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Measurement of heat stress response in pigs using an *in-vitro* model

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Research Organisation
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Executive Summary

The objective of this project was to develop a simple *in-vitro* test which could be used to obtain a phenotype which indicated an individual animal's response to heat stress. This test could potentially overcome the current limitations to obtaining phenotypes which reflect heat stress response, which include: 1) small scale climate controlled facilities which enable heat stress to be applied, but on relatively few animals, and 2) use of indirect information from changes in performance data with season in an industry setting, which can be ambiguous (ie seasonal effects are not just about temperature) and which is not available for progeny of all sires at all times of the year. These limitations restrict the ability to select animals with improved tolerance to heat stress in commercial breeding programs. Developing an *in-vitro* test which overcomes these limitations could improve this aspect of breeding program development and also have application in designed experiments for quantifying the impacts of heat stress on performance and welfare.

The first part of the project involved the development of the *in-vitro* test for samples which could be obtained from live animals housed in thermo-neutral (not heat stressed) conditions. Alternative strategies were investigated using samples such as cheek cells and white blood cells, which could be obtained from live animals, as well as other resources such as primary cell lines (pig kidney, lung and testes cells) to establish how robust the test was to different types of samples. Cells scraped from the pig's cheek were the least invasive, but viability was less than 10%. The cell apoptosis assays proved unsuitable for obtaining specific and robust *in-vitro* heat stress phenotypes. Consequently, quantitative reverse transcriptase PCR (qRT PCR) was used to measure the effect of heat on transcription of heat shock protein 70 (HSP70). This required concurrently identifying a gene which did not respond to heat stress (beta-actin), in order to produce a robust phenotype for samples which typically vary in the amount of RNA present. Blood samples (specifically white blood cells) were found to be a suitable source of cells for this test, whereas cheek cells and hair follicles were unsuitable for this purpose.

In the second part of the project, we sampled more animals (20 grower-finishers) before and during heat stress to conduct the *in-vitro* test. These animals were also subjected to a heat stress challenge using the climate controlled facilities at the Elizabeth Macarthur Agricultural Institute (EMAI). The heat stress challenge was applied using a diurnal exposure to heat, to better mimic the common pattern of an animal's exposure to heat stress in an Industry setting (ie increasing heat during the daytime, followed by reductions in temperature at night). The animal's physiological response to the developing heat stress was recorded over 5 days, by measuring respiration rate and rectal temperature three times per day, and total feed intake once per day. These variables were also recorded during the week prior to the application of heat stress. The associations between the *in-vitro* test results and the physiological response of individual animals to heat stress was assessed by calculating the correlations between these variables.

Compared to the reference week (experimental animals housed under thermo-neutral conditions), feed intake increased, on average, during the week of heat stress, demonstrating that the level of daily feed intake measured on growing animals can be a poor indicator of the physiological impact of short term, diurnally experienced, heat stress. That is, feed intake would be expected to reduce under heat stress, but this was not observed in our study, on average. Similarly, the majority of animals had a very small change in rectal temperature with increasing heat stress, on average. Both these outcomes might have occurred because lower overnight temperatures allowed full recovery of animals from the heat stress applied during the daytime, and also a cooler time night time period

when animals could compensate for patterns of reduced daytime feed intake with increased night time intake.

In contrast, changes in respiration rate and the *in-vitro test* results for HSP70 were both large in magnitude, and variable between animals. In addition, when blood samples were obtained from heat stressed animals, the HSP70 response from the *in-vitro* test was relatively higher than the same response measured from samples taken when the animals were housed in a thermo-neutral environment. Variation in HSP70 induction measured under thermo-neutral versus heat stress conditions suggests that the magnitude of the average HSP70 response could be used to quantify whether animals were heat stressed at the time of sampling, but this application should be confirmed with a larger sample of animals.

The HSP70 response of an individual animal sampled for testing under thermo-neutral conditions was negatively correlated ($r=-0.49$, p-value=0.027) with its own respiratory response to heat stress. That is, animals with a high HSP70 response had a lower increase in respiration rate as heat stress developed each day, suggesting they were more tolerant of the applied heat stress. Since high HSP70 is thought to be beneficial for an animals tolerance to heat stress, this outcome is supported by other literature in this area. However, HSP70 expression under thermo-neutral conditions was not correlated with expression under heat stress in the same animals. Therefore, more work is required to assess the repeatability of the HSP70 test phenotype both within and across time periods of thermo-neutral and heat stress conditions prior to further application of this test in industry or experimental settings.

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2. Background to Research

Heat stress has numerous consequences for reproductive, health and production performance in pigs through effects on reproductive hormones, immune responsiveness and feed intake (Morrow-Tesch, McGlone et al. 1994, Prunier, de Braganca et al. 1997, Hansen 2009, Williams 2009). Research in dairy cattle (Ravagnolo and Misztal 2000, Misztal, Aguilar et al. 2010) and differences between selection lines of pigs in their tolerance to heat stress (as measured by changes in performance traits) suggest active selection for improved tolerance is possible (Bloemhof, van der Waaij et al. 2008, Lewis and Bunter 2011). However, there are several limitations to collecting data in the field, reviewed extensively elsewhere. These include limitations resulting from differences in expression between age classes (and therefore timing of recording), the environment provided and its modification, as well as limited applicability of species- specific phenotypes related to heat stress tolerance for other species (Misztal 2017). For example, hair and shedding attributes of cattle are simple traits related to heat tolerance, which have no similar phenotype in pigs.

Even in hot environments breeding companies are frequently unable to routinely generate data in the field all year round which can characterise individual animals for their response to heat stress. Due to normal turnover of breeding animals, not all sires or dams produce progeny which are recorded under seasonal conditions which create heat stress challenges and, therefore, a proportion of animals will remain unchallenged and unrecorded (either directly or through indirect measures) for their relative tolerance to heat stress under field conditions. In addition, housing and facilities can be modified to minimise the heat stress challenge received by animals in order to maximise both welfare and production as much as possible (Lucas, Randall et al. 2000). Thus, a nucleus farm might have limited capability for routinely recording the performance of all animals under heat stress conditions. The alternative is to challenge animals to heat stress using controlled climate facilities. However, access to these facilities is limited and does not enable sufficient throughput of individuals for large scale applications, such as phenotyping for breeding programs.

In addition to the primary limitation of relying on natural heat challenge for field data, we have demonstrated previously how difficult and ineffective it is generally to use performance trait phenotypes (eg lifetime growth rates, reproduction or longevity outcomes, lactation intakes etc) as indirect indicators of heat stress for individual animals. This is because numerous factors (not just heat stress) can affect the observed phenotypes of individuals for traits which are also sensitive to heat stress (Bunter, Lewis et al. 2009, Lewis, Hermesch et al. 2010, Lewis and Bunter 2011). Moreover, the magnitude of heat stress experienced can alter which performance phenotypes are indicative of poorer tolerance to heat stress (Misztal, 2017). Therefore, one should not rely solely on absolute performance data to indicate to what extent an individual animal is heat stressed outside a controlled experimental scenario. In addition to limited capability within nucleus herds, the reliance on indirect phenotyping provided by performance data to indicate heat stress tolerance is a second major limitation to accurately evaluating heat stress both in the field and for experimental studies.

These two limitations together suggest that an independent and quantitative measurement of the magnitude of heat stress at the physiological level is required for field applications. The general methodology to obtain a direct measure of heat stress based on cell apoptosis has been investigated successfully for applications in Barramundi (Jerry *et al*, 2010) and for bacteria by Dr Alison Collins (unpublished). Other alternatives, such as using electrical conductivity to detect the extent of cell

injury from heat stress, have been proposed for plants (Alsadon, Wahb-allah et al. 2006), but are less adaptable for animal applications.

In this study we proposed to develop *in-vitro* techniques for evaluating individual animals for their response to heat stress using samples from both heat stressed and non-stressed cells from live animals. A standardised *in-vitro* strategy to assess heat stress impact at an individual level provides opportunities for applications not only in genetics, but also for other research areas. For example, an *in-vitro* model might help to refine heat stress studies (eg identifying when heat stress is present in industry settings to trigger sample collection, or refining procedures for climate control facilities) and could also identify differences among individuals (or individual tissues in experimental scenarios) when a standard *in-vitro* heat stress challenge occurs. Moreover, *in-vitro* capacity is potentially much larger than the physical capacity of climate controlled facilities (ie many more data points on animals and tissues could be obtained). The project was intended to provide a research tool first, but keeps in mind that developing a low cost high-throughput procedure could enable more industry-based applications.

3. Objectives of the Research Project

This project set out to:

1 To develop an innovative test procedure using an *in-vitro* model that can quantify an individual's response to heat stress, which will potentially provide a tool for future multi-disciplinary research into heat stress.

2 To demonstrate that the *in-vitro* test results were correlated with other physical measures of an animal's response to heat stress in an experimental setting.

4. Introductory Technical Information

In previous studies involving Barramundi (Jerry *et al*. 2010), the ratio of live to dead cells measured by flow cell cytometry was used to infer population differences in their heat stress response. This measurement could therefore be used to replace more expensive phenotyping strategies (eg swim tanks) for assessing heat tolerance in Barramundi. However, the cytometry technique was facilitated by the ease with which cell populations from tissue samples of fish can be disassociated, and also required specialised, non-portable laboratory equipment. Therefore, techniques to assess heat tolerance, based on quantifying cell death, required modification for pigs. Moreover, in order to understand the biological mechanisms of heat stress, large numbers of samples need to be tested in the field, and therefore a more portable and high throughput method is needed. Feasible phenotyping strategies also need to accommodate the types of tissues or samples which can be obtained easily from live pigs.

Previously, EMAI demonstrated that a live:dead qPCR (quantitative PCR) for bacterial species correlated well with ratios of live to dead cells counted with traditional culture techniques (unpublished). Methodology using qPCR is fast and well suited to high throughput testing. EMAI have also developed a very simple method that only requires a heating block and light box to heat stress cells and separate live from dead cells. Samples can then be safely frozen and transported to the lab for later qPCR. Alternatively, the expression of heat shock protein 70 (HSP70) in heat stressed cells can be quantified by Reverse Transcriptase PCR in pig tissues, as a candidate phenotype for evaluating individual animals for their response to heat stress. Other studies have indicated that HSP70 expression can be correlated with physiological measures indicative of resistance to heat stress (Rout *et al*., 2016; Archana *et al*., 2017). The physiological impact of heat stress on mammals is frequently quantified using measures such as respiration and heart rates, as well as rectal temperature and feed intake.

5. Research Methodology

5.1 Step 1: Develop PCR assays to quantify the effect of heat on cell viability (apoptosis) and/or RNA expression of heat shock protein 70 (HSP70).

Cells can respond to heat stress in multiple ways depending on the length and severity of the heat stress event. Cells may respond by activating pathways to assist in survival (heat shock protein 70, HSP70), or they may initiate cell death pathways to remove damaged cells (apoptosis). In this project we measured the effect of heat stress on both apoptosis and on RNA expression of HSP70.

5.1.1 *Cell apoptosis assays*

Cell apoptosis was measured both visually and by molecular biology methods using different assays. Viable cells have an intact cell membrane which prevents the entry of large dye molecules such as trypan blue, whereas dead cells allow entry of the dye. Microscopic examination of trypan blue stained cells demonstrated that heating cells at 38.0°C, 40.5°C, 42.7°C and 44.8°C for 60 mins had only a minimal effect on the viability of human epithelial cells (HT29-MTX) (Figure 1).

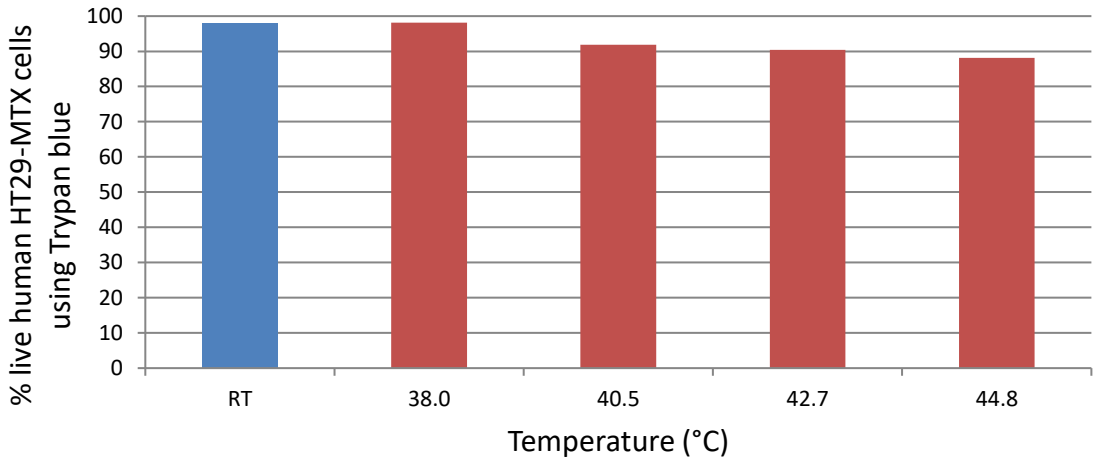

Figure 1: Viability of HT29-MTX cells after heat for 60mins at varying temperatures; RT= room temperature. Trypan blue exclusion by cells was used to quantify cell viability (single replicate per temperature).

Dead cells can also be quantified with the fluorescent dye PMA. The disrupted membranes of dead cells allow PMA to enter, where it binds to double-stranded DNA and prevents amplification during the polymerase chain reaction (PCR). The proportion of live (impermeable to PMA) and dead (DNA bound to PMA) cells can therefore be quantified by quantitative PCR amplification of a target gene found in all cells (ie. beta-actin). Heat treatment (40.5°C for 60 mins) of PMA treated cells drastically reduced cell viability from 96% to 20% alive (Figure 2), which is in stark contrast to the less sensitive counting of trypan blue stained cells. However, at temperatures above 42.7°C, cell permeability to PMA and cell death decreased and viability appeared to increase. This may be due to the induction of protective cell mechanisms like induction of HSP70 that has an inhibitory effect of cell apoptosis (Jäättelä, Wissing et al. 1998), reducing cell permeability and therefore reducing PMA uptake. The decrease in cell permeability above 42.7°C was observed on two separate occasions. Measuring cell

permeability and apoptosis at temperatures above 40.5°C may be measuring both apoptosis and the anti-apoptotic effect of HSP70.

These preliminary investigations of two simple assays to assess cell viability or apoptosis under heat stress were unable to provide a distinct measure of only cell viability in response to heat stress. Therefore, a more specific measure of the cell's response to heat stress, ie HSP70 induction or production needed to be developed to ultimately identify pigs more tolerant to heat.

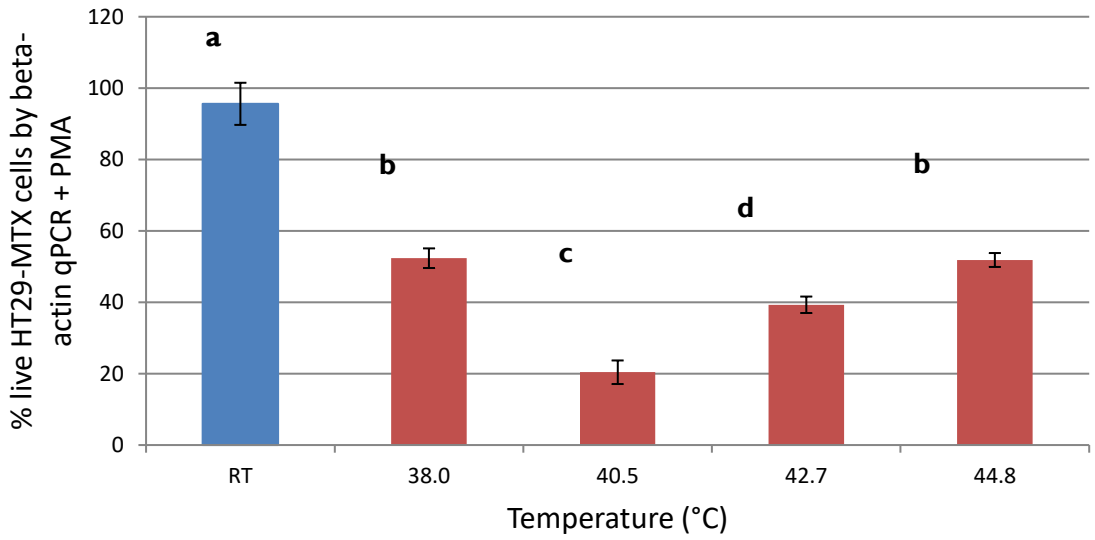

Figure 2: Viability of HT29-MTX cells after heat stress over 60 mins at varying temperatures, duplicated over 2 days (mean ± SEM). Cell viability measured by beta-actin PCR in PMA stained cells. [a,b,c] Bars with different superscripts are significantly different ($p<0.05$).

5.1.2 Heat shock protein 70 assay

A quantitative reverse transcriptase PCR (qRT PCR) was developed to measure the effect of heat on transcription of heat shock protein 70 (HSP70) RNA. However, in order to compare HSP70 induction between animals, another RNA target, not induced by heat was required to normalise the HSP70 levels between samples and between animals. Nygard *et al.* (2007) found that beta-actin was the most stable ubiquitous RNA target across 17 different pig tissues, including kidney, lung and small intestine, making it a good choice for normalisation of HSP70. Expression of HSP70 RNA required prior RNA extraction from cells (TRIzol, Invitrogen), followed by reverse transcriptase qPCR (RTqPCR) of both beta-actin and HSP70 by previously published methods (Yu *et al.* 2007). Primary pig tissue cells (lungs, kidney and testes) were used to optimise the qRT PCR assay for HSP70.

Beta-actin expression remained relatively constant in pig lung and pig testes cell lines despite heat shock of up to 44.8°C for 60 mins (Figure 3A and 3B), confirming that it would be an appropriate internal control to normalise HSP70 results for these cell lines. Conversely, the pig kidney cells had decreased expression of beta-actin after heating (Figure 4C), suggesting that they are more susceptible to heat shock. The internal location of the kidney may make it less tolerant to heat stress.

Fig. 3A: Pig lung - beta-actin

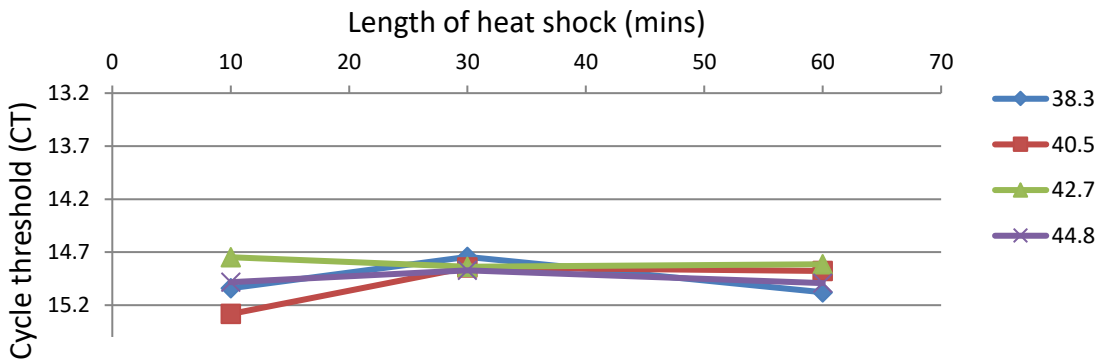

Fig. 3B: Pig testes - beta-actin

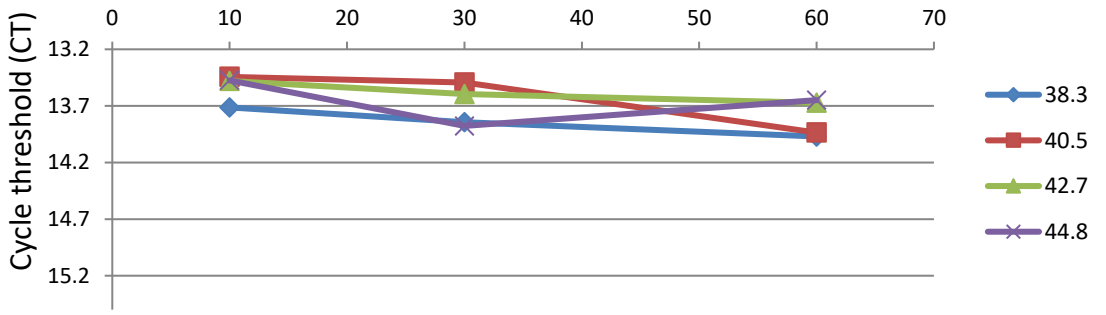

Fig. 3C: Pig kidney - beta-actin

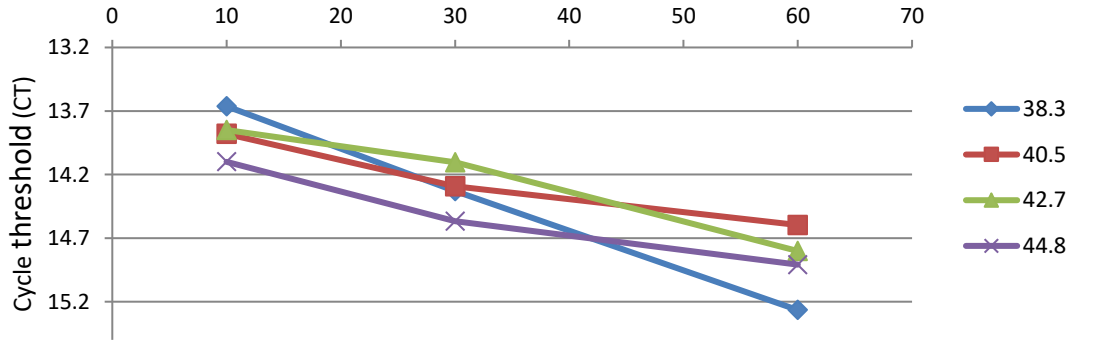

Figure 3: Effect of heat stress on beta-actin RNA expression over time in pig (3a) lung; (3b) testes and (3c) kidney cells in triplicate (mean). Increasing cycle threshold is equivalent to decreasing beta-actin expression.

The expression of HSP70 RNA was highly responsive to heat in all of the pig cell cultures, both in absolute terms and relative to the expression of beta-actin. The expression of HSP70 RNA increased with increasing temperatures of 38.3°C, 40.5°C and 42.7°C (Figures 4A-C). The expression of HSP70 also increased over time (10, 30 and 60 mins) at the same temperatures (Figure 4). The only exception was reduced HSP70 levels in pig lung cells at 42.7°C compared to 40.5°C after 60 minutes (Figure 4A). The plateau in HSP70 RNA levels after 60 minutes in some cases suggests that a shorter heat stress interval would be better to identify variation between individuals. Induction of HSP70 decreased in lung and kidney cells once temperatures reached 44.8°C, but remained stable in pig

testes cells. In-vitro heat stress at greater than 42.7°C appeared to create some unusual results, and this might be due to the impact of cell death on assay results. It is probable that *in-vitro* stress needs to be limited to within reasonable biological limits.

Fig. 4A: Pig lung - HSP70 RNA

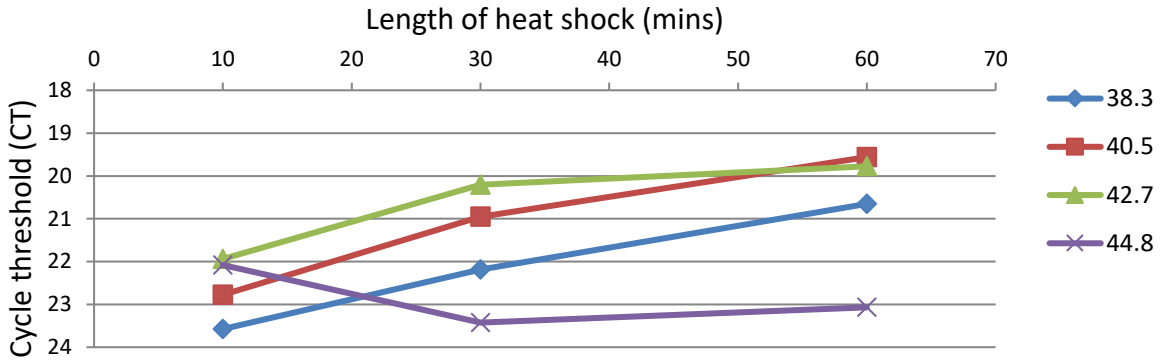

Fig. 4B: Pig testes - HSP70 RNA

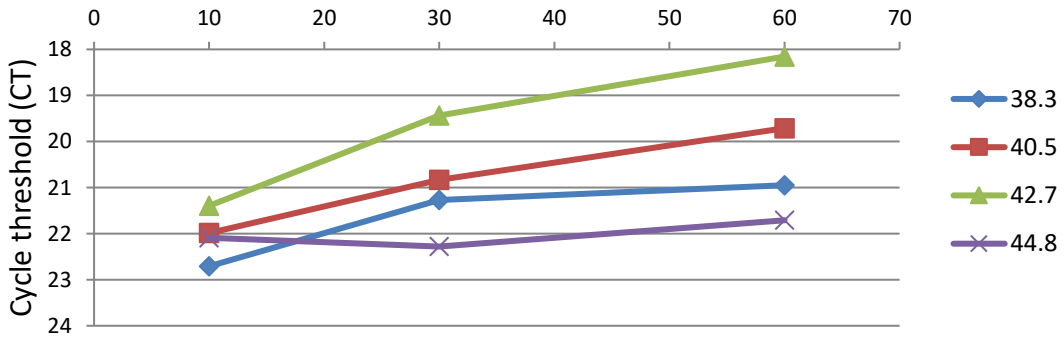

Fig. 4C: Pig kidney - HSP70 RNA

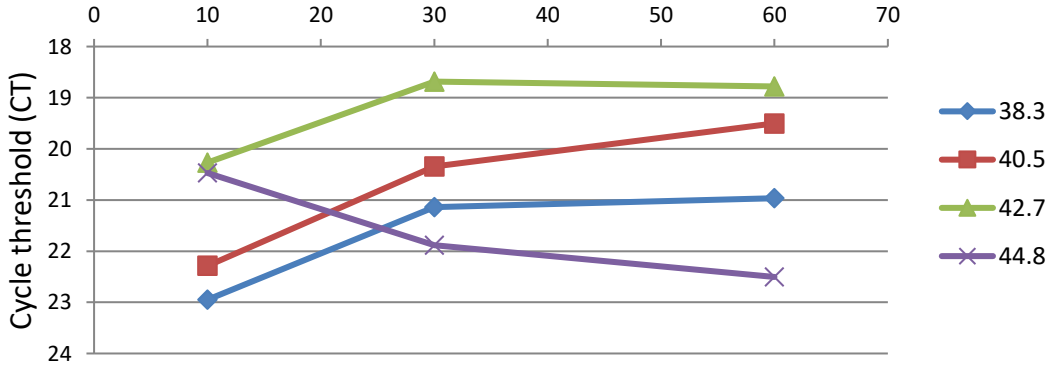

Figure 4: Effect of heat stress on HSP70 RNA expression over time in pig (4a) lung; (4b) testes and (4c) kidney cells in triplicate (mean). Increasing cycle threshold is equivalent to decreasing HSP70 RNA expression.

In order to normalise the concentration of RNA between cell preparations and overcome differences in cell numbers between samples, HSP70 expression levels were divided by beta-actin

expression levels. Heat stress caused the expression of HSP70 (relative to beta-actin) to increase with increased temperature and time (Figure 5A and B).

Fig. 5A: Pig kidney - HSP70/beta-actin

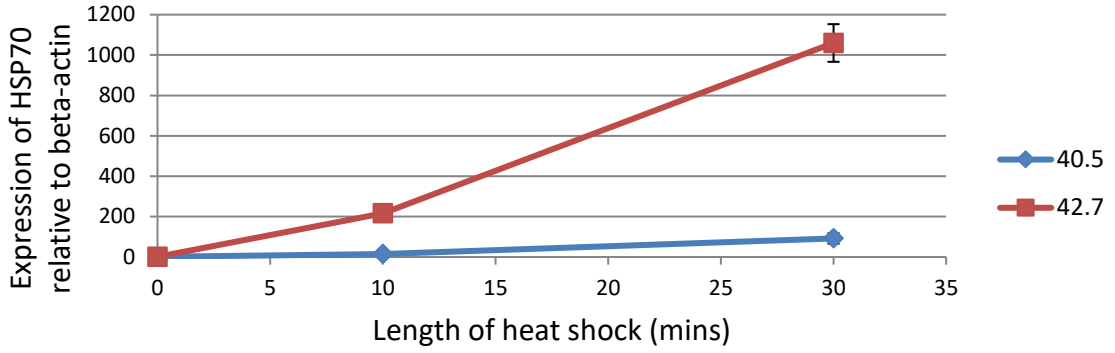

Fig. 5B: Pig lung - HSP70/beta-actin

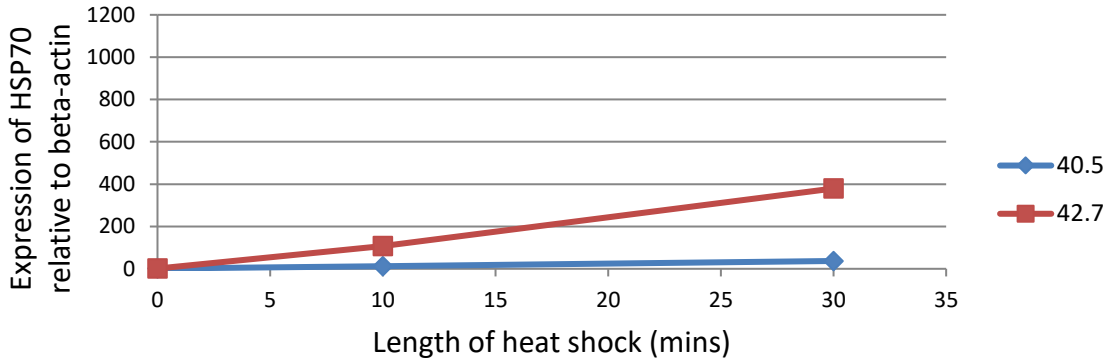

Figure 5: The magnitude of increased HSP70 RNA expression relative to beta-actin in pig (5a) kidney and (5b) lung cells in response to heat stress. Expression levels on y axis are relative to a baseline concentration of RNA in non-stressed cells at time zero. Performed in triplicate.

It was clear from these studies that in the majority of cases heat stress at 42.7°C for 10 minutes was sufficient to induce a detectable induction of HSP70 relative to a baseline concentration of HSP70 RNA in non-stressed cells. However, in some cases, a larger response was detected after heating cells for 30 minutes.

5.1.3 Thermotolerance

Thermotolerance is the ability of cells to become resistant to heat stress after a prior sub-lethal heat exposure. Induction of HSP70 is associated with the development of tolerance to heat, with thermo-tolerant cells producing less HSP70 after secondary heat stress compared with previously unstressed cells (Ryan *et al.*, 1991). It has been proposed that cells can regulate the levels of HSP70 in response to repeated challenges. Acquired thermotolerance is transient, and its duration depends upon the

severity of the initial heat stress. The potential for pigs to develop thermotolerance to heat stress may impact on our ability to identify pigs more tolerant to heat stress, especially if blood is collected from pigs recently heat stressed.

In order to investigate thermotolerance in pigs, we needed to develop an *in-vitro* assay where cells were heat stressed twice with a cooling period in between. In a series of experiments, we investigated thermotolerance in pig kidney cells after an initial heat stress of 42.7°C for 10 mins, followed by recovery for periods of one or five hours at 37°C, and a second heat stress event. As previous studies had demonstrated that HSP70 continues to be produced after the heat stress event is over, we included control cells maintained at 37°C over the total experiment period of either one or five hours, to measure changes in HSP70 RNA levels over time without heat stress.

As previously demonstrated, we found that HSP70 expression was induced 3-fold greater in cells rested for 1 hour after heat stress (relative expression is about 90), compared to cells rested for an hour before heat stress (relative expression is about 30; Figure 6A), indicating that cells continue to produce HSP70 after the heat stress event, even though the heat has been removed. An additional heat stress event after one hour recovery did not significantly affect the expression of HSP70. This suggests that a longer period of recovery is required to test for thermotolerance in pig kidney cells, to allow HSP70 levels to drop back to baseline levels.

In contrast, resting cells for 5 hours after heat stress was long enough for elevated HSP70 expression to return to normal, with no significant difference in HSP70 levels between cells rested for five hours or not rested after the initial heat stress (4.5 fold HSP70 induction relative to 6 fold induction, Figure 6B). A second heat stress event in cells rested for 5 hours did lead to a further induction of HSP70 (4.5 versus 16 fold induction of HSP70, Figure 6B), suggesting that HSP70 levels had dropped over the rest period and secondary heat could induce HSP70 RNA again; hence the cell didn't develop sustained thermo-tolerance.

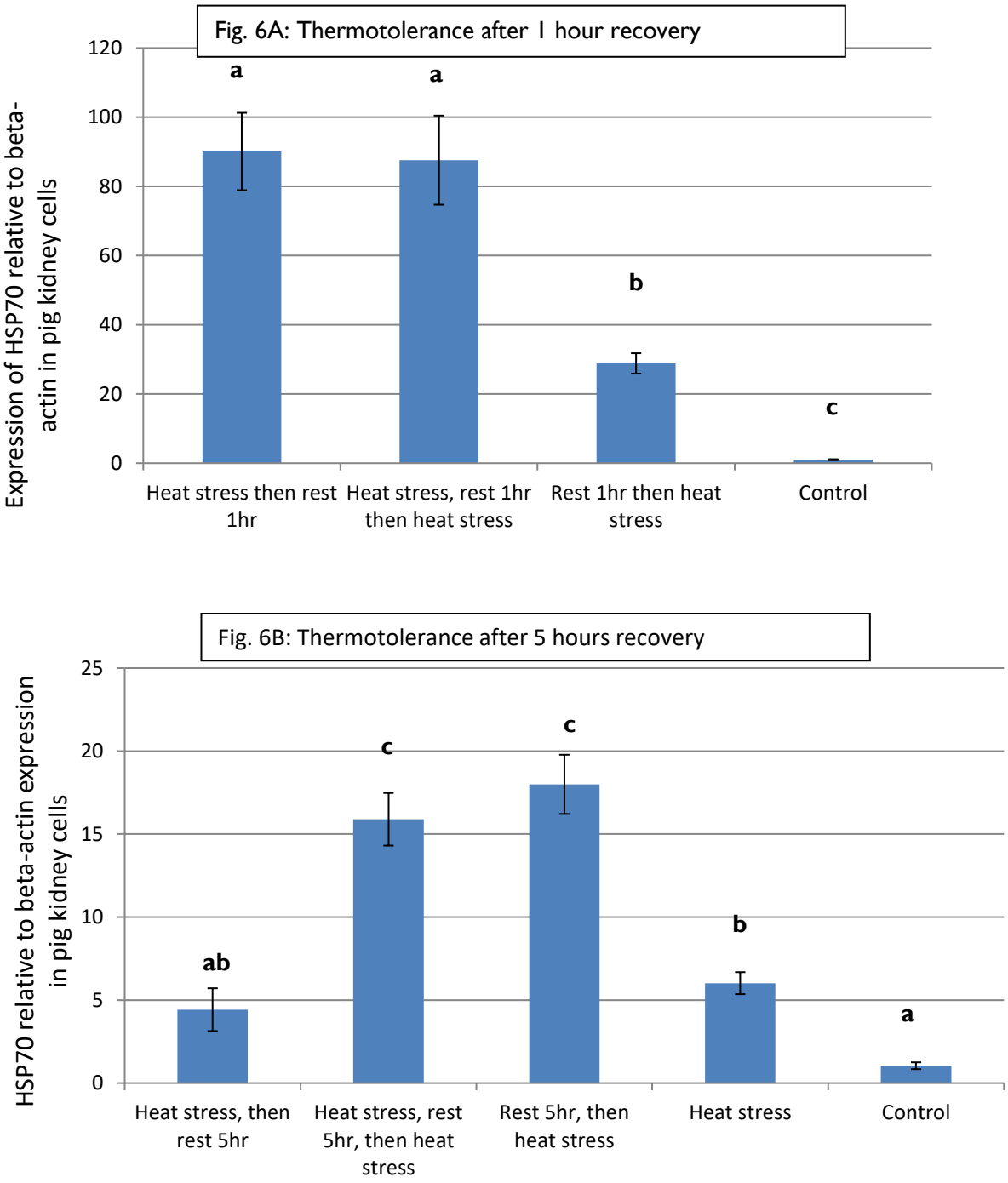

Figure 6: Thermotolerance in pig kidney cells heat stressed, allowed to recover for one (6a) or five hours (6b) then re-stressed; expressed as the magnitude of HSP70 RNA expression relative to beta-actin (Mean ± SEM of triplicate samples). Controls are non-stressed cells.

5.2 Step 2: Evaluate the suitability of different pig sample types (blood, hair and cheek cells) to demonstrate the effect of heat stress on pig cells.

Once the above assays were developed, a range of sample types from live pigs were collected (hair, cheek cells and white blood cells) to measure the response of individual pig cells to heat stress. While cheek cells are a convenient and non-invasive sample type, heat stressing cheek cells at 42.7°C for 10 mins did not significantly increase HSP70 RNA levels (normalised against beta-actin) relative to cheek cells kept at room temperature (control) (December 2017 progress report). This may be complicated by the fact that only 10% of scraped cheek cells were viable.

Hair follicles were also easy to obtain, but cells needed to be disaggregated with trypsin to produce single cell suspensions. Heat stress again did not reliably induce HSP70 RNA, regardless of whether intact or disaggregated hair follicles were used (December 2017 progress report).

Collection of blood samples was more invasive than collection of cheek or hair cells. However, mononuclear cells (lymphocyte and monocyte cells) proved to be a better source of RNA for heat stress studies. The mononuclear cells were rapidly separated from red blood cells by density gradient centrifugation in CPT blood tubes (Becton Dickinson). Mononuclear cells were heat stressed at 42.7°C for 10 and 30 minutes and induction of HSP70 RNA was measured by quantitative RT-PCR. As different numbers of mononuclear cells were present in each blood sample, an internal control for each pig was used, which comprised a non-heat stressed cell sample. The expression levels of HSP70 and beta-actin in heat stressed cells were both normalised against the control levels of each target (beta-actin or HSP70) in non-stressed cells from each pig prior to calculating the relative expression of HSP70.

Blood was collected from a small sample of finisher pigs to investigate variation in HSP70 expression between pigs. The expression of HSP70 relative to beta-actin increased significantly after heat treatment for both 10 and 30 minutes in all pigs tested (Figure 7). There was also a significant increase in HSP70 after 30 mins compared to both the control and 10 mins of heat stress. Greater variability between pigs in relative HSP70 induction was observed after 30 minutes heat stress compared to 10 minutes; however, the standard error of the mean was also larger between replicates. The expression of HSP70 was therefore responsive to an *in-vitro* heat stress challenge. The change in expression of HSP70 from an *in-vitro* heat stress challenge was subsequently examined for individual animals the week before and during an imposed heat stress and correlated to their physiological response (see Step 3).

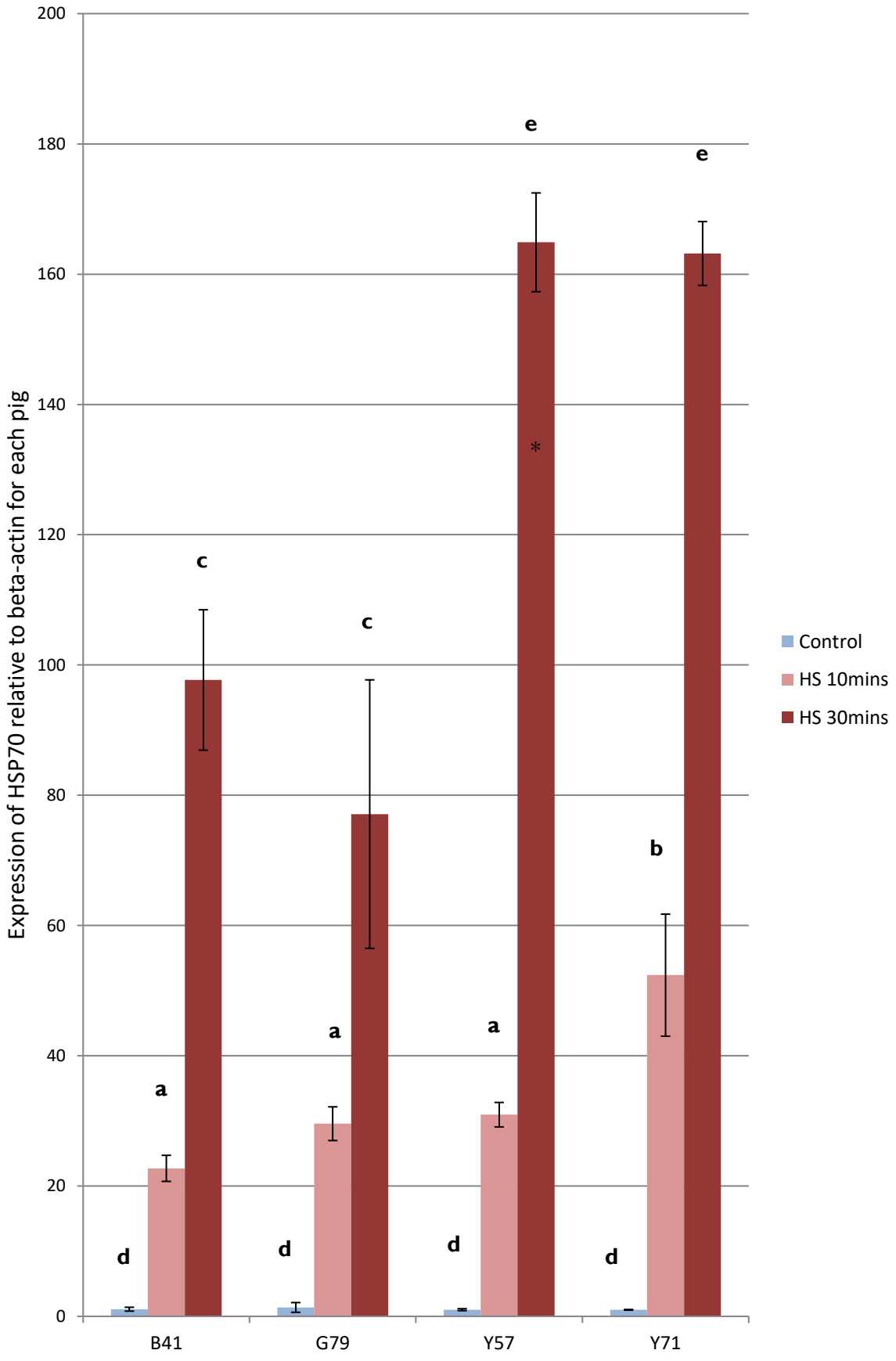

*Figure 7: Expression of HSP70 RNA relative to beta-actin in mononuclear cells from four grower pigs before and after heat stress at 42.7°C for 10 and 30 minutes (Mean ± SEM of triplicates). *One outlying replicate removed.*

5.3 Step 3: Evaluating the association between in-vitro heat stress quantification and physiological measures of heat stress recorded on live animals

Following development of the *in-vitro* heat stress model, the effects of elevated temperature on pig cell stress (HSP70 expression) was assessed from blood samples taken on 20 live animals prior to and during exposure to a heat stress challenge in a climate controlled facility.

The *in-vitro* test data (relative HSP70 RNA expression) was obtained using white blood cells with a 30 minute exposure to heating at 42.0^{0}C, prior to freezing for later assay. Each sample was assayed in triplicate for both beta-actin and HSP70 expression. The resulting cycle-threshold (Ct) values were used to calculate relative expression levels using the 2^-delta delta method (Rao, Huang et al. 2013), after elimination of outlier values. That is, the levels of HSP70 RNA expression were reported relative to beta-actin expression (a molecule not induced by heat) in the same samples.

Heat stress was induced in 20 live finisher pigs housed individually in two rooms within a temperature controlled environment. The physiological effects of heat stress on these individuals were quantified through individual measurement of average daily feed intake, respiration rate (3 x per day) and rectal temperature (3 x per day). Baseline levels for each individual were obtained within a period of thermal comfort (21^{0}C) from days -7 to day 0. On D0, room temperature was increased from 21°C by 3^{0}C at 8am, 11am and 12.30pm, up to a maximum of 30 degrees. Room temperature was held above 24^{0}C for close to 9 hours/day over the following 5 days. Electronic data loggers were used to record room temperatures, and to send an SMS alarm notification if temperatures went outside the set range. Relative humidity was also monitored throughout the two week period.

Temperatures were reduced overnight to alleviate the effects of heat stress on feed intake and body temperature, and to mimic the more normal pattern of temperature changes which occur during the duration of naturally occurring heat stress events. Clinical signs of heat stress were measured daily as outlined above, and HSP70 RNA expression was measured in blood collected at day -6 and at the end of the heat stress period (day 5).

The associations between *in-vitro* heat stress phenotype (a change in HSP70 expression) and physiological measures of heat stress recorded on the live finisher pigs were investigated using mixed linear and non-linear models, where appropriate. The significance of pair-wise contrasts was assessed assuming the Tukey-Kramer adjustment for multiple comparisons. The associations between heat stress response phenotypes (*in-vitro* or physiological) of individual pigs were evaluated using simple Pearson correlations.

6. Results

6.1 Controlled heat stress challenge

6.1.1 Environmental control

Data summarised from the data loggers show the extent of control in room temperatures and relative humidity over the duration of the trial. Pigs were housed within their thermal comfort zone in week 1, but were subjected to diurnal heat stress over five days in week 2. Relatively small differences were apparent between rooms (Figure 8).

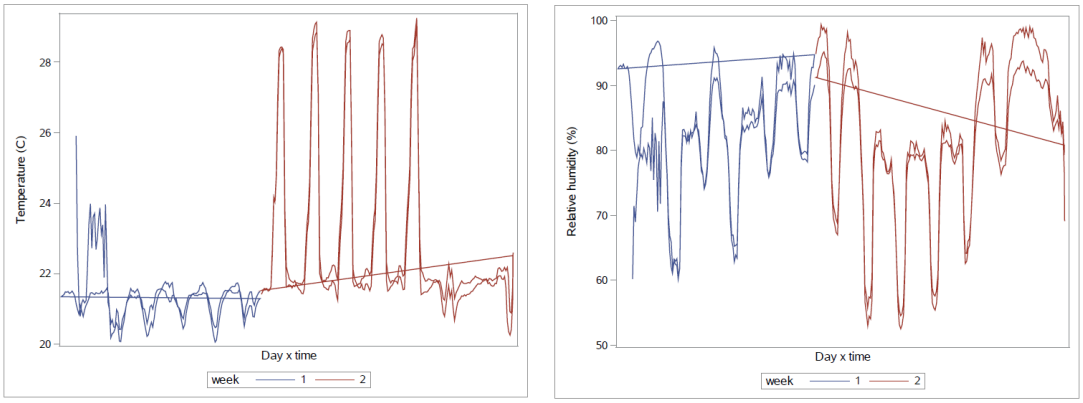

Figure 8. Change in temperature and relative humidity over days and time (by room)

6.1.2 Significant model terms for respiration rate, rectal temperature and average daily feed intake

The significance of model terms from PROC MIXED (SAS), accounting for repeated observations per pig as a random term, for respiration rate and rectal temperature is shown in Table 1. Gender was not a significant factor affecting these traits.

Table 1. P-values for model terms associated with respiration rate and rectal temperature (N=600) accounting for repeated observations per pig per day (random term: pig x time)*

	Week	Room	Time	Week x Room	Week x Time	Room x Time
Respiration rate	<0.0001	0.0338	<0.0001	0.0037	<0.0001	ns
Rectal temp.	<0.0001	0.0101	0.0170	0.0004	<0.0001	ns

*Number of observations=animals x times/day x days = 20x3x10 = 600; ns: not significant (p>0.05)

Least squares means for respiration rate and rectal temperature by week and time are presented in Table 2. In this group of pigs, the average respiration rate was approximately 25 breaths per minute

under thermo-neutral conditions, regardless of time of day. Respiration rate increased with room temperature during week 2, almost doubling by 12.30pm and approximately 2.5 times higher by 4.30pm, relative to the base levels observed in week 1. During the week where heat stress was induced, there was a trend for morning respiration rates to become slightly elevated, because room temperatures did not fully reduce to below 21^{0}C overnight (Figure 8). Therefore, the response in respiration rate with temperature change for individual pigs was calculated using week 2 data.

Similarly, rectal temperature remained unchanged regardless of the time of day in week 1. However, rectal temperature was unexpectedly lower before mid-afternoon ($p<0.05$), on average, in week 2 relative to week 1, but increased ($p<0.05$) to the same levels as week 1 by 4.30pm in week 2 (Table 2).

Table 2. Least squares means for respiration rate and rectal temperature (N=600) by week and time of day, accounting for repeated observations per pig per day (random term: pig x time)*

		Week 1			Week 2		
		8.30am	**12.30pm**	**4.30pm**	**8.30am**	**12.30pm**	**4.30pm**
Respiration rate	/min	24.5[a]	24.7[a]	25.6[a]	28.8[a]	46.2[b]	63.8[c]
Rectal temperature	^{0}C	39.0[a]	39.0[a]	39.1[a]	38.8[b]	38.8[b]	39.0[a]

[a] Means with different superscripts (within row) are significantly ($p<0.05$) different

Gender was a significant factor affecting feed intake ($p<0.05$), and pigs were subsequently grouped by gender and room to evaluate changes in feed intake between weeks. Despite the elevated room temperatures in week 2, on average pigs ate significantly ($p=0.0030$) more food/day in the second week compared to the first week. However, due to large variability between gender-room groups ($p=0.0002$) and between pigs in daily feed intake over time, group x week only approached significance ($p=0.0705$). The change in feed intake by week-group is shown in Table 3. The diurnal heat stress challenge applied in week 2 did not produce a significant decline in average feed intake.

Table 3. Least squares means for average daily feed intake (kg/day) by week, accounting for repeated observations per pig (random term: pig; N=160)*

	Room-gender group			
	3F	**3M**	**4F**	**4M**
Week 1	2.62±0.22	2.90±0.18	1.48±0.25	2.94±0.16
Week2	2.94±0.22	2.99±0.18	2.09±0.25	2.94±0.16
Sig of week (p-val)	ns	ns	0.068	ns

*Number of observations = pigs x days = 20 * 8 = 160

6.1.3 Identifying 'relatively' heat stressed animals

Based on the results above, the relative response of individual animals to the heat stress applied in week 2 was quantified by calculating changes to respiration rates and rectal temperatures as temperature increased during each day in week 2. This change is represented as the individual linear regression of respiration rate (RRATE) or rectal temperature (RECTT) on time, averaged over the 5 days of recording (N=15 records per pig). In addition, the change in average feed intake between weeks (DeltaFI) was calculated for each pig. Individual pigs relatively more adversely affected by heat stress were considered to be those whose respiration rate or rectal temperature increased relatively more during the heat stress challenge, and/or whose average feed intake reduced from week 1 to week 2, on average.

Raw data characteristics for variables describing each individual pig's physiological or *in-vitro* response to heat stress are shown in Table 4. Changes in respiration rate and rectal temperature were highly variable amongst pigs (cv=35% and 65% respectively), while the response in the relative expression of HSP70 tended to be even more variable (range in cv: 72 to 250%). Samples taken on day -5 (HSP-5: pre- heat stress challenge) had a lower relative response to the *in-vitro* heat challenge compared to the response observed in blood samples taken from animals which were actively physiologically affected by heat stress (HSP+6: post heat stress challenge).

Table 4. Characteristics of the data for physiological traits representing heat stress response of finisher pigs (N=20)

Trait	Abbreviation	Unit	Mean (SD)	Min	Max
		Physiological response			
Change in respiration rate[1]	RRATE	/min	17.5 (6.05)	8.73	27.4
Change in rectal temperature[1]	RECTT	^{0}C	0.120 (0.078)	-0.034	0.282
Change in feed intake between weeks	DeltaFI	kg/day	0.182 (0.504)	-0.60	1.20
		In-vitro response			
Relative[2] expression of HSP70 (day -5)	HSP-5		95.2 (98.0)	27.3	387
Relative[2] expression of HSP70 (day +6)	HSP+6		155 (112)	19.8	428
Change in expression[2] of HSP70	DeltaHSP		59.7 (149)	-276	389

[1] Regression coefficient; [2] HSP70 expression relative to beta-actin gene expression

Correlations between physiological and *in-vitro* measures are shown in Table 5. Pigs with a high feed intake in week 1 (housed under thermo-neutral conditions) also had a high feed intake in week 2 (under daily heat stress), but the correlation was significantly less than 1, demonstrating an altered ranking for feed intake between weeks. The correlation between HSP-5 and HSP+6 was essentially zero. Therefore, animals would rank very differently for HSP70 expression based on samples taken at these two time points (before and during heat stress). Despite this, the correlations between HSP70 expression and values for the other traits were consistent in direction for samples taken both before and during heat stress. Pigs with higher HSP70 expression at normal temperatures appeared not to respond as much physiologically to heat stress. However, no significant correlation was observed between HSP70 expression (day +5) and change in respiration rate during periods of heat stress. Pigs with a stronger increase in respiration rate due to temperature also had a higher average feed intake and a positive deltaFI in week 2. However, pigs with high FI in week 1 had a reduced change in feed intake (deltaFI), suggesting that the heat stress effect on feed intake was more pronounced in pigs with a higher feed intake.

Table 5. Pearson correlations between in-vitro (HSP-5, HPS+6 and deltaHSP) and physiological measures (RRATE, RECTT and deltaFI) of heat stress (correlation 1st row; p-value 2nd row)

	HSP+6	deltaHSP	RRATE	RECTT	Wk1FI	Wk2FI	deltaFI
HSP-5	0.004	-0.66	-0.49	-0.09	-0.15	-0.22	-0.05
	0.99	0.002	0.027	0.72	0.54	0.34	0.82
HSP+6		0.75	-0.12	-0.25	-0.12	-0.16	-0.02
		0.0001	0.61	0.29	0.62	0.48	0.92
deltaHSP			0.27	-0.13	0.01	0.02	0.02
			0.24	0.58	0.98	0.93	0.94
RRATE				0.080	0.07	0.54	0.50
				0.74	0.78	0.01	0.03
RECTT					0.12	-0.01	-0.17
					0.60	0.96	0.46
Wk1FI						0.67	-0.59
						0.001	0.006
Wk2FI							0.20
							0.39

7. Discussion

7.1 Physiological response to diurnal heat stress

On average, pigs significantly increased their respiration rate, but not their rectal temperature, as temperature increased and heat stress developed. The magnitude of change in respiration rate with temperature was large, making respiration rate a clear indicator of developing heat stress. The magnitude of increase in respiration rate was similar to that reported in a review by Campos *et al.* (2017). However, the diurnal pattern of heat stress applied in our study would be expected to have an impact on changes in physiological responses, by enabling a recovery period. Only some pigs developed an increase in rectal temperature by the end of each day of heat stress. We suggest that the increased respiration rate combined with observed behavioural changes, such as an altered diurnal pattern of feed intake, increased water consumption and increased resting (data not available) was sufficient for most pigs to maintain close to normal rectal temperatures during each day in week 2, while the lower overnight temperature enabled a recovery period.

Based on daily feed intake observations over 10 days, the diurnal heat stress induced in this study was not sufficient to produce a clear signal with respect to an expected reduction in daily feed intake due to heat (Campos, Le Floch et al. 2017). The majority of pigs made up for reduced feed intake during the day by increasing intake during the cooler night time conditions. Pigs were indeed observed to recommence eating as soon as room temperatures were cooled at the end of each day in week 2 (Alison Collins, pers. comm.). Only five pigs had a lower average daily feed intake in week 2 compared to week 1. Three of these five pigs had recorded an above average increase in rectal temperature during the day time, supporting reduction in intake with increasing heat stress.

In fact, the finisher pigs subjected to heat stress within the controlled environment facility increased intake, on average, between weeks 1 & 2 under *ad-libitum* feed delivery. This was likely because a) they became more familiar with the housing environment and experimental monitoring, and b) continued to grow over the trial period. Therefore, a tendency for reduced feed intake due to heat stress might also have been masked by an under lying trend towards increased feed intake with age/weight. Further, pigs with clinical signs of excessive heat stress (increasing rectal temperature, altered behaviour, altered manure and/or drinking patterns) were treated with Vytrate®. This individual treatment might also have reduced the impact of heat stress on feed intake changes due to heat stress, but was required to maintain pig welfare during the study period.

Overall, for all physiological measures a proportion of pigs displayed adverse responses to the heat stress conditions, either through increased respiration rate (all pigs), or in some pigs, increased rectal temperature or reduced feed intake. The strong response in respiration rate to temperature, combined with substantial variation amongst individuals in this response, improved the relative utility of this measurement to quantify the short term response of individual animals to heat stress.

7.2 Associations between the relative expression of HSP70 to an in-vitro challenge with observed physiological responses

Pigs with a high relative response for HSP70 at day -6 had a reduced change in respiration rate, without a significant change in feed intake, implying better tolerance to the applied heat stress. This outcome was consistent with the studies of Rout *et al.* (2016), who showed that heat tolerant goats (assessed by reduced respiration and cardiac rates under heat stress) showed increased HSP70 expression in liver and kidney tissues. This result also suggests that *in-vitro* HSP70 expression could be a suitable phenotype which represents individual variation in heat stress response.

However, the test (HSP70 response to *in-vitro* heat stress) to obtain this phenotype appeared sensitive to the circumstances of sampling. Relative to phenotypes obtained from earlier samples, a completely different ranking in phenotype for HSP70 expression was observed for the same animals blood sampled during their exposure to heat stress. A greater mean response in HSP70 expression occurred in white blood cells extracted from samples collected during the last day of the heat stress period. Our *in-vitro* heat stress studies in primary pig kidney cells showed that cells continued to produce HSP70 after the heat stress event ceased. Therefore an increased expression overall might be expected from samples obtained from heat stressed animals. What was not expected was the re-ranking of individual pigs for their HSP70 expression based on results from samples obtained before or during the heat stress time period. We have no comprehensive explanation for this outcome, but it creates some uncertainty as to the suitability of using a white blood cell sample to measure an *in-vitro* HSP70 response to heat stress when an animal is sampled in a heat stressed state.

The average change in feed intake between weeks was not negative, demonstrating that overall in this experiment, feed intake alone was a poor indicator for the development of heat stress in this group of animals. We propose this was the case because animals were still growing and, due to the diurnal pattern of heat stress experienced, could on average alter their behaviours to maintain or increase feed intake to support continued growth in the week of heat stress. However, larger scale studies demonstrate clear reductions of feed intake in sustained hot seasonal field conditions (Lewis and Bunter 2011) and in experiments with sustained heat stress (Campos, Le Floch et al. 2017). Similarly, there were individual pigs in our data which reduced their intake, or did not increase their intake to expectation, during the week of heat stress. These pigs also commonly showed other symptoms of heat stress, such as increased respiration rate (the most reliable indicator of physiological response to short term heat stress) or increased rectal temperature.

8. Implications & Recommendations

The study was successful in its objective to develop an *in-vitro* test which produced phenotypes representing an individual pig's response to heat stress. The test required blood sampling and is therefore invasive, but could potentially be combined with blood sampling for other purposes. Procedures involving more easily accessible cells, such as cheek cells, or potentially simpler technology, proved unsuccessful. Technologies such as PCR, on which this test was based, do offer a high throughput technology for larger scale phenotyping.

The resulting *in-vitro* test (HSP70 response to heat stress), based on samples obtained during thermo-neutral conditions, was correlated with the pigs respiration rate response to increasing heat. Therefore, the test met our objective to obtain a phenotype related to heat stress response of individuals using samples obtained under thermo-neutral conditions, because the requirement of subjecting animals to a heat stress situation is a major limitation to obtaining data for the heat stress responses on a large number of animals. However, this test also appeared sensitive to the circumstances of sampling. When samples were obtained from heat stressed animals, the phenotypes of individuals from this test were uncorrelated with results from the test applied to samples obtained prior to heat stress. This indicates that the same test applied at two different time points is not measuring the same phenotype, and/or the phenotype is not repeatable. Results of the test applied on samples obtained during heat stress were also uncorrelated with an animal's physiological response to that heat stress. This lack of consistency in results for this *in-vitro* test could be limiting for field implementation, unless the cause(s) of inconsistency can be identified and/or remedied, or used to determine the most appropriate conditions for sampling. For example, if the lack of consistency solely arose because samples from heat stressed animals were compromised, then this would limit blood sampling to a time when animals are not heat stressed, which creates another problem for year-round phenotyping in the presence of natural heat challenges. Alternatively, if the test phenotype is not reproducible, and/or inconsistently related to long term outcomes after acclimation, then the test also has only limited value. Inconsistent results might also have occurred by chance, due to the small scale of the study.

Appropriate procedures for the *in-vitro* test developed from this small-scale study should be investigated further prior to larger-scale testing. Further work should look at the repeatability of ranking individual animals for the *in-vitro* tests based on samples obtained from groups of animals which are or are not experiencing heat stress (eg repeated summer and winter samples). Further, *in-vitro* test results should also be related to the longer term response to heat stress, in a larger sample of animals, to establish implications of the *in-vitro* test phenotype for both production and welfare outcomes.

9. Technical Summary

Method developments in this project include the adaptation of RNA extraction methods for pig white blood cells, cheek cells and hair follicles. High throughput technologies were also developed for white blood cell isolation, heat treatment, RNA extraction and quantitative amplification of beta-actin and HSP70 RNA. In-vitro heat stress conditions were modified from the literature to maximise expression of the HSP70 gene in pig tissues and blood.

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