First published in the *Journal of Animal Science*, volume 83, issue 8 (2005).
Published by the American Society of Animal Science.

The heritability of the expression of two stress-regulated gene fragments in pigs[1]

C. A. Kerr*[2], K. L. Bunter†, R. Seymour*, B. Shen*, and A. Reverter*

*CSIRO Livestock Industries, Queensland Bioscience Precinct, St Lucia, QLD 4067, Australia; and
†Animal Genetics and Breeding Unit, University of New England, Armidale, NSW 2351, Australia

ABSTRACT: Pigs reared in commercial production units sometimes encounter stressors that significantly decrease growth performance. It is hypothesized that response to stress challenges could potentially be used as selection criteria. This study aimed to investigate, in a commercial setting, the heritability of two target genes previously shown to be induced in response to stress, and related to growth performance, in an experimental situation. Blood samples (n = 2,392) were collected from three separate breeding lines of pedigreed and performance-tested boars between 24 to 25 wk of age. The expression levels of a novel fragment, '29a,' and the calcitonin receptor gene (CTR) were quantified using quantitative real-time PCR (qRT-PCR) on a subset (n = 709) of the blood samples. Gene expression levels were corrected for the efficiency of PCR reactions and also computed directly from threshold cycle (Ct) values. Resulting data showed a skewed nonnormal distribution of expression levels for the target genes relative to the endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and were highly variable. Analyses were subsequently performed using untransformed and log-transformed data, with outliers identified and deleted in edited data sets. Regardless of the transformation or editing procedures for outliers applied, there was negligible genetic variation for the expression of target genes relative to GAPDH. In contrast, repeatabilities of replicate samples were generally high (between 0.54 and 0.67). Absolute expression levels for GAPDH and 29a were lowly heritable (h^2 of about 0.04), although estimates did not exceed their SE. Subsetting the data according to whether the target gene had a higher or lower level of expression than GAPDH was then performed using the relevant Ct values. In the subset where the target gene was more highly expressed than GAPDH, a moderate estimate of heritability (0.18 ± 0.10) for the log-transformed absolute expression level of 29a was obtained, whereas the estimate for its expression relative to GAPDH was lower (0.09 ± 0.07). Estimates of heritability did not increase in the subset of low expression data. The limitations of using gene expression measures as potential selection criteria in commercial situations are discussed.

Key Words: Gene Expression, Heritability, Real-Time Polymerase Chain Reaction, Stress, Swine

Introduction

Pigs raised in intensive production units are likely to encounter various stresses (Black et al., 2001). Stress factors can unbalance metabolic homeostasis, directing the demand for nutrients and energy for essential physiological processes away from growth (Heetkamp et al., 1995). Previous studies have demonstrated that the stress factors of climate, such as air temperatures away from the thermoneutral zone (Quiniou et al., 2000; Collin et al., 2001); disease challenges such as *Actinobacillus pleuropneumoniae* (Wallgren et al., 1999; Kerr et al., 2003); and psychological-social factors, such as grouping of unfamiliar cohorts (Gonyou and Stricklin, 1998; Hyun et al., 1998a; de Groot et al., 2001), can have a detrimental effect on growth performance. The growth rate and efficiency of feed use by growing pigs housed individually in ideal experimental environments is generally greater than that of their commercial group-housed counterparts (de Haer and de Vries, 1993; Black et al., 1994). Consequently, the decreases in the effects of stress factors are expected to significantly increase pig growth performance.

[1]The authors acknowledge the Australian Pork Limited for financial support. The research assistance of B. Hines, M. Taniguchi, M. O'Grady, R. Daniel, J. Gough and T. Vuocolo, and operational guidance by G. Harper is gratefully acknowledged. The assistance of B. Luxford, C. Bennett and the Research and Development staff at QAF Meat Industries, who collected and prepared samples on site, also is gratefully acknowledged. In addition, we thank J. Black for his guidance in coordinating the project.

[2]Correspondence: 306 Carmody Rd. (phone: +61 7 3214 2326; fax: +61 7 3214 2288; e-mail: Caroline.Kerr@csiro.au).

Received March 8, 2005.
Accepted April 29, 2005.

It is hypothesized that knowledge of the level of expression of previously identified stress-regulated gene fragments could provide information for a breeding objective that places value on resilience to stress factors, such that selected animals will perform better in commercial environments. The aim of this study is to investigate whether the expression levels of gene fragments that have been previously shown (Kerr et al., 2004) to respond to stress challenges could be used as selection criteria under commercial conditions. Heritabilities were estimated for quantitative real-time PCR (**qRT-PCR**) gene expression data on the calcitonin receptor gene (**CTR**) and a novel fragment (shown as **'29a'**) using data obtained from blood samples taken from animals tested under commercial conditions, where variable levels of disease challenge and/or stressors exist.

Materials and Methods

Animal Experiment Details

Between April 2002 and May 2003, blood samples were collected from individuals representing three separate breeding lines of pedigreed and performance-tested pigs, located at QAF Meat Industries, Corowa, Australia. The performance-testing procedures involved measuring feed intake using electronic feeders. The boars commenced their performance testing at approximately 70 kg, remained on test for 8 wk (including 1 wk of settling into feeders), and completed their test at approximately 110 kg live weight. Feed intake was restricted according to starting BW during the 7 wk of the performance test. The level of feed restriction throughout the test period was approximately 85% of projected ad libitum intake. The average age off test was 175 d, or between 24 and 25 wk of age. Regardless of their health or performance, blood samples from all boars (n = 2,392) were then collected upon completion of this performance-testing procedure. The diet throughout the performance test period was a complex commercial diet based on wheat with five protein meal sources of animal and vegetable origin, providing amino acids in excess of expected requirements.

Pigs were restrained by nasal snare, and blood samples were collected via venipuncture into tubes containing 150 μL of 15% EDTA, and placed on wet ice. As soon as possible, the whole blood (3 mL) was mixed with 3 mL of denaturing solution (4 *M* guanidinium isothiocyanate, 0.02 *M* sodium citrate, and 0.5% *N*-lauroyl-sarcosine; Sigma-Aldrich, Sydney, Australia) before freezing at –80°C on-site. Samples were subsequently placed on dry ice for transport to the laboratory (CSIRO Livestock Industries, Queensland Bioscience Precinct, Brisbane, Australia), where they were stored at –80°C until further processing. The general health of individual animals during performance testing was recorded based on their need for medication. Similarly, notes on the health status of each animal at the end of performance testing (at sampling for gene expression levels) were made.

Analysis of CTR and 29a Gene Expression

A subsample containing 758 blood samples was selected for gene expression analysis. These samples were chosen to ensure good contemporary and sire progeny group sizes (more than 10 to 20 animals per group), thereby maximizing the information content for the estimation of heritabilities, using otherwise limited data. For each individual, gene expression values were determined using qRT-PCR procedures on RNA extracted from the mixed leukocyte populations in whole blood samples.

A schematic diagram describing the flow and layout of the experiment from blood samples to qRT-PCR is described in Figure 1. Blood samples were aliquoted into a 96-well format for RNA extraction, and total RNA was extracted with an RNeasy 96 kit (Qiagen, Basal, Switzerland) according to the manufacturer's protocol, but using 150 μL of the blood/denaturing solution mix as the starting material and including the optional DNAse step to ensure genomic DNA elimination. The RNA eluate was stored at –80°C in a 96-well format. Complementary DNA (**cDNA**) was synthesized from the RNA-adapted version of the Omniscript RT kit (Qiagen), following the suggested incubation conditions, but with different constituent concentrations. The RNA (8 μL) was preincubated with Omniscript reverse transcriptase (0.25 μL) and Oligo dT (100 ng) at 70° for 3 min. The reaction was chilled and deoxynucleotide triphosphates (2.00 μL), 10× buffer (2.00 μL), and water (6.75 μL) were added before the mixture was incubated at 37°C for 60 min, followed by 75°C for 10 min to complete the synthesis. The cDNA was then diluted 1:5 with DNAse/RNAse free water and stored at –80°C ready for qRT-PCR analysis.

The previously reported (Kerr et al., 2004) CTR and glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**, the endogenous control) conventional PCR primers were screened for their suitability in a SYBR green qRT-PCR protocol using Primer3 software (Rozen and Skaletsky, 2000; http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The resulting CTR and GAPDH primers appear in Table 1. Similarly, primers were designed for the fragment (29a), shown to be differentially expressed in response to high ambient heat challenge (our unpublished observations). The BLASTN (Altschul et al., 1997) results for 29a are shown in Table 2, demonstrating an alignment to a number of porcine expressed sequence tags; however, it is also significant to note that the amplified fragment aligned to porcine short interspersed nuclear elements (**SINE**). As a consequence, a number of precautionary steps (see below) were taken to ensure genomic contamination was detected for elimination from the samples and the data. The resulting primers for 29a also are shown in Table 1.

All primers were used at a final concentration of 300 n*M*. The SYBR Green qRT-PCR assay was performed in 5-μL volumes consisting of 1 μL of cDNA, 0.5 μL of H_2O, 2.5 μL of SYBR Green PCR master mix (Applied

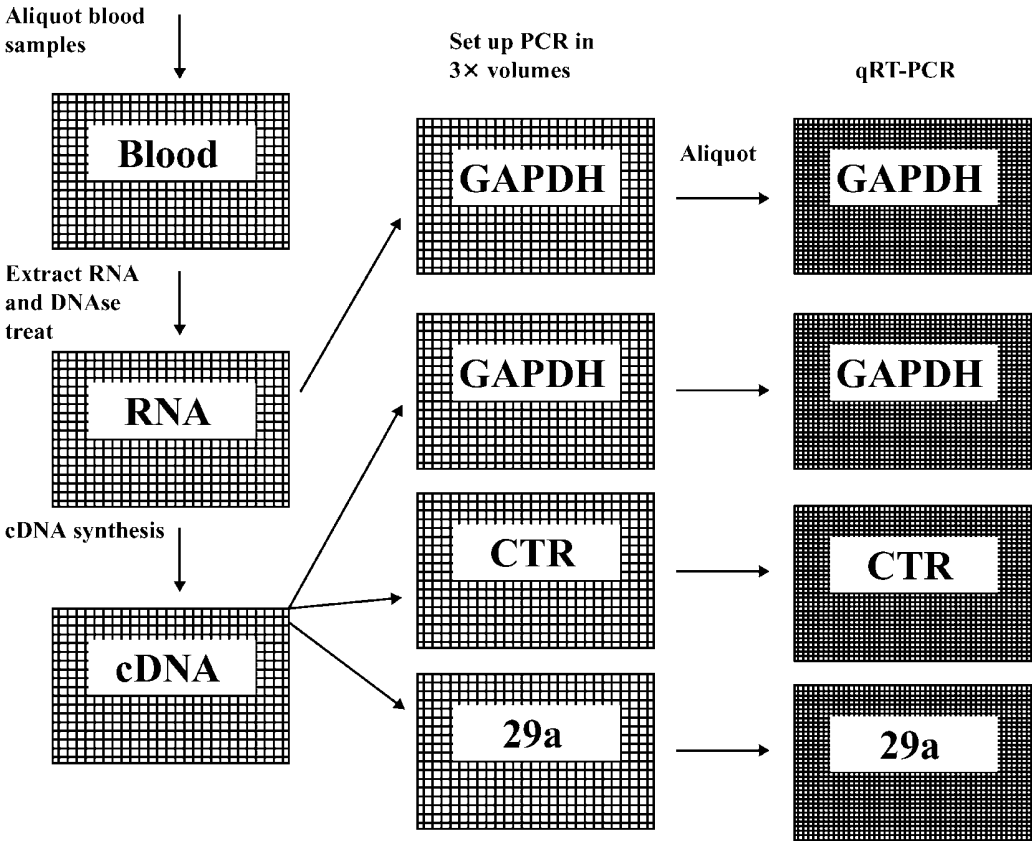

Figure 1. A schematic diagram demonstrating the sample flow and the protocol used. Blood samples (758) were aliquoted into eight 96-well format plates (Plate 1 = 1 to 96; Plate 2 = 97 to 193, etc.). The RNA was extracted, cDNA synthesized and the PCR set-up (in 3× volumes per well) from both a dilution of RNA (1 in 12.5) and cDNA for GAPDH, and cDNA for 29a and CTR, maintaining this format. The 3× PCR volumes were aliquoted into separate, consecutive, triplicate wells on a 384-well plate (i.e., Plate 1 = Samples 1, 1, 1, to 96, 96, 96) for amplification by qRT-PCR. For each of the eight aforementioned 96-well plates with blood samples, there were eight corresponding 96-well plates of RNA and eight of cDNA. There were 32 PCR set-up plates of 96 wells (eight plates of RNA and cDNA four times) and 32, 384-well qRT-PCR plates. Note: each plate also contained the same negative and positive controls (the latter was used to produce a standard curve). CTR = calcitonin receptor gene; 29a = a novel fragment; GAPDH = porcine glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR = quantitative real-time PCR.

Biosystems, Foster City, CA), and 0.5 μL of both the sense and antisense primers (both added at 3 μ*M*) in triplicate on a 384-well PCR plate. Amplification was carried out under the following cycle conditions: Step 1, 50°C for 2 min; Step 2, 95°C for 10 min; Step 3, 95°C for 15 s; Step 4, 60°C for 1 min; Step 5, repeating Steps 3 and 4 (40 times); Step 6, 95°C for 2 min; Step 7, 60°C for 15 s; and Step 8, ramp up slowly to 95°C. Each 384-well plate qRT-PCR consisted of negative (water) and positive (known cDNA) samples as controls. The ABI 7900HT sequence detection system (PE Applied Biosystems) RT-PCR machine and software (SDS version 2.2) were used to perform the qRT-PCR and collect the PCR data.

Examining the amplification plots assessed the production of the PCR amplicon (Figure 2). The product

Table 1. Primer design for quantitative real-time polymerase chain reaction to amplify a novel fragment (29a), the calcitonin receptor gene (CTR), and porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH, used as an endogenous control) and their location on the cDNA sequence

Gene	Sense primer	Antisense primer	Location
CTR	5′-accagttcttcctgacagtg-3′	5′-tggtatgggggtaacttttg-3′	315-455
29a	5′-gtgtacactgcggtagatg-3′	5′-ccatgcacacaaatccattc-3′	194-322
GAPDH	5′-acatcaagaaggtggtgaag-3′	5′-attgtcgtaccaggaaatgag-3′	110-260

Table 2. The 10 highest sequence similarity BlastN results for the fragment 29a against the National Center for Biotechnology Information expressed sequence tags database

Accession	Definition	E-value[a]	Identity
BI327058	Infected porcine bone marrow cDNA library *Sus scrofa* cDNA, mRNA sequence	10^{-147}	98%
BI336286	Porcine Spleen cDNA library *Sus scrofa* cDNA, mRNA sequence	4.00×10^{-49}	96%
AW326278	MARC 2PIG *Sus scrofa* cDNA 5′, mRNA sequence	7.00×10^{-23}	85%
CF788024	MARC 4PIG *Sus scrofa* cDNA 3′, mRNA sequence	2.00×10^{-20}	89%
BQ338456	NN1111 *Homo sapiens* cDNA, mRNA sequence	2.00×10^{-20}	86%
AJ652983	*Sus scrofa* cDNA clone C0005182_D15, mRNA sequence	4.00×10^{-18}	85%
CN154163	MARC 4PIG *Sus scrofa* cDNA 3′, mRNA sequence	4.00×10^{-18}	85%
CD777460	*Avicennia marina* leaf cDNA library similar to fused-ccdB (*Escherichia coli*), mRNA sequence	4.00×10^{-18}	98%
BI360785	MARC 2PIG *Sus scrofa* cDNA 5′, mRNA sequence	4.00×10^{-18}	85%
BP150580	Full-length enriched swine cDNA library, adult ovary *Sus scrofa*	2.00×10^{-17}	83%

[a]The theoretically expected number of false hits per sequence query.

dissociation curves (represented in Figure 3) assessed the quality of individual well PCR amplifications, and the standard plots (Figure 4) were used to determine the efficiency of PCR amplification for each 384-well plate qRT-PCR. Precautionary steps were taken to examine and eliminate possible contaminating genomic DNA. This was done by performing qRT-PCR with RNA for GAPDH diluted 1:12.5 concurrently with qRT-PCR for GAPDH using cDNA. Genomic DNA contamination was defined by at least two of the triplicates in the RNA-only analysis (no cDNA synthesis) RT-PCR giving a signal, and a difference in average threshold cycle (**Ct**) value between GAPDH on cDNA and GAPDH on RNA exceeding eight cycles. Eight cycles represents a 256-fold difference in transcript levels, but is an arbitrary cut-off point.

Data were obtained for each sample (animal) in triplicate, including raw Ct values for each amplicon (GAPDH, CTR, and 29a). Threshold cycle values are the cycle number at which the fluorescence generated within the PCR reaction crosses a defined threshold (i.e., the point at which a sufficient number of amplicons have accumulated to differ statistically from baseline levels). Values of Ct were used to compute normalized expression levels for the target genes relative to expression levels for GAPDH (the endogenous control) using the Q-Gene worksheet (Muller et al., 2002). Mean normalized expressions for 29a and CTR were computed for each individual according to Eq. 3, whereas the normalized expression for each replicate was according to Eq. 1 of Muller et al. (2002). In each case, the efficiency of the PCR reaction was accounted for in the computation of normalized expression values. It should be noted, however, that the term normalized only refers to the depiction of gene expression as a relative value, rather than any process of normalizing the resulting distributions for these data. Thus, to avoid confusion, these traits are referred to hereafter as relative expression levels for CTR and 29a (rCTR and r29a).

Data Editing and Models Used

Data analyzed were either mean expression levels or replicate values. Records from 49 individuals with evidence of genomic contamination were excluded from analyses of mean relative expression levels, leaving records of 709 animals. A proportion of these remaining animals had no PCR signal for one or more replicates of the target and/or housekeeping genes. Thus, the number of replicates contributing to the mean relative expression values varied from one to three, making direct analysis of replicate values more appealing. For the analysis of replicate values, records for replicates with genomic contamination also were deleted. Furthermore, replicates with no signal for GAPDH were deleted as they indicated poor cDNA quality because this gene should have been expressed in all animals regardless of a stress challenge (Kerr et al., 2004). Mean or replicate values with no signal for target genes were assumed to have zero expression.

The remaining data were analyzed with Proc Univariate of SAS (SAS Inst., Inc., Cary, NC) to investigate distributions for each trait, and for detecting and deleting outliers. Outliers were considered to be those records that deviated by more than three interquartile ranges (obtained from the 25th and 75th percentile values) from the mean. For r29a, the resulting ranges in edited normalized expression values were $0 \leq$ mean r29a ≤ 60 or $0 \leq$ r29a ≤ 61 for replicate samples; however, for rCTR, this criterion would have removed data from all individuals or replicates with nonzero values for gene expression, so outlier editing on this basis was pursued no further.

Data were subsequently analyzed with and without editing for outliers. Following initial analyses of data edited as above, parameters were reestimated for replicate data only after the following steps:

Log transformation of relative expression data, to improve normality of the data, followed by editing of outliers (as above) after this transformation.

Calculation of absolute and relative gene expression variables directly from threshold cycle (Ct) values, followed by log transformation and editing of outliers (as above). This computation removed Q-Gene worksheet corrections for the efficiency of amplification.

Subsetting of the data into low and high relative expression groups using the reported Ct values for target

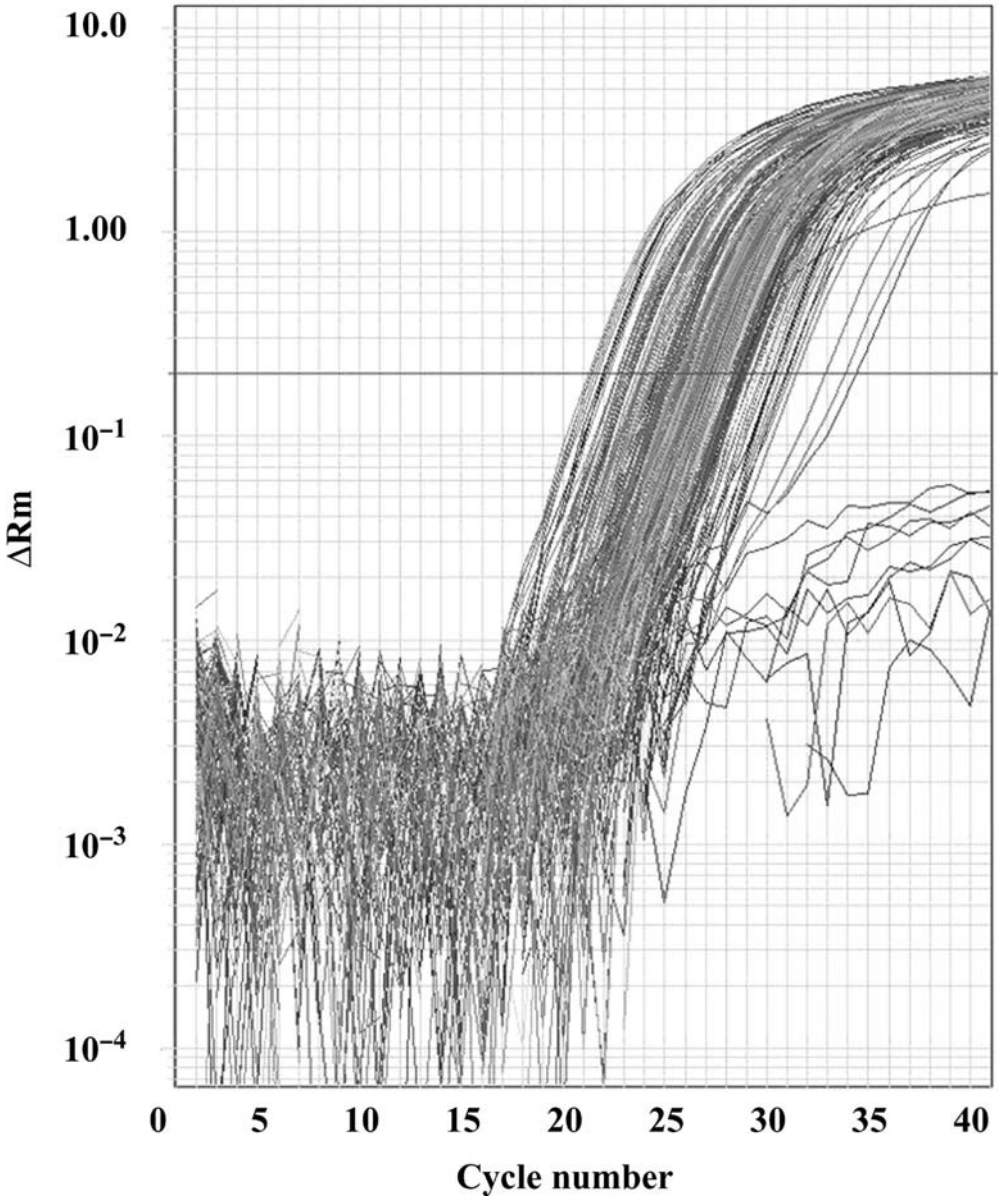

Figure 2. An example of an amplification plot for novel fragment 29a showing all samples on a representative plate in triplicate. The point at the change in amplification (ΔRm) for each reaction (plot) threshold line (the horizontal line) indicates the each plot's cycle threshold (Ct) value.

and housekeeping genes. Expression values were calculated as per point 2.

No PCR-related systematic factors were fitted in analytical models. Such factors were not present in the data provided, and accounting for them (e.g., day-to-day variation in PCR results) was considered unnecessary due to internal standardization of qRT-PCR procedures (G. S. Harper, CSIRO Livestock Industries, Brisbane, Australia, personal communication). Systematic effects that influenced the performance traits (feeder group and line) were considered for gene expression traits. Further, the effect of an animal's health status at the end of performance testing, when the blood was collected, on gene expression traits was evaluated. Approximate *F*-tests were conducted to assess the significance of these systematic effects, and only those that were significant ($P < 0.05$) were retained in models for parameter estimation.

Fixed and random effect models were developed and parameter estimates were obtained using ASREML software (Gilmour et al., 1999). Parameter estimates were obtained under an animal model, attributing each record to an individual. Additional random terms, such as common litter effects, were initially considered for mean relative expression traits; however, the data were poorly structured to estimate both additive genetic and common litter effects. Consequently, common litter effects were not included in further analyses of data repli-

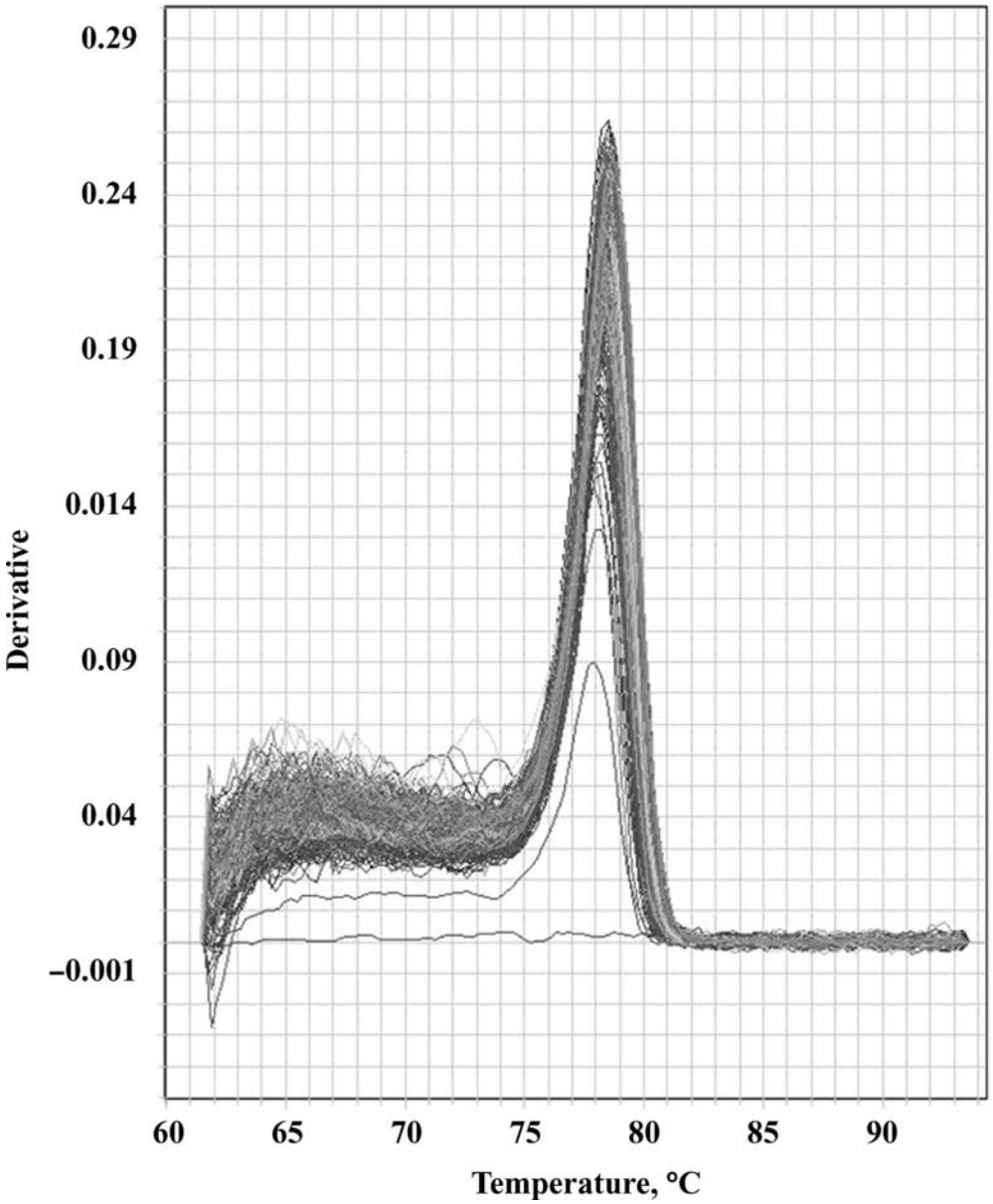

Figure 3. A dissociation curve for novel fragment 29a on a representative plate containing triplicates. All samples had similar dissociation plots, indicating the same product was produced and no primer dimers were present.

cates. The pedigree constructed for the complete data set was used in the analysis of gene expression data.

Results

Characterization of Data

After editing for genomic contamination, gene expression values for 709 animals, performance tested in 30 electronic feeder groups, were analyzed. These animals were the progeny of 28 sires and 377 dams (i.e., 709/28 = 25 progeny per sire, on average), arising from 421 litters. There were no sire-offspring combinations in which both animals had gene expression data recorded. The 3,274 animals included in the pedigree file represented pedigree predominantly from two generations only.

Mean Relative Expression Values. Characteristic values for relative expression traits (mean r29a and mean rCTR) with and without editing for outliers (mean r29a only) are presented in Table 3. The distributions of data for mean relative gene expression traits (obtained from the Q-gene worksheet) were not normally distributed. Trait distributions were characterized by extreme positive outliers (before outlier editing) and frequency peaks at the zero expression level. For example, 5% of individuals were listed with no signal for mean r29a, whereas 89% of individuals had no signal for mean rCTR. Editing for outliers in this instance achieved only marginal improvements in data distributional proper-

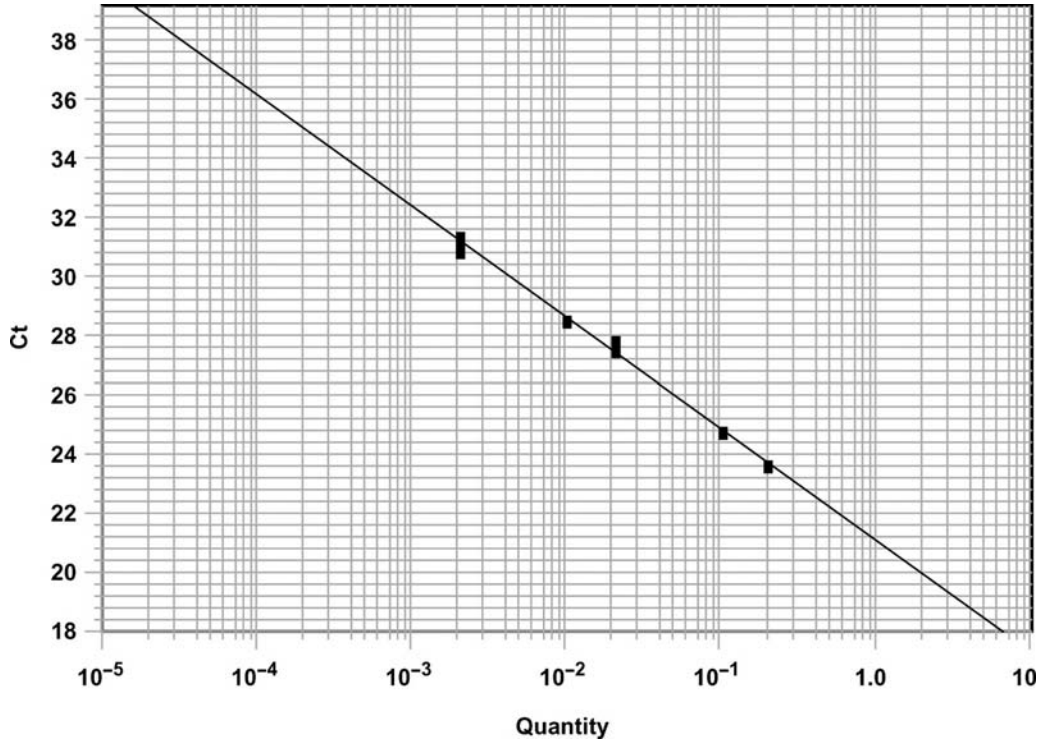

Figure 4. The standard curve for the quantitative real-time PCR using the 29a primers on a representative plate for a known cDNA diluted 1:5, 1:10, 1:50, 1:100, and 1:500. These standards were used for all plates.

ties (e.g., CV were decreased; Table 3). Mean relative expression levels for 29a and CTR were, on average, 12.9 and 2.41 times higher respectively than expression levels for GAPDH in the edited data.

Sample Replicates. Data characteristics for sample replicates also are shown in Table 3 (r29a and rCTR). As for mean values obtained from the Q-gene worksheet, relative expression values from replicate data were not normally distributed, even after deletion of replicates exhibiting genomic contamination and following deletion of outliers. Nonetheless, such editing decreased CV compared with unedited data. As expected, variability of data for replicates was larger than variability of mean values.

Expression Levels Calculated from Ct Values. Absolute gene expression data were generated directly from Ct

Table 3. Characteristics of the data for gene expression levels of the gene fragments (29a and calcitonin receptor gene [CTR])

Trait[a]	Data[b]	No.	Mean (SD)	Minimum	Maximum	CV
Mean relative expression values: Q-gene worksheet						
Mean r29a	Original	709	51.2 (305)	0	6,110	597
	Edited	661	12.9 (11.6)	0	59.4	90
Mean rCTR	Original	709	2.41 (43.1)	0	944	1,786
Relative expression values from replicates: Q-gene worksheet						
r29a	Original	2,152	54.6 (520)	0.006	18,315	952
	Edited	1,887	10.6 (11.0)	0.006	59.03	104
rCTR	Original	2,121	3.54 (71.8)	0	2,775	20,282
	Edited	1,851	0.004 (0.022)	0	0.274	550
Absolute expression levels: Calculated from Ct values (log-transformed)						
lGAPDH	Edited	2,041	18.0 (2.18)	13.2	27.4	12.1
l29a	Edited	2,006	18.2 (2.00)	13.7	27.4	11
lCTR	Edited	234	22.6 (1.96)	17.1	27.4	8.67
Relative expression levels: Calculated from Ct values (log-transformed)						
l29a-lGAPDH	Edited	1,964	0.26 (0.78)	–2.91	3.47	300
lCTR-lGAPDH	Edited	234	5.77 (3.03)	–6.65	12.5	52.5

[a]CTR = calcitonin receptor gene; 29a = a novel fragment; GAPDH = porcine glyceraldehyde-3-phosphate dehydrogenase; Ct = threshold cycle.
[b]Original = genomic contamination excluded but data not edited for outliers; Edited = outliers deleted.

Table 4. Estimates of heritabilities (h^2), common litter effects (c^2), the repeatability of replicated samples (r^2), and the phenotypic variance (σ^2p) for gene expression traits

Trait	Data[a]	No.	h^2	c^2	r^2	σ^2p
Mean relative expression values: Q-gene worksheet						
Mean r29a	Original	709	0.03 ± 0.07	0.76 ± 0.05	—	125,832
	Edited	661	0.003 ± 0.05	0.06 ± 0.07	—	93.3
Mean rCTR	Original	709	B[b]	B	—	1,862
Relative expression values from replicates: Q-gene worksheet						
r29a	Edited	1,887	0.04 ± 0.05	—	0.67 ± 0.05	69.1
	Edited	1,887	B	0.10 ± 0.05	0.62 ± 0.05	69.3
	Log-edited	2,003	B	—	0.64 ± 0.05	0.94
rCTR	Edited	1,851	0.03 ± 0.04	—	0.55 ± 0.04	0.0006
	Edited	1,851	B	0.05 ± 0.03	0.54 ± 0.04	0.0006
	Log-edited	232	B	—	0.94 ± 0.01	6.17

[a]Original = genomic contamination excluded but data not edited for outliers; Edited = outliers deleted; Log-edited = outliers detected for deletion after log transformation; CTR = calcitonin receptor gene.
[b]B = became fixed to zero boundary; — = not fitted in model for analysis.

values, ignoring the estimated differences in amplification efficiencies used by the Q-gene worksheet, followed by a log transformation (lGAPDH, lCTR and l29a). Relative expressions of the log-transformed data for CTR and 29a were then given by lCTR-lGAPDH and l29a-lGAPDH, respectively. Characteristics of these data after editing for genomic contamination and outliers also are shown in Table 3. Assuming that zero results for GAPDH are truly erroneous, GAPDH and the 29a gene fragment were expressed in all animals to some degree within edited data, whereas many animals exhibited zero expression for CTR. Relative to typical coefficients of variation for performance traits, CV were relatively low (≤12%) for log-transformed absolute expression levels (lGAPDH, lCTR, and l29a). In contrast, expression levels for the target genes expressed relative to the housekeeping gene (lCTR-lGAPDH and l29a-lGAPDH) were considerably more variable (CV ≥ 50 or 300%, respectively).

Significant Systematic Effects

Gene expression traits were significantly affected by contemporary group, which was based on entry dates to the electronic feeding system. This contemporary group definition combined animals physically grown and bled together. Thus, any systematic differences in environmental conditions and disease challenges during performance test (not deliberately imposed), along with external factors that may have influenced the quality and amount of RNA in the blood samples, were contained within this contemporary group definition. In contrast, breeding line was not significant for gene expression traits. Observable health status of the animal at the end of performance testing also was not significantly associated with gene expression values, although the majority of animals were classified as healthy.

Estimates of Genetic Parameters

Mean Relative Expression Values. Estimates of genetic parameters for mean relative gene expression traits are shown in Table 4. Heritability estimates were low and insignificant for both target genes. For r29a, estimates of common litter effects and phenotypic variances differed substantially between the original data (only records with genomic contamination deleted) and data with outliers removed (edited).

Sample Replicates. All results from analyses of replicate data are presented for data where outliers were edited (Table 4). As with results for mean values, estimates of variation in relative expression traits due to additive genetic or common litter effects were low and not significantly different from zero. Furthermore, results from mean and replicate data suggest that there is negligible genetic variation for these target genes in their relative expression levels.

In contrast, repeatabilities of replicate samples for untransformed gene expression traits were generally high (0.54 to 0.67; Table 4). Phenotypic variances were lower compared with estimates for mean relative gene expression traits (Table 4). High sampling correlations between additive genetic and common litter effects resulted in repartitioning of variation between these sources according to the analytical model, as illustrated by boundary estimate(s) when both sources of variation were included in models for analysis (Table 4).

The log transformation of relative gene expression values before editing decreased the number of records subsequently removed as outliers (e.g., n = 2,003 for log-edited vs. n = 1,887 for edited distributions; Table 4). Regardless, heritability estimates for relative expressions of target genes were not improved by a log transformation alone. The repeatability of samples from animals with nonzero rCTR values (whether untransformed or log-transformed) was considerably higher than the repeatability across all values.

The repeatability of Ct values for GAPDH, 29a, and CTR also were estimated to evaluate whether PCR data for target genes were as robust as PCR data for GAPDH. The repeatability of Ct values for GAPDH was 0.87 ± 0.05, whereas repeatability of Ct values for 29a and CTR were much less (0.65 and 0.54, respectively). This result suggests that either PCR conditions were not

Table 5. Estimates of heritabilities (h^2), the repeatability of replicated samples (r^2), and the phenotypic variance (σ^2p) for log-transformed gene expression traits[a]

Trait	No.	h^2	r^2	σ^2p
Values calculated as 2^{Ct} – log-transformed				
lGAPDH	2041	0.04 ± 0.05	0.87 ± 0.05	3.74
l29a	2006	0.03 ± 0.05	0.93 ± 0.05	2.64
lCTR	234	B[b]	0.83 ± 0.03	3.12
l29a-lGAPDH	1964	B	0.71 ± 0.02	0.52
lCTR-lGAPDH	234	B	0.87 ± 0.02	6.32
High expression target subsets ($Ct_t < Ct_h$)				
Values calculated as above (2^{Ct} – log-transformed)				
l29a	1391	0.18 ± 0.10	0.79 ± 0.10	2.47
LCTR	225	B	0.81 ± 0.04	2.89
l29a-lGAPDH	1381	0.09 ± 0.07	0.48 ± 0.07	0.22
lCTR-lGAPDH	225	B	0.87 ± 0.03	4.94
Values are log (r29a or rCTR)				
log(r29a)	1712	B	0.66 ± 0.02	0.83
log(rCTR)	206	B	0.91 ± 0.02	4.26
Low expression target subsets ($Ct_h < Ct_t$)				
Values calculated as above (2^{Ct} – log-transformed)				
l29a	730	B	0.56 ± 0.04	18.1
l29a-lGAPDH	770	B	0.70 ± 0.03	0.34
lCTR-lGAPDH	9	S[c]	S	—
Values are log (r29a or rCTR)				
log(r29a)	291	0.01 ± 0.04	0.46 ± 0.04	0.06
log(rCTR)	26	B	0.77 ± 0.04	0.85

[a]Initial expression levels calculated directly from threshold cycle values (2^{Ct} = 2 raised to the power of Ct), along with log-transformed relative expression levels for comparison. CTR = calcitonin receptor gene; 29a = a novel fragment; GAPDH = porcine glyceraldehyde-3-phosphate dehydrogenase; Ct = threshold cycle.
[b]B = became fixed to zero boundary.
[c]S = singular (no information for estimation).

fully optimized for the target genes, or that the consistency of samples for target genes was less than the consistency of samples for GAPDH, leading to greater random variation between results for replicate samples from the same animal. Differences in the accuracy of Ct values between target and housekeeping genes directly influences the accuracy of calculating relative expression levels. Low repeatabilities for target genes decrease the accuracy of comparisons between individuals, potentially affecting heritability estimates for relative expressions of the target genes.

Expression Levels Calculated from Ct Values. Computing absolute gene expression values directly from Ct values gave higher repeatability estimates for sample replicates (see lGAPDH, l29a, and lCTR; Table 5), similar repeatability estimates for the derived relative expression traits (e.g., l29a-lGAPDH, Table 5 vs. r29a, Table 4), along with similar (CTR) or lower (29a) estimates of phenotypic variances (see results for log-edited data; Table 4). These results suggest that the adjustments for differences in the efficiency of PCR reactions (via the Q-gene worksheet) increased residual variances for relative gene expression traits; however, common litter effects were not included in these models for parameter estimation, either.

The log of absolute gene expression levels for GAPDH and 29a were lowly heritable (h^2 of about 0.04), although estimates did not exceed their SE (Table 5). In contrast, heritability estimates for log CTR, or the log relative expressions of 29a and CTR, went to the zero boundary of the parameter space. This finding could suggest that the changes in amplification efficiency were important for comparing relative expression values, particularly when different PCR reactions (e.g., 29a and GAPDH) for the same samples occur on different plates, as was the case in this study. Nonetheless, these results also were entirely consistent with those presented in Table 4, where amplification efficiencies were accounted for in the calculation of relative expression values.

Subsetting the data according to whether the target gene had a higher or lower level of expression than the housekeeping gene was then performed using the relevant Ct values. In the data subset where the target gene (29a or CTR) was more highly expressed than GAPDH, a moderate estimate of heritability (0.18 ± 0.10) for the absolute (log-transformed) expression of the 29a fragment was obtained (Table 5). Furthermore, low estimates of heritability were apparent for l29a-lGAPDH (0.09 ± 0.07); however, these parameter estimates do not differ significantly from zero when the magnitude of the associated standard error is considered. In addition, phenotypic variation was decreased in the subsample relative to the complete data, although the estimate of heritability was increased. In addition, common litter effects may inflate heritability estimates for gene expression traits, if these are pres-

ent. In the low-expression target gene subset, parameter estimates remained unchanged.

Discussion

Overall, the relative expression levels for both target genes were lowly heritable, estimates of repeatability were high and phenotypic variances low. Log-transforming the data increased the repeatability of CTR. The log of absolute values for 29a was lowly heritable (0.04 ± 0.05); however, in the subset of data where the target gene was more highly expressed than the endogenous control (GAPDH), there was a moderate heritability (0.18 ± 0.10) for log-transformed 29a data. In this situation, data were limited, as indicated by large standard errors for heritability estimates. Subsequently, the estimation of covariances between the log-transformed 29a expression levels and other traits would be highly inaccurate, and estimation was not performed.

Breeding for improved immune system response and/or resistance to stress relies on identifying suitable indirect and heritable measures that are indicative of an animal's capacity to appropriately deal with environmental stressors (including disease conditions). This is necessary because deliberate disease challenges facilitating direct selection strategies are implausible for pig breeding operations. Thus, indirect selection for the expression of immune capacity is a more promising alternative. Generalized immune or stress responsiveness is typically measured through various immunological assay traits, many of which are heritable and will respond to selection (Moser et al., 2004). However, effective indirect selection using such traits depends on good information about different components of the immune system and their relationships with specific disease conditions, along with general competence and performance traits (reviewed by Knap and Bishop, 2000).

Molecular genetic approaches also have been used to select for immune and against stress traits (reviewed by Mormede et al., 2002), for example, by using the halothane-susceptibility gene (Fuji et al., 1991). Further, QTL have been identified for neuroendocrine responses to a "novel environment" stress test, but are yet to be related to performance traits (Desautes et al., 2002). In a similar manner, actual gene expression traits also could be considered as suitable candidate measurements (Gladney et al., 2004), facilitating indirect selection for immune responsiveness. However, we are unaware of any studies that establish whether the expression levels for genes regulated in response to stressors in experimental situations (e.g., CTR and 29a, above) are heritable when gene activity is measured under commercial conditions, which do not impose deliberate stress challenges.

The interactions between the physiological response pathways to stress are extensive and highly complex (for reviews, see Johnson, 1997; Blalock, 1999; Reichlin, 1999), and all systems (hypothalamic-pituitary-adrenal, adrenomedullary-sympathetic nervous and immune systems) are stimulated and act synergistically, regardless of the stress-inducing situation (Besedovsky and del Rey, 1996; Johnson, 1997). The gene fragments evaluated in this study, 29a and CTR, were identified based on this assumption. Differential display polymerase chain reaction methodology (dd-PCR) was used to identify porcine peripheral leukocyte genes that altered in expression when pigs were exposed to stressors such as psychological-social stress, high ambient temperatures, and disease challenges. For example, Kerr et al. (2004) demonstrated that *Actinobacillus pleuropneumoniae* (**App**) challenge altered the expression of the receptor for the Ca^{2+} channeling hormone calcitonin. With further investigation, it was revealed that the expression levels of the calcitonin receptor (CTR) gene reflected changes in growth performance associated with alteration in ambient temperature and App challenge, suggesting that calcitonin receptor expression represented a mechanism through which endocrine and immune systems interact to affect growth (Kerr et al., 2004). Furthermore, 29a has been shown (our unpublished observations) to be upregulated in response to respiratory disease challenge (*Mycoplasma hyopneumoniae* and *Pasteurella multocida*) in group-housed, pregnant sows, and downregulated under "ideal" housing conditions (experimental controls).

As a result of the aforementioned study, the low expression level of the calcitonin receptor gene in this study was not expected. In contrast to the experimental situation, pigs used in this study were chronically and/or variably exposed to the same stressors (e.g., disease challenges, physiological-social, high ambient temperatures, etc.) under commercial conditions. Thus, low CTR activity levels may have indicated a low degree of heat stress and/or APP challenge close to the sampling event, a differential challenge status of individuals, and/or adaptation of animals to the prevailing environment. Furthermore, the responsiveness of the target genes to stress challenges other than temperature, respiratory disease, and the mixing of unfamiliar cohorts, is unknown. Additional analyses are required to demonstrate whether there was any correlation between gene activity levels and growth performance obtained under commercial conditions.

Pigs in a commercial situation can encounter multiple concurrent stress challenges (Hyun et al., 1998b; Bornett et al., 2000; Kerr et al., 2005). Therefore, the results obtained here also could be a function of the different effects of chronic vs. acute stress challenges. A comparison between multiple concurrent stress challenges that can occur in a commercial environment and the acute challenges often applied in an experimental situation would need to be investigated to support this supposition. Furthermore, inadvertent introduction or deliberate removal of disease challenges typically occurs over time in commercial pig breeding operations. Because this research involved a single time point sampling, there seems to be no clear strategy that would

address the implications of a changing health status for recording stress resistance or immuno-competence measures on live animals where deliberate challenge procedures are not imposed. Nonetheless, it may be feasible to use other stress challenges in a commercial situation, such as the grouping of unfamiliar cohorts. These issues must be addressed when developing tools for determining stress and disease status of individual animals in a commercial situation.

A BLASTn search (i.e., matching nucleotides) on the NCBI database matched the 29a fragment sequence to expressed sequence tags (mostly porcine) of unknown function. Closer examination of the alignments has indicated a SINE pattern. If this is the case, then it is an expressed SINE. This could potentially indicate that the expression levels measured for 29a may reflect expression of a number of genes as opposed to a single gene. The concept of stress traits being under polygenic control has been previously suggested from selection studies (Mormede et al., 2002). Furthermore, it has been demonstrated in mice that mammalian SINE can behave like regulated cell stress genes and be shown to be part of a vital response to stress (Li et al., 1999). In addition to stress factors having a detrimental effect on growth, they also stimulate both the endocrine and immune systems through the release of hormones and cytokines (Besedovsky and del Rey, 1996; Johnson, 1997). Therefore, as the response to a stress challenge generally involves a complex cascade of physiological events, and SINE expression has been shown to be associated with a stress response, it is conceivable that 29a is part of a SINE associated with stress pathways; however, further work is required to support this theory.

Relative expression values indicate the multiplicative change in gene expression level relative to expression levels for the GAPDH endogenous control, or reference, gene. All things being equal, expression levels of a target gene relative to the reference gene are directly proportional to their relative amounts of mRNA (Muller et al., 2002), whereby GAPDH acts as an internal control for mRNA quantity and quality in this study. Relative expression levels for 29a were very high and were very low for CTR, on average; however, the mean relative expression level for positive CTR samples was approximately 23.7 (SD 119) confirming a high expression level relative to GAPDH when this gene was "activated." Unfortunately, this value was based on data for 116 individuals only, 39 of which had samples affected by genomic contamination. Thus, valid non-zero rCTR results were only available for 77 animals. Over all sample replicates, Ct values for CTR were not detectable for 78.1% of samples. For both CTR and 29a, very high expression levels were ultimately removed as outliers under the imposed editing procedures, but less so for log-transformed data. Transformation of the data obtained from the Q-gene worksheet (or similar software) and detection of outliers for removal was generally necessary, and should be conducted before hypothesis testing.

It is possible that more random error occurs in PCR gene expression data when expression levels are low. This phenomenon is potentially supported by the lower repeatability estimate for l29a (absolute expression level) in the low expression class, although record numbers are relatively low, giving rise to fewer replicates per animal. Errors in expression values will result in higher estimates of residual variance, and thereby lower estimates of heritability. In this scenario, using only high expression data could be expected to improve parameter estimates, as was the case in this study. That is, the expression of 29a may be lowly heritable (0.09 ± 0.07) under the prevailing collection protocol and commercial conditions. Providing the removal of low expression data does not constitute selection, and is simply a strategy to remove sample and PCR related errors, this editing strategy would seem appropriate; however, this speculation is unproven.

Alternatively, high levels of gene expression could indicate which animals were challenged by stressors close to the sampling event. This suggests that the assessment of genetic variation in gene activity levels for genes with stress related activation might be more successful if it followed a specific challenge event, as usually occurs in experimental situations. This is certainly the motivation for procedures used to select for variation in resistance to gastrointestinal worms, established through post-challenge fecal egg counts, in the Australian sheep industry (Pollott et al., 2004). However, in the commercial pig-breeding situation, use of deliberate disease challenges to generate data for selection criteria is unlikely. An alternative approach would be to apply noncontagious stressors (e.g., mixing pigs) before sampling animals for any stress related measures, although production constraints exist for this strategy as well.

The exact influence of extracting RNA from a mixed leukocyte population in whole blood compared with RNA extracted only from T-lymphocytes (as in Kerr et al., 2004) on results is unknown. Analysis of hematology data (not presented), however, showed that there was genetic variation between individuals in both their leukocyte counts and the types of leucocytes present, which potentially could have influenced results obtained here. Further analyses indicate that correlations between log 29a expression values and total leukocyte or lymphocyte counts were not significantly different from zero. Consequently, accounting for differences in counts of peripheral blood cell types on gene expression levels was not required.

It also is possible that the use of a data subsample could have affected parameter estimates for the gene expression traits. However, estimates of genetic parameters for ADG, using only data from animals included in the gene expression data subsample, were close to expectations and similar to those obtained from the complete data set. Use of a limited subsample therefore

would not seem to be an issue for the parameter estimates obtained for the gene expression traits.

A single control (GAPDH) also was used in this study. Although this gene is commonly used as a control in qRT-PCR, its expression is known to vary. Vandesompele et al. (2002) demonstrated that out of 10 such housekeeping genes, GAPDH had the fourth most stable expression for leucocytes. It is possible that expression instability of GAPDH could have affected our results; however, the feasibility of using more control genes was generally limited by efficacy vs. cost. Zero values for GAPDH in replicates were assumed to be erroneous, as they possibly indicated degraded RNA, offering a possibility for identifying errors in PCR results.

Finally, there were a number of technical and logistical challenges associated with this study. This was particularly the case for the on-farm collection, transport and/or storage procedures of samples. The procedures followed are plausible for commercial swine operations. Better procedures in this area are available, but they would come at a significantly higher cost.

The expression levels of two gene fragments (CTR and 29a) previously identified under experimental conditions (Kerr et al., 2004) to respond to acute stressors (ambient temperature changes and APP challenge), and which were related to growth performance, were variable but generally of negligible heritability when samples were collected under commercial conditions. There was, however, a suggestion that in a high-expression-level subset of the data, absolute and relative target gene expression levels were heritable. Assuming similar errors in PCR data at low and high gene expression levels, this finding implies that genetic variation for gene activity levels may only be observed in stress-challenged or otherwise high-gene activity animals. Such data would then only become available at a high cost, through deliberate challenge events or through nonuse of large volumes of low gene expression data.

Providing a disease challenge event to generate useful data is typically implausible in breeding operations. In this situation, the generation and use of gene activity data, or other physiological measures, to breed for improved stress resistance or immuno-competence is subsequently compromised. Identifying an effective, commercially acceptable, and reversible stress challenge to apply in a commercial nucleus herd situation (e.g., grouping of unfamiliar cohorts) before obtaining phenotypic measures of stress tolerance may provide a suitable alternative. Evaluation of prospective approaches to breeding for stress and/or disease tolerance must be transferable from experimental to commercial (breeding herd) situations if selection for improved performance is to be achieved.

Literature Cited

Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

Besedovsky, H. O., and A. del Rey. 1996. The cytokine-HPA axis feedback circuit. Zeitschrift Rheumatologie 59:26–30.

Black, J. L., G. T. Davies, and L. R. Bradley. 1994. Future needs in swine research. Pages 87–102 in Livestock Production for the 21st Century: Priorities and Research Needs. Wageningen Pers, Wageningen, The Netherlands.

Black, J. L., L. R. Giles, P. C. Wynn, A. G. Knowles, C. A. Kerr, M. R. Jones, A. D. Strom, N. L. Gallagher, and G. J. Eamens. 2001. A Review—Factors limiting the performance of growing pigs in commercial environments. Pages 9–36 in Manipulating Pig Production VIII, Australasian Pig Sci. Assoc., Werribee, Australia.

Blalock, J. E. 1999. Propiomelanocortin and the immune-neuroendocrine connection. Ann. N. Y. Acad. Sci. 885:161–172.

Bornett, H. L. I., C. A. Morgan, A. B. Lawrence, and J. Mann. 2000. The effect of group housing on feeding patterns and social behaviour of previously individually housed growing pigs. Appl. Anim. Behav. Sci. 70:127–141.

Collin, A., J. van Milgen, S. Dubois, and J. Noblet. 2001. Effect of high temperature on feeding behaviour and heat production in group-housed young pigs. Br. J. Nutr. 8:63–70.

de Groot, J., M. A. W. Ruis, J. W. Scholten, J. M. Koolhaas, and W. J. A. Boersma. 2001. Long-term effects of social stress on antiviral immunity in pigs. Physiol. Behav. 73:145–158.

de Haer, L. C. M., and A. G. de Vries. 1993. Feed intake in growing pigs. J. Anim. Prod. 54:95–104.

Desautes, C., J. P. Bidanelt, D. Milant, N. Iannuccelli, Y. Amigues, F. Bourgeois, J. C. Caritez, C. Renard, C. Chevalet, and P. Mormede. 2002. Genetic linkage mapping of quantitative trait loci for behavioral and neuroendocrine stress response traits in pigs. J. Anim. Sci. 80:2276–2285.

Fuji, J., K. Otsu, F. Zorzato, S. de Leon, V. K. Khama, J. E. Weiler, P. J. O'Brian, and D. H. Mac Lennan. 1991. Identification of a mutation in the porcine ryanodine receptor associated with malignant hyperthermia. Science 253:448–451.

Gilmour, A. R., B. R. Cullis, S. J. Welham, and R. Thompson. 1999. ASREML Reference Manual. Biometrics Bulletin No. 3, NSW Agric., Orange, Australia.

Gladney, C. D., G. R. Bertani, R. K. Johnson, and D. Pomp. 2004. Evaluation of gene expression in pigs selected for enhanced reproduction using differential display PCR and human microarrays: I. Ovarian follicles. J. Anim. Sci. 82:17–31.

Gonyou, H. W., and W. R. Stricklin. 1998. Effects of floor area allowance and group size on the productivity of growing/finishing pigs. J. Anim. Sci. 76:1326–1330.

Heetkamp, M. J. W., J. W. Schrama, L. de Jong, J. W. G. M. Swinkels, W. C. P. Schouten, and M. W. Bosch. 1995. Energy metabolism in young pigs as affected by mixing. J. Anim. Sci. 73:3562–3569.

Hyun, Y., M. Ellis, and R. W. Johnson. 1998a. Effects of feeder type, space allowance, and mixing on the growth performance and feed intake pattern of growing pigs. J. Anim. Sci. 76:2771–2778.

Hyun, J., M. Ellis, G. Riskowski, and R. W. Johnson. 1998b. Growth performance of pigs subjected to multiple concurrent environmental stressors. J. Anim. Sci 76:721–727.

Johnson, C. A. 1997. Inhibition of growth by pro-inflammatory cytokines: An integrated view. J. Anim. Sci. 75:1244–1255.

Kerr, C. A., G. J. Eamens, J. Briegal, P. A. Sheehy, L. R. Giles, and M. R. Jones. 2003. Effects of combined *Actinobacillus pleuropneumoniae* challenge and change in environmental temperature on production, plasma insulin-like growth factor I (IGF-1) and cortisol parameters in growing pigs. Aust. J. Agric. Res. 10:1057–1064.

Kerr, C. A., L. R. Giles, M. R. Jones, and A. Reverter. 2005. Effects of grouping unfamiliar cohorts, high ambient temperature and stocking density on live performance of growing pigs. J. Anim. Sci. 83:908–915.

Kerr, C. A., K. O. Mathews, L. R. Giles, and M. R. Jones. 2004. Effects of combined *Actinobacillus pleuropneumoniae* challenge and change in environmental temperature on calcitonin receptor

expression levels in growing pigs. Aust. J. Agric. Res. 55: 727–732.

Knap, P. W., and S. C. Bishop. 2000. Relationships between genetic change and infectious disease in domestic livestock. BSAS Occasional Publ. 27:65–80.

Li, T. H., J. Spearow, C. M. Rubin, and C. W. Schmid. 1999. Physiological stresses increase mouse short interspersed element (SINE) RNA expression in vivo. Gene 239:367–372.

Mormede, P., H. Courvoisier, A. Ramos, N. Marissal-Arvy, O. Ousova, C. Desautes, M. Duclos, F. Chaouloff, and M. P. Moisan. 2002. Molecular genetic approaches to investigate individual variations in behavioral and neuroendocrine stress responses. Psychoneuroendocrinology 27:563–583.

Moser, R. J., A. Reverter, C. A. Kerr, K. J. Beh, and S. A. Lehnert. 2004. A mixed-model approach for the analysis of cDNA microarray gene expression data from extreme-performing pigs after infection with *Actinobacillus pleuropneumoniae*. J. Anim. Sci. 82:1261–1271.

Muller, P. Y., H. Janovjak, A. R. Miserez, and Z. Dobble. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32:1372–1379.

Pollott, G. E., L. J. E. Karlsson, S. Eady, and J. C. Greeff. 2004. Genetic parameters for indicators of host resistance to parasites from weaning to hogget age in Merino sheep. J. Anim. Sci. 82:2852–2864.

Quiniou, N., S. Dubois, and J. Noblet. 2000. Voluntary feed intake and feeding behaviour of group-housed growing pigs are affected by ambient temperature and body weight. Livest. Prod. Sci. 63:245–253.

Reichlin, S. 1999. Neuroendocrinology of infection and the innate immune system. Recent Prog. Horm. Res. 54:133–181.

Rozen, S., and H. J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. Pages 365–386 in Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ.

Vandesompele, J., K. de Preter, F. Pattyn, B. Poppe, N. van Roy, A. de Paepe, and F. Speleman. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 3:0034.1–0034.11.

Wallgren P., T. Segall, A. Morner, and P. Gunnarsson. 1999. Experimental infections with *Actinobacillus pleuropneumoniae* in pigs — I. Comparison of five different parenteral antibiotic treatments. J. Vet. Med. Series B Infect. Dis. Vet. Publ. Health 46:249–260.

First published in the *Journal of Animal Science*, volume 83, issue 8 (2005).
Published by the American Society of Animal Science.