane potential was maintained. However, as shown in the Chapter 4, the proton leak kinetics of isolated skeletal muscle mitochondria were unchanged in the wild-type sham and septic groups.

So, how does one explain these findings? The lack of concordance in the above findings may be as a result of:

1. Different environmental conditions in the intact cell vs. isolated mitochondria: UCP3 activity is regulated by free-fatty acids and products of ROS. Therefore, the results obtained in whole cell preparations ($\Delta\psi_m$ measurements using TMRM) may be very different to those from isolated mitochondria (proton leak kinetics) where mitochondria are removed from their innate environment and free-fatty acid levels kept to a minimum.
2. UCP3 has a function unrelated to the proposed proton conductance channel across the inner mitochondrial membrane but may affect, for instance, substrate uptake.

It is clear from the above that further work is necessary to make sense of the TMRM measurements made in complex tissues such as the diaphragm muscle preparation. Manipulation of $\Delta\psi_m$ by switching

substrates or adding a variety of inhibitor compounds, in particular, carboxyatractyloside to inhibit the ANT complex (to assess proton leak through it), and guanosine diphosphate to inhibit UCP3 dependent proton conductance (Parker, Affourtit et al. 2008), may help in understanding some of the more complex bioenergetic interactions in the mitochondria.

Free radicals mediate skeletal muscle contractile dysfunction (Callahan, Stofan et al. 2001; Griffiths and Hall 2010) while mitochondria isolated from *Ucp3*$^{(-/-)}$ mice produce more ROS (Vidal-Puig, Grujic et al. 2000) and suffer more oxidative damage to proteins (Brand, Pamplona et al. 2002). However, in this study, the diaphragm strips isolated from *Ucp3*$^{(-/-)}$ mice did not exhibit worsened muscle function compared to the wild-type septic mice where UCP3 protein abundance was increased. It must also be noted that despite the increase in UCP3 protein abundance in the septic wild-type mice, mitochondrial ROS production was significantly higher in septic wild-type mice compared to the wild-type sham group.

Mitochondrial ROS production and proton leak measurements in isolated mitochondria were not measured in this study as no significant phenotype difference was found between *Ucp3*$^{(-/-)}$ and their wild-type counterparts. This lack of phenotypic difference may simply be as a result of development of alternative compensatory mechanisms/pathways in the *Ucp3*$^{(-/-)}$ mice. However, in the original description of these mice, no compensatory increase in mRNA abundance of the other uncoupling proteins or anti-oxidant mechanisms were reported (Vidal-Puig, Grujic et al. 2000).

5.5 Conclusion

The absence of UCP3 had no effect on long-term survival from sepsis in this mouse model, nor does it alter metabolic, cardiovascular or skeletal muscle function. Even though mitochondrial transmembrane potential was maintained in the septic *Ucp3*$^{(-/-)}$ mice when compared to wild-type septic mice, this did not result in any clear whole-body bioenergetic consequence or evidence of worsened ROS damage to the contractile apparatus.

CHAPTER 6: GENERAL DISCUSSION

Severe sepsis is the result of systemic inflammation due to infection and often leads to multi-organ failure (MOF) and death (Angus, Linde-Zwirble et al. 2001). The pathophysiological mechanisms underlying MOF are multifactorial and complex (Abraham and Singer 2007). A detailed knowledge of metabolic changes in sepsis and how animal models compare to the human form of the disease is still lacking.

In this project, a reproducible mouse model of sepsis and MOF was developed and characterized. Physiological changes including whole animal metabolism, O_2 delivery, myocardial and respiratory muscle function were subsequently examined. Further studies investigated mitochondrial function in freshly isolated *ex vivo* tissues followed by interrogation of the function of isolated mitochondria and their ability to generate a trans-membrane potential ($\Delta\psi_m$), phosphorylate ADP, produce ROS and their proton leak.

6.1 Metabolic suppression in sepsis

In Chapter 2, I reported the verification in this mouse model of a clinical scoring system used for defining severity of sepsis in rats (Brealey, Karyampudi et al. 2004). This score correlated with biochemical indices of organ dysfunction and with the degree of metabolic suppression observed. However, the rapid onset and severity of metabolic suppression was surprising. Oxygen consumption dipped to

one-third of the starting value within 10 hours of the onset of sepsis, with a switch to lipid metabolism. It was accompanied by hypothermia, myocardial dysfunction and a low cardiac output state. Of note, artificial warming of the severely septic mice to restore their core temperatures to baseline values (>36°C) did not alter oxygen consumption, even though cardiac output (and thus oxygen delivery) had doubled. This implied that neither the developing hypothermia, nor the reduced oxygen delivery resulting from the low cardiac output state were directly responsible for the metabolic suppression seen in septic mice. It does confirm that hypothermia affects cardiovascular function (Polderman 2009), resulting in a lower heart rate and stroke volume in these mice. This important effect should be considered in *in vivo* studies of myocardial dysfunction in mice with sepsis.

A reduction in VO_2 and a switch to lipid metabolism were also seen in the starved sham mice but without alterations in body temperature. The reduction in VO_2 was less profound than that seen in septic mice. Since septic mice also stop eating and switch to lipid metabolism, it could be argued that part of the metabolic suppression and response seen in the septic animals may be related to starvation behaviour.

Metabolic suppression has been described in a variety of organisms under unfavourable external situations such as cold, food shortage, desiccation and hypoxia (Boutilier and St-Pierre 2002). The strategies developed by these animals to cope with such extreme states of energy stress are similar and termed “cross-tolerance” (reviewed by (Staples and Buck 2009)). For example, neurons of hibernating ground

squirrels tolerate hypoxia and hypoglycaemia better than neurons taken from non-hibernating rats (Frerichs and Hallenbeck 1998). These coping strategies fundamentally result in a balanced reduction in energy production and utilization, and can be tissue specific in their form and magnitude (Staples and Buck 2009).

A number of processes consume cellular ATP. The proportion of energy consumption by each of these processes varies depending on the organ or tissue (Rolfe and Brown 1997). The major consumers of energy are membrane pumps, e.g. the Na^+/K^+-ATPase (accounting for 40-70% in brain and kidney), Ca^{2+}-ATPase (up to 30% in the heart), protein synthesis (up to 70% in the gastrointestinal tract) and proton leak (up to 50% in skeletal muscle). At times of energy stress, a hierarchical suppression response may be seen in these metabolic processes, whereby the most vital processes (e.g. Na^+/K^+-ATPase activity) remain functional while processes less crucial for short-term survival (e.g. protein synthesis) become suppressed (Wieser and Krumschnabel 2001). Reduction in ion transport and protein synthesis, but not proton leak, have been reported in hibernating animals (Staples and Brown 2008). Down-regulation of protein synthesis has been reported extensively in sepsis (Lang, Frost et al. 2007), and may serve to reduce ATP demand.

A reduced energy supply in the form of ATP has been implicated as a trigger of metabolic suppression. This has been suggested following observed reduction in aerobic capacity of skeletal muscle of hypoxic hibernating frogs (St-Pierre and Boutilier 2001), and impaired substrate kinetics of the hepato-pancreas of aestivating snails (Bishop, St-Pierre et

al. 2002). Similar findings were found in starvation-induced torpid mice (Brown, Gerson et al. 2007).

I observed impaired substrate kinetics in mitochondria isolated from skeletal muscle taken from mice starved for 24h. These changes may have been the result of torpor or hibernatory responses. No such change in substrate kinetics were observed in the septic mice. Although a mild degree of impairment in mitochondrial phosphorylation kinetics was seen, the ability of the mitochondria to generate ATP was unlikely to be significantly affected. These results suggest that the metabolic suppression seen in these septic mice may be the result of other regulatory processes that may either reduce demand for ATP or impose control over substrate oxidation and trafficking into the mitochondria thereby regulating respiratory chain function and mitochondrial ATP synthesis.

Potential candidates of such regulatory mechanisms are nitric oxide (discussed in chapter 4), pyruvate dehydrogenase kinase-4 (PDK4) and hypoxia inducible factor-1α (HIF-1α). Nitric oxide is produced in excess amounts in sepsis (Boczkowski, Lanone et al. 1996; Brealey, Brand et al. 2002), and can inhibit mitochondrial respiration (Boczkowski, Lisdero et al. 1999; Brown and Borutaite 2001). Direct inhibition of the respiratory chain by NO is reversible and in direct competition with O_2. While this inhibition is likely to be effective *in vivo* where intramitochondrial levels of oxygen tension are very low, it will be lost when mitochondria are isolated and exposed to room air. This may potentially explain the lack of alteration in substrate kinetics seen in

isolated mitochondria taken from septic mice. State 3 and 4 respiration of isolated mitochondria fed through complex I were also unaffected in the septic mice, implying that there was minimal Complex I inhibition.

PDK-4 has been linked with hibernation states by phosphorylating and inactivating the pyruvate dehyrdrogenase complex. Pyruvate decarboxylase (PDC) decarboxylates pyruvate to acetyl CoA and connects glycolysis to the citric acid cycle of the mitochondria. Inactivation of PDC by PDK-4 results in a metabolic switch away form glucose/carbohydrate metabolism to lipid metabolism (Andrews 2004). Alamdari *et al* (Alamdari, Constantin-Teodosiu et al. 2008) reported increased PDK-4 mRNA expression in rats infused with LPS for 24 hours. This was accompanied by a 65% reduction in pyruvate dehydrogenase activity and high muscle lactate levels.

HIF-1α is a cellular transcriptional activator that becomes more abundant and activated in hypoxia and upregulates expression of genes encoding for glycolytic enzymes and glucose transporters (Boutin and Johnson 2007). It also inhibits entry of pyruvate into the TCA cycle, limiting mitochondrial respiration and diverting pyruvate metabolism to lactate (Kim, Tchernyshyov et al. 2006; Papandreou, Cairns et al. 2006). In this way, it switches aerobic to anaerobic metabolism. Recent evidence has shown that LPS can independently increase HIF-1 levels under normoxic conditions. Elimination of the HIF-1 gene from the myeloid lineage of mice resulted in reduced cytokine release, a reduction in their hypothermic and hypotensive response to LPS, and improved survival (Peyssonnaux, Cejudo-Martin et al. 2007).

Many models have been used to investigate pathophysiological processes in sepsis. However, little has been published on how closely these models mimic the human form of the disease. The early metabolic suppression observed in the septic mice in my model clearly differs from findings reported in human sepsis where VO_2 is initially elevated (Kreymann, Grosser et al. 1993), and most patients with sepsis present with pyrexia (Clemmer, Fisher et al. 1992). Of note, VO_2 returns to normal 'healthy' levels with the most severe manifestations of sepsis, and a rebound increase occurs during the recovery phase (Kreymann, Grosser et al. 1993). While hibernatory responses are present in animals tolerant of energy stress, they are not in general recognized as a feature in humans or other animals intolerant of such conditions. However, there are parallels in humans, such as myocardial hibernation that occurs with persisting ischaemia but improves on restoration of adequate perfusion (Camici, Prasad et al. 2008). Even in sepsis, the loss of organ function is mainly reversible with minimal cell death evident in organs examined at post-mortem (Hotchkiss, Swanson et al. 1999; Langenberg, Bagshaw et al. 2008). Furthermore, there is a very low requirement for long-term renal replacement therapy in septic survivors who developed acute renal failure (Noble, MacKirdy et al. 2001; Bagshaw, Uchino et al. 2007). However, it must be noted that without the advent of modern intensive care and multi-organ support, many patients who develop MOF would likely die. It is therefore not clear how cellular energetic suppression in these organs would have a survival benefit for humans. Although it can be argued that if cells were to continue to respire and act "normally", a larger number of

them would die and in this way compromise their ability to recover. If we knew the cellular triggers that send such cells into dormancy, by reversing them, we may be able to restore cellular and organ function more rapidly.

In my mouse model of sepsis, severity of metabolic suppression was associated with increased mortality. It could be argued that the animal's response to the bacterial insult was a reflection of "cross-tolerance", whereby a variety of stimuli result in the same hypometabolic hibernatory response. However, in these septic mice, with ongoing bacterial dissemination and proliferation, it resulted in the death of the animal. Alternatively, the metabolic suppression observed may be an evolutionary response to allow isolation and death of the sick animal to save the rest of the herd. Those able to manifest a more moderate hibernatory response have the ability to survive and return to health.

6.2 Mitochondrial membrane potential and diaphragm function

The finding that $\Delta\psi_m$ of intact diaphragm muscle strips were significantly lower in the septic mice, yet addition of oligomycin to block ATP production resulted in a rise in $\Delta\psi_m$ is intriguing. At steady state, mitochondrial membrane potential represents the balance between flux of electrons through the respiratory chain and demand for ATP by the cell (Chandel, Budinger et al. 1997). One explanation for the lower $\Delta\psi_m$ values in the septic diaphragms may be a higher ATP demand in these

cells, resulting in a larger consumption of the proton-motive force. A large bulk of this energy demand would be used for the maintenance of ionic gradients (by Na^+/K^+- ATPase and Ca^{2+}-ATPase), and for contractile protein synthesis.

The diaphragm is a significant muscle of respiration, and will be working hard to achieve gas exchange in a sick animal with distended abdomen, pleural effusions and likely lower compliant lungs. As shown in Chapter 3, the ability of the septic diaphragm muscle strips to generate and maintain force and power output were significantly impaired and would have imposed further strain on function. This was demonstrated by the experiments where repetitive stimulation of the septic diaphragm strips resulted in earlier fatigue related to the 2^{nd} (mitochondrial) phase of the fatigue pattern (see fig 3.12).

The diaphragm is likely to be one of only a few muscles where demand for ATP remains high. The onset of metabolic down-regulation was associated with almost complete cessation of movement of the mice and the loss of oxygen consumption spikes (presumed to be movement related). Skeletal muscle constitutes a large proportion of the animal's body mass and O_2 consumption; thus, a large part of the metabolic suppression seen in the septic mice may be as a result of metabolic suppression in skeletal muscle.

6.3 Uncoupling protein-3 in sepsis

In Chapter 5, I showed that the absence of UCP3 did not result in any functional, metabolic or survival benefit/disadvantage in mice with sepsis. $\Delta\psi_m$ of diaphragm muscle strips isolated from septic *Ucp3*$^{(-/-)}$ mice were nevertheless preserved. This was the only phenotypic variation in sepsis between these knockout mice and wild-type mice. However, its importance is not clear and was not investigated further as there was no clear functional consequence.

No significant phenotype difference has been reported in *Ucp3*$^{(-/-)}$ mice compared to wild-type mice (Vidal-Puig, Grujic et al. 2000; McDonald, Walker et al. 2008; Barclay, Woledge et al. 2009). Most of the evidence for UCP3 function points to involvement in fatty acid metabolism and limitation of ROS production (Costford, Seifert et al. 2007). In my model of sepsis, the change in respiratory exchange ratio (RER) implied a predominance of fatty acid metabolism, which may have resulted in the upregulation of UCP3 in the skeletal muscle of wild-type septic mice at 24h. However, no differences in proton conductance of skeletal muscle mitochondria were observed in septic mice when compared to fed or starved sham mice. ROS production was higher in mitochondria taken from septic and starved sham mice. Therefore, I was unable to find a significant role for UCP3 during sepsis, nor any metabolic consequence of its up-regulation or absence.

6.4 Live-cell imaging

A major aim of this project was to develop advanced live-cell imaging techniques using fluorescent markers to study cellular physiology. At the start of this project, there had been several reports of live-cell imaging in organs such as the kidney using multi-photon microscopy (Peti-Peterdi 2005; Sipos, Toma et al. 2007). However, it soon became clear that there were many technical challenges that had to be overcome to achieve this, even in freshly isolated tissues and whole organs. These ranged from problems with organ/tissue isolation, preservation and preparation, to dye loading, movement artefact and temperature maintenance. While most of these were eventually overcome, problems of inconsistent dye loading (especially with the cytosolic Ca^{2+} dyes) and movement artefact remained.

To my knowledge, the TMRM fluorescence experiments presented here are the first time $\Delta\psi_m$ has been measured in living intact diaphragm muscle strips. I spent considerable effort in obtaining liver slices and optimizing storage and imaging conditions but, due to lack of time, further work with this tissue was abandoned (data not shown).

The development of genetically modified mice with Ca^{2+} reporter genes (Hasan, Friedrich et al. 2004), as well as green fluorescent protein (GFP)-based indicators that can be used to transfect whole tissues (Rudolf, Mongillo et al. 2003; Pozzan and Rudolf 2009), may resolve dye-loading problems and represent avenues to be explored in the future.

6.6 Future work

Comprehensive human studies investigating temporal changes in whole body metabolism and incorporating measurements of cardiac output, O_2 delivery, organ support in the form of ventilation, renal replacement and nutrition, as well as the use of sedatives, steroids, vasopressors and inotropes, are long overdue. Supplementation of these studies with measurement of metabolism at the tissue level (skeletal muscle) using oxygen probes and *in vivo* measurements of $\Delta\psi_m$ may be ambitious, but together with modular kinetic analysis of isolated mitochondrial function, will help our understanding of the balance of energy production and consumption in multi-organ failure. Changes in the abundance of regulatory factors such as PDK-4 and HIF-1α will provide clues about regulatory mechanisms of mitochondrial and glycolytic pathways.

The metabolic response of larger rodents (rats) to faecal peritonitis mimics human physiology more closely and thus warrants closer inspection. Further studies should investigate changes in $\Delta\psi_m$ in vital organs such as the liver and kidneys and correlate these results with skeletal muscle to understand the variations in response of different tissues/organs to sepsis. The effect of inhibition of NO production on whole animal metabolic rate and $\Delta\psi_m$ in living tissues will help our understanding of regulation of mitochondrial function by NO.

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