Breeding Focus 2018 – Reducing Heat Stress

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Preface

"Breeding Focus 2018 – Reducing Heat Stress" is the third workshop in the series. The Breeding Focus series was developed to provide an opportunity for exchange between industry and research across a number of agricultural industry sectors. With this goal in mind, workshops have included presentations across the livestock and aquaculture industries to take participants outside their area of expertise and encouraged them to think outside the box. This year we increased the scope even further by also inviting presentations from the cropping and horticulture industries. Since the topic of heat stress has recently gained increased attention, we will discuss a wide range of aspects associated with heat stress, such as the physiology of heat stress and phenotypic indicators, genetic approaches and industry impacts.

Heat stress in animals describes a situation where an animal is exposed to high temperatures and unable to dissipate body heat, which causes an increase in body temperature. In the short term, an animal will react to heat stress with behavioural strategies (e.g. seeking shade, panting) to reduce the heat load. With prolonged excessive heat load, feed intake is reduced and production losses occur. Under extreme circumstances, excessive heat load can lead to death. In plants, heat stress can be defined as irreversible damage to plant function and development as a consequence of hot temperatures. Environmental causes of heat stress in plants and animals include high temperatures and high humidity over a long period of time, which is exacerbated by low cloud cover and high solar radiation.

With raising average temperatures, agricultural industries are faced with the challenge to manage potential impacts of heat stress on their crops, their pasture base and welfare and production of their livestock or aquaculture species. Management strategies such as shade and irrigation are effective but costly and, depending on the severity of climatic conditions, may have limited success. Susceptibility of organisms to heat stress can vary due to factors such as age and general health, but also genetic factors, such as breed or variety. Further, as we will hear during the workshop, genetic variation exists within breeds that enables genetic approaches to address heat stress in plants and animals. Selective breeding provides a long term approach that facilitates improvement of the physiology of plants and animals to cope with excessive heat load. The challenge here is to obtain cost-effective phenotypes to describe heat stress.

The chapters of this book discuss where the current climate is trending, and outlines opportunities for the crop, orchard, livestock and aquaculture industries to describe and measure heat stress, all with the focus on genetic improvement.

We would like to thank everyone who has contributed to this event for their time and effort: the authors for their contributions to the book and presentations, the reviewers who all readily agreed to critique the manuscripts. We would like to express a special thanks to Kathy Dobos for her contributions into the organisation of this workshop and the publication. Thank you!

Susanne Hermesch and Sonja Dominik
Armidale, September 2018

The challenge of improving tolerance to heat stress in livestock species

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Abstract

Heat stress has numerous detrimental consequences for reproduction, health, production performance and welfare of pigs and other livestock species. To select for improved tolerance to heat stress, it is necessary to obtain phenotypes for individuals which identify genetic variation in tolerance specifically to heat stress. Performance trait phenotypes, recorded in environments considered thermally benign or consistent with a heat stress induction, have been used to infer heat stress tolerance phenotypes. However, these are indirect measures for heat tolerance which can be difficult to disentangle from other factors which contribute to variation amongst individuals in performance phenotypes. Moreover, the necessary data structures are unlikely to be available for all animals which must be evaluated. Phenotypic indicators associated with heat stress tolerance may also not be widely applicable across breeds or species, or equally relevant across different levels of exposure to heat stress. In this paper we discuss developing a direct test for heat stress tolerance based on assays for cell death and/or an *in-vitro* induction of a cellular response in heat shock protein 70 (HSP70). Using blood samples from 20 grower pigs obtained before a heat stress event imposed using climate controlled facilities, we demonstrated a moderate correlation (-0.49) between HSP70 response and the respiration rate response of pigs to increasing ambient temperature. However, ranking of animals for the HSP70 response was not consistent when retested using samples taken from the same animals in a heat stressed state. More work is required to establish whether *in-vitro* tests could play a role in providing phenotypes which enable selection for improved tolerance to heat stress.

The consequences of heat stress

Heat stress has numerous detrimental consequences for reproductive, health and production performance in pigs and other livestock species through effects on reproductive hormones, immune responsiveness and feed intake (Morrow-Tesch *et al.* 1994; Prunier *et al.* 1997; Hansen 2009; Williams 2009). The consequences of heat stress for production outcomes are reasonably well quantified, and the conditions under which animals experience heat stress well

defined. For most of our livestock species, the thermal comfort (TCZ) and thermal neutral (TNZ) zones have been described for important growth or production stages. These zones describe temperature ranges over which an animal is in thermal comfort and performance is optimal (TCZ), or the lower and upper temperatures (as defined by the TNZ) outside which animals must expend energy to maintain thermal homeostasis. The TCZ/TNZ varies by species and also by age group within species. A schematic which illustrates the types of responses which occur as ambient temperature changes is shown in Figure 1.

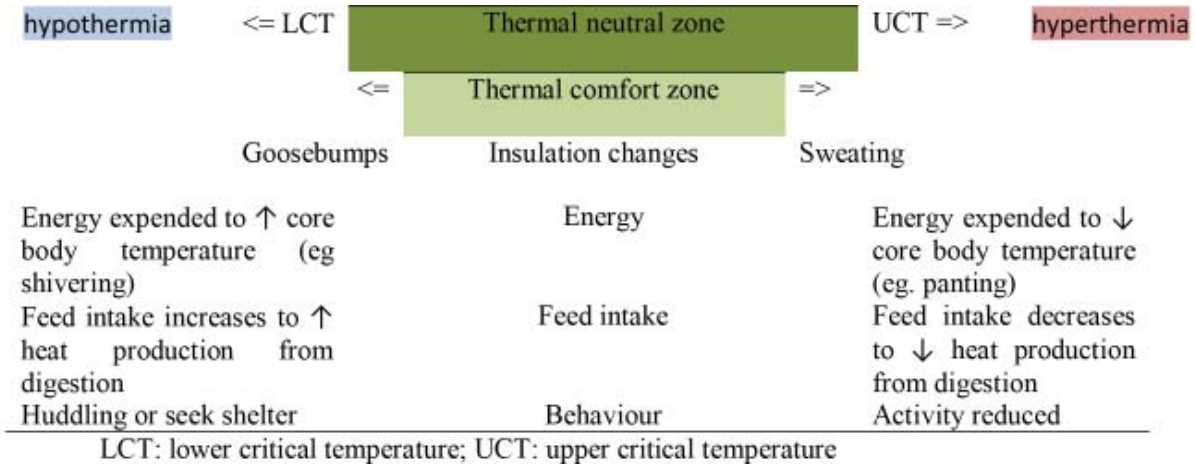

Figure 1. A schematic to illustrate responses in homeo-therms (eg humans and livestock species) which occur in response to the changes in ambient temperature

For temperatures above the TNZ, the heat load received by animals from their environment can exceed their ability to dissipate heat, and animals will then experience heat stress. Animals essentially must expend energy to maintain thermal homeostasis which, at a minimum, reduces production efficiency. At temperatures above the upper critical temperature (UCT) of the TNZ, a key input for production traits (ie feed intake) will also decline as animals attempt to control increases in their core body temperature by reducing the metabolic heat load associated with digestion. Further, heat stress has ongoing consequences for cellular and metabolic processes independent of feed intake changes. These physiological responses to heat stress mean that "growth, production, reproduction and health are not priorities any more in the metabolism of heat-stressed animals" (Slimen *et al.* 2016). Therefore, above the UCT, animals can experience significant production losses from heat stress, the overall impact of which will depend on the severity and duration of the heat stress event. However, as with other characteristics, individual variation exists in the overall response of individuals to heat stress. Moreover, animals have the capacity to tolerate or adapt to on-going higher ambient temperatures. These attributes may contribute to individual variation in their heat stress response. Overall, it is reasonable to speculate that if individual variation in response and/or adaptation to heat stress is genetic in nature, then avenues potentially exist to select for improved tolerance to heat stress generally. This recognition has progressed most quickly for dairy cattle, and the first Australian Breeding Values for heat tolerance are now available (Anonymous 2017).

Identifying genetic variation in heat stress

Research in dairy cattle (Ravagnolo and Misztal 2000; Misztal *et al.* 2010) and differences between selection lines of pigs in their tolerance to heat stress conditions suggest that active selection for improved tolerance to heat stress is possible (Bloemhof *et al.* 2008; Lewis and Bunter 2011). These studies essentially used changes in performance phenotypes with temperature or season as an indirect estimate of tolerance to heat stress. However, there are several limitations to collecting data which can be used to infer heat stress tolerance in this way. 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. Further, 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 *et al.* 2000). Thus, a nucleus farm might have limited capability for routinely recording the performance of all animals under heat stress conditions. Other limitations include differences in expression between age classes (and therefore optimising the timing of recording) 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 of individuals, but which have no similar phenotype in pigs. An alternative is to challenge animals to heat stress using controlled climate facilities. However, access to these facilities is limited, climate controlled facilities are expensive to operate and they generally do 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, which typically varies throughout the year, 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 *et al.* 2009; Lewis *et al.* 2010; Lewis and Bunter 2011). Indirect measures might be inaccurate when heat stress events are confounded with other factors that systematically affect performance (eg photoperiod) or which are difficult to disentangle at an individual level (eg variation in the genetic merit for the performance trait itself). Moreover, the timing and 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 performance data to indicate to what extent an individual animal is heat stressed outside a controlled experimental scenario. 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 quantitative measurement of the magnitude of response to heat stress at the physiological level is required

for field applications. The remainder of this paper will concentrate on describing development of an *in-vitro* test intended to directly quantify an individual's response to heat stress.

Developing a direct measure to quantify an individual's response to heat stress

In our study we proposed to develop *in-vitro* techniques for evaluating individual animals for their response to heat stress using samples of both heat stressed and non-stressed cells obtained 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), and an *in-vitro* challenge for cells is ultimately more welfare friendly than subjecting live animals to heat stress events. The project was intended to provide a research tool first, but was mindful of the opportunity to develop a low cost high-throughput procedure which could enable more industry-based applications, such as phenotyping for breeding programs.

At a cellular level heat stress ultimately causes cell death (Samali *et al.* 1999). 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, this 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. The ratio of live:dead qPCR (quantitative PCR) for bacterial species also correlated well with ratios of live to dead cells counted with traditional culture techniques (EMAI, unpublished). Methodology using qPCR is fast and well suited to high throughput testing, and EMAI had also developed a very simple method that only required a heating block and light box to heat stress cells and to separate live from dead cells. Samples can then be safely frozen and transported to the lab for later qPCR. Therefore, this approach was investigated for pig cells obtained from various tissues, using samples which are only available *post mortem* (eg lung, kidney, testes) as well as those more readily accessible for live animals (skin, hair, blood samples).

In addition to quantifying cell deaths, the expression of heat shock protein 70 (HSP70) in heat stressed cells can also be quantified by Reverse Transcriptase PCR in pig tissues, as a candidate phenotype for evaluating individual animals for their response to heat stress. Initiation of heat shock proteins helps protects cells from heat stress, and failure to initiate heat shock proteins appears to be a cause of cell deaths (Samali *et al.* 1999). 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). Therefore, the expression of HSP70 resulting from an *in-vitro* heat stress challenge was also investigated as an alternative phenotype representing response to heat stress. We subsequently confirmed the suitability of HSP70 expression as an indicator of physiological response using data from 20 grower pigs, recorded during a heat stress event applied using climate controlled facilities at EMAI. 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.

Assay results

Cell apoptosis (death) assays. Two simple assays designed to assess cell viability or apoptosis under heat stress were unable to provide a specific measure of cell viability in response to heat stress. The first assay, using microscopic examination of visible staining (of dead cells) by Trypan blue, failed to reliability identify differences between cells heat stressed at different temperatures. The second assay, based on qPCR to amplify propidium monoazide (PMA) treated cells, was unreliable because heat stress of PMA treated cells differentially reduced cell viability with temperature, affecting qPCR results. Therefore, these assays were not pursued further.

HSP70 expression. Heat stress at 42.7°C for 10 minutes was sufficient to induce a detectable response of HSP70 relative to a baseline concentration of HSP70 RNA in non-stressed cells in lung (depicted, Figure 2), kidney and testes cells. However, these cells are not particularly convenient for sampling from live animals, requiring further investigation of more accessible sample sources.

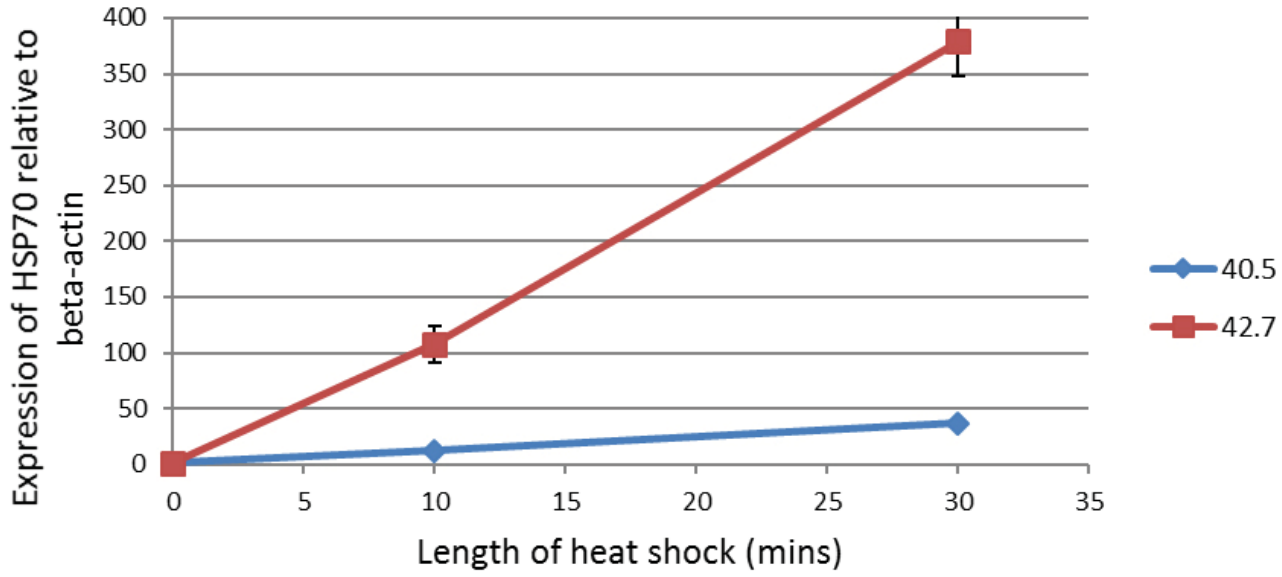

Figure 2: The magnitude of increased HSP70 RNA expression relative to beta-actin expression (acting as a control gene) in pig lung cells in response to heat stress. Expression levels on y axis are relative to a baseline concentration of RNA in non-stressed cells at time zero. Performed in triplicate.

Samples of cheek cells and hair follicles proved unsuitable sources for the HSP70 assay. However, mononuclear cells (lymphocyte and monocyte cells) obtained from blood samples proved to be a suitable source of RNA for heat stress studies. Mononuclear cells 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 (Figure 3). The HSP70 response was also observed to vary between the grower pigs in this preliminary study. Therefore, this assay was used as the direct phenotype assumed to represent an animals response to heat stress.

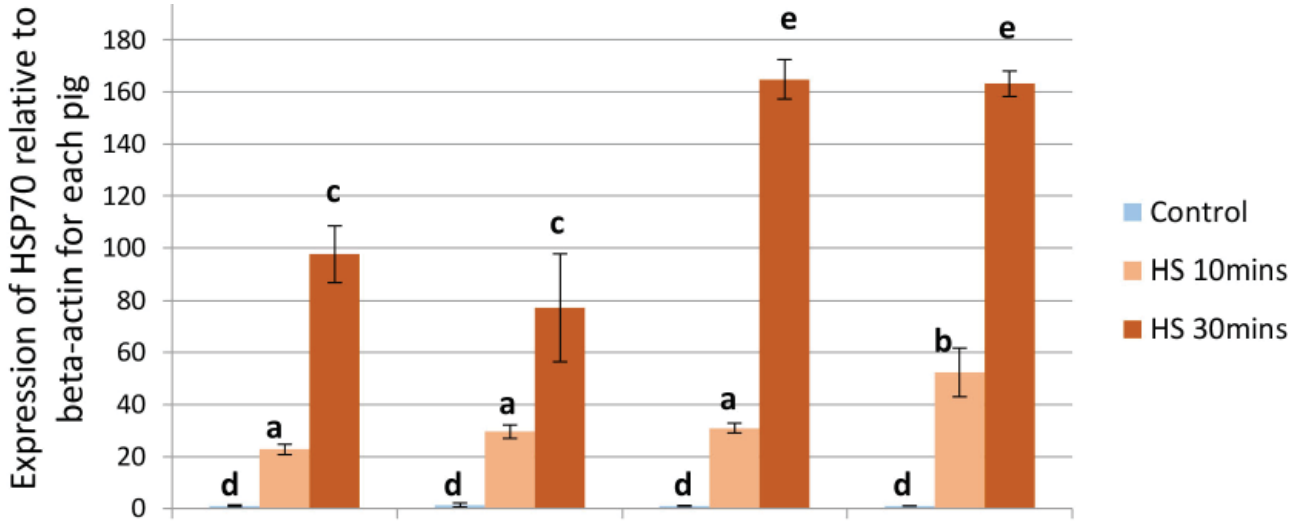

Figure 3: Expression of HSP70 RNA relative to beta-actin in mononuclear cells from four grower pigs before and after heat stress at 42.7°C for 10 and 30 minutes (Mean ± SEM).

Correlations between HSP70 response and physiological response to heat stress.

Heat stress was subsequently 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 (21^0C) from days -7 to day 0. On D0, room temperature was increased from 21°C by 3^0C at 8am, 11am and 12.30pm, up to a maximum of 30 degrees. Room temperature was held above 24^0C for close to 9 hours/day over the following 5 days (see Figure 4). However, 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 over the course 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 -5 (HSP-5) and close to the end of the heat stress period at day 6 (HSP+6).

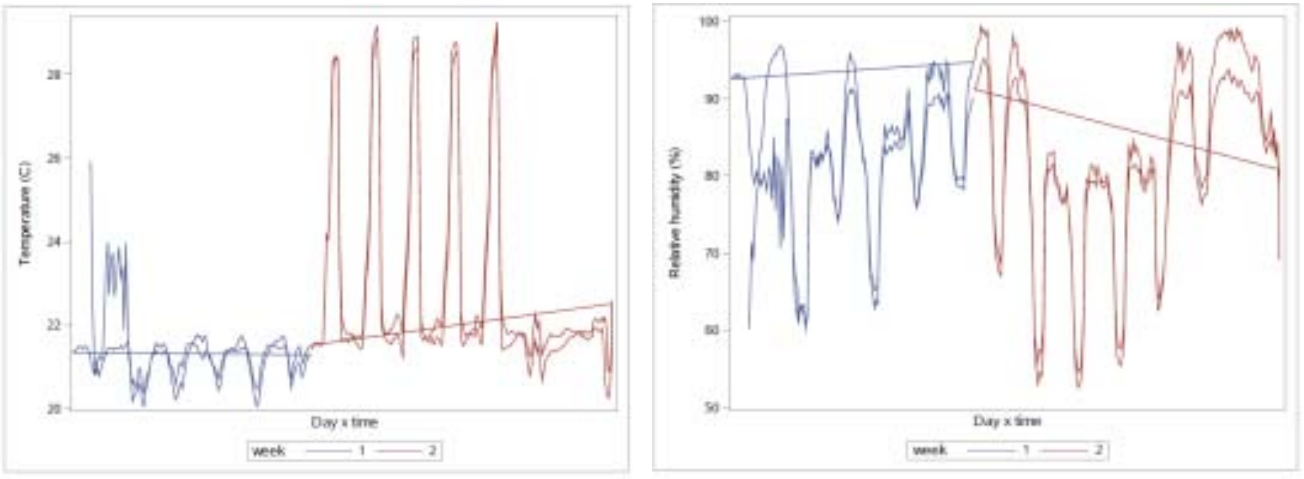

Figure 4. Change in temperature and relative humidity over days and time (by room)

Figure 4 nicely demonstrates the accurate control that can be achieved in climate control facilities to induce a controlled level of heat stress. Further, this study also demonstrated that heat stress applied diurnally did not have a significant impact on reducing average feed intake or increasing core body temperature in these grower pigs over a five day period (not shown). In contrast, respiration rate more than doubled (28.8 vs 63.8 respirations/minute) as room temperature increased to the maximum temperature of around 30°C each day. Consequently, the change in respiration rate with increasing temperature was used as the indicator of physiological response to increasing heat stress, while the HSP70 response was recorded from blood samples taken before (HSP-5) and during (HSP+6) exposure to heat stress (Table 1). The relative response of HSP70 in the *in-vitro* test (exposure of samples to 42.0°C for 30 mins) was higher in the HSP+6 compared to the HSP-5 samples, suggesting that the HSP70 response increased in response to animals experiencing prior periods of heat stress.

Table 1. Characteristics of the data for physiological traits representing heat stress response of finisher pigs (N=20)

Trait	Abbreviation	Mean (SD)	Min	Max
Change in respiration rate[1]	RRATE	17.5 (6.05)	8.73	27.4
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

[1] Regression of respiration rate on time; [2] HSP70 expression is relative to beta-actin gene expression

Correlations of RRATE with HSP-5 or HSP+6 were -0.49 (p=0.03) and -0.12 (p=0.61), demonstrating that animals with a higher relative HSP70 response to an *in-vitro* heat stress challenge had a lower physiological response to ambient heat stress, observed through changes in respiration rate. Therefore, the *in-vitro* test met our objective to obtain a phenotype related to heat stress response of individuals using samples obtained under thermal 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, HSP-5 and

HSP+6 phenotypes were uncorrelated (0.004, p=0.99), which was not expected. This means that the *in-vitro* test based on HSP70 expression might be sensitive to the timing of sampling with respect to previous exposure to heat stress events. This indicates that the same *in-vitro* 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 not significantly correlated with an animal's physiological response to that heat stress.

The lack of consistency in results for the HSP70 *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 very small scale of the study. Therefore, further investigation of the *in-vitro* test for HSP70 response is required before larger scale phenotyping occurs in field applications. Nevertheless, these preliminary results suggest that the search for direct, rather than indirect, measures of the response to heat stress might yield new phenotypes suitable for providing a direct measurement of heat stress response for individual animals.

Conclusions

- Heat stress is known to impact on the performance and welfare of animals.
- Performance trait phenotypes can be used to infer individual variation in the response to heat stress given appropriate data structures, but are indirect measures only.
- Heat stress applied in a diurnal pattern failed to induce a significant reduction in feed intake, or a significant increase in core body temperature in this study. Diurnal variation in temperature might reduce the impact of heat stress over short term periods. In contrast, a reliable response in respiration rate was observed with increasing temperature.
- To obtain a direct measure of response to heat stress, an *in-vitro* assay was developed, based on a heat shock protein 70 response (HSP70).
- The HSP70 responses in samples taken from growers before the heat stress event were moderately correlated with an animal's physiological response to heat stress, assessed through changes in respiration rate.
- The ranking of animals for HSP70 response differed for samples taken before and during the heat stress event. Therefore, timing of sampling altered the *in-vitro* phenotype.

- 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). In addition, it would be useful to quantify if health status (eg fever), or other factors, had any significant impact on phenotypes derived from the *in-vitro* test.
- *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.

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