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ORIGINAL ARTICLE

Single nucleotide polymorphisms associated with thermoregulation in lactating dairy cows exposed to heat stress

S. Dikmen[1], X.-z. Wang[2], M.S. Ortega[3], J.B. Cole[4], D.J. Null[4] & P.J. Hansen[3]

1 Department of Animal Science, Faculty of Veterinary Medicine, University of Uludag, Bursa, Turkey
2 College of Animal Science and Technology, Southwest University, Chongqing, China
3 Department of Animal Sciences, University of Florida, Gainesville, FL, USA
4 Animal Genomics and Improvement Laboratory, Agricultural Research Service, USDA, Beltsville, MD, USA

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Correspondence
P. J. Hansen, Department of Animal Sciences, University of Florida, PO Box 110910, Gainesville, FL 32611-0910, USA.
Tel: +1 352 392 5590;
Fax: +1 352 392 5595;
E-mail: hansen@animal.ufl.edu

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Summary

Dairy cows with increased rectal temperature experience lower milk yield and fertility. Rectal temperature during heat stress is heritable, so genetic selection for body temperature regulation could reduce effects of heat stress on production. One aim of the study was to validate the relationship between genotype and heat tolerance for single nucleotide polymorphisms (SNPs) previously associated with resistance to heat stress. A second aim was to identify new SNPs associated with heat stress resistance. Thermotolerance was assessed in lactating Holsteins during the summer by measuring rectal temperature (a direct measurement of body temperature regulation; $n = 435$), respiration rate (an indirect measurement of body temperature regulation, $n = 450$) and sweating rate (the major evaporative cooling mechanism in cattle, $n = 455$). The association between genotype and thermotolerance was evaluated for 19 SNPs previously associated with rectal temperature from a genomewide analysis study (GWAS), four SNPs previously associated with change in milk yield during heat stress from GWAS, 2 candidate gene SNPs previously associated with rectal temperature and respiration rate during heat stress (*ATPA1A* and *HSP70A*) and 66 SNPs in genes previously shown to be associated with reproduction, production or health traits in Holsteins. For SNPs previously associated with heat tolerance, regions of BTA4, BTA6 and BTA24 were associated with rectal temperature; regions of BTA6 and BTA24 were associated with respiration rate; and regions of BTA5, BTA26 and BTA29 were associated with sweating rate. New SNPs were identified for rectal temperature ($n = 12$), respiration rate ($n = 8$) and sweating rate ($n = 3$) from among those previously associated with production, reproduction or health traits. The SNP that explained the most variation were *PGR* and *ASL* for rectal temperature, *ACAT2* and *HSD17B7* for respiration rate, and *ARL6IP1* and *SERPINE2* for sweating rate. *ARL6IP1* was associated with all three thermotolerance traits. In conclusion, specific genetic markers responsible for genetic variation in thermoregulation during heat stress in Holsteins were identified. These markers may prove useful in genetic selection for heat tolerance in Holstein cattle.

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Introduction

Heat stress can compromise physiology and production of domestic animals because of the physiological adaptations employed to prevent hyperthermia [e.g. reduced feed intake (Rhoads *et al.* 2009)] and the adverse consequences of failure to regulate body temperature [e.g. inhibition of embryonic development at elevated temperatures (Sakatani *et al.* 2012)]. In dairy cattle, heat stress can reduce milk yield (Keister *et al.* 2002), depress fertility (Flamenbaum & Galon 2010) and disrupt immune function (Elvinger *et al.* 1992). As shown for milk yield (Ingraham *et al.* 1979) and fertility (Gwazdauskas *et al.* 1973, the magnitude of heat stress effects on cow function is related to the magnitude of the increase in rectal temperature induced by heat stress. Rectal temperature during heat stress is heritable in dairy cattle, with an estimate of 0.17 (Dikmen *et al.* 2012), so genetic selection for thermoregulation should reduce effects of heat stress on production.

There is a negative genetic correlation between heat tolerance and milk yield in dairy cattle (Dikmen *et al.* 2012). Identification of genetic markers for heat tolerance that are not related to milk yield would allow selection for heat tolerance independent of selection against milk yield. Genetic markers for heat tolerance have been identified using a genomewide association study (GWAS) for milk yield responses to heat stress (Hayes *et al.* 2009) and rectal temperature during heat stress (Dikmen *et al.* 2013). In addition, single nucleotide polymorphisms (SNPs) associated with rectal temperature and respiration rate during heat stress have been identified in several candidate genes including *ATPA1A* (Liu *et al.* 2011), heat-shock protein 70 (*HSP70A*) (Deb *et al.* 2013), *HSP90AB1* (Charoensook *et al.* 2012) and *PPARA* (Fang *et al.* 2014).

There were two goals of this study. The first was to validate the relationship between genotype and heat tolerance for SNPs previously identified as being associated with resistance to heat stress. This task was undertaken because genetic markers in one study are often not predictive in other studies (Ioannidis *et al.* 2011). The SNPs evaluated included several identified by GWAS that were associated with milk yield responses to heat stress in Holstein and Jerseys (Hayes *et al.* 2009) and to rectal temperature during heat stress in Holsteins (Dikmen *et al.* 2013). Candidate gene SNPs evaluated were for *ATPA1A*, associated with rectal temperature, respiration rate and milk yield during summer in Chinese Holsteins (Liu *et al.* 2011) and *HSP70A*, associated with rectal temperature, respiration rate and milk yield during hot weather in Frieswal cows (Deb *et al.* 2013). The second goal was to identify new SNPs related to thermotolerance. The SNPs screened were those that had previously been associated with various production, reproduction and health traits in Holsteins (Cochran *et al.* 2013a,b).

Materials and methods

Animals and housing

Animal use was approved by the University of Florida Institutional Animal Care and Use Committee (Approval No. 2012-03578). The study was conducted with a total of 625 lactating Holstein cows at the University of Florida Dairy Unit (Hague, Florida; 29°46′ N and 82°24′ W). Cows were housed in sand-bedded freestall barns equipped with sprinklers (Rain Bird Manufacturing, Glendale, CA, USA) and fans (J&D Manufacturing, Eau Claire, WI, USA) that were programmed to become activated when dry-bulb temperature exceeded 21.1°C. Sprinklers were placed above the feed bunk and were also arranged around the fan so that water was directed into the airstream. When activated, fans and sprinklers operated continuously and sprinklers were activated for 1.5 min at 6-min intervals. Feed was provided twice daily and water was available *ad libitum*. Cows were milked twice daily between 0800–1000 h and 2100–2300 h.

Traits used to characterize thermotolerance

Thermotolerance was assessed during the hottest part of the day in summer by measuring three traits related to regulation of body temperature during heat stress. Rectal temperature is a direct measurement of a cow's ability to prevent hyperthermia during heat stress. Respiration rate, which increases during heat stress as a physiological mechanism to increase evaporative cooling (McLean 1963), also indicates a cow's ability for thermoregulation. Increased respiration rate is associated with reduced ability to prevent hyperthermia (Da Silva *et al.* 2015). Sweating rate was measured as another physiological adaptation to reduce heat stress through increased evaporative heat loss. More metabolic heat is lost by sweating at high air temperatures than by respiration (McLean 1963; Da Silva *et al.* 2015). Increased capacity for cutaneous evaporation has been associated with decreased rectal temperature during heat stress (Da Silva *et al.* 2015).

Data collection

The experiment was conducted during June–August in 2013 and 2014. On each day, a population of approximately 50 cows was used to measure rectal temperature, respiration rate, sweating rate, parity, milk yield and days in milk. Measurements of rectal temperature, respiration rate and sweating rate were obtained between 1400 and 1600 h while cows were resting in a freestall. Rectal temperatures were recorded using a digital GLA M750 thermometer (GLA Agricultural Electronics, San Luis Obispo, CA, USA). Sweating rate was measured on the right side of the rump with a Vapometer device (Delphin Tech. Ltd., Kuopio, Finland). Respiration rate was determined by visual observations of flank movements for 1 min. Milk yield was recorded twice daily using Afi-Lab real-time milk analyzer (Afimilk, Kibbutz Afikim, Israel).

Measurements of dry-bulb temperature, relative humidity, dew point temperature and black globe temperature were measured at 1-min interval between 1400 and 1600 h using a HOBO-V2 (black globe temperature) or U12 data logger (other variables) (Onset Company, Bourne, MA, USA) that was located at a height of 3 m from the ground at a position in the centre of the barn where cows were housed. Rectal temperature was matched with the environmental measurements taken at the closest minute at which environmental variables were recorded. Data on rectal temperature, respiration rate, sweating rate and milk yield were matched with data on parity and days in milk at the day of measurement.

Cows were subjected to measurement of rectal temperature, respiration and sweating rate, a variable number of times, from 1 to 11 per cow. Overall, rectal temperatures were recorded a total of 2157 times on 623 cows, respiration rates were recorded 2162 times on 625 cows, and sweating rate was recorded 2143 times on 622 cows.

Genotyping

Samples of whole blood collected from coccygeal vessels were used to determine genotype for a total of 19 SNPs previously associated with rectal temperature from a GWAS study (Dikmen *et al.* 2013), four SNPs previously associated with change in milk yield during heat stress from GWAS (Hayes *et al.* 2009), 2 candidate gene SNPs previously associated with rectal temperature and respiration rate during heat stress (*ATPA1A* and *HSP70AA*) (Liu *et al.* 2011; Deb *et al.* 2013) and 66 SNPs in genes that were previously associated with reproduction, production or health traits in Holsteins (Cochran *et al.* 2013a,b). SNPs previously associated with reproduction, production or health traits were examined for opportunistic reasons because most of the study herd had been genotyped for these SNPs. Identification and minor allele frequency of each SNP are listed in Tables S1 and S2 (Supporting information).

DNA extraction and genotyping was performed by Neogen (Lincoln, NE, USA). Genotyping was performed using the Sequenom MassARRAY® system (iPLEX GOLD; Sequenom, San Diego, CA, USA) according to the manufacturer's instructions. The technique is based on the analysis of DNA products using matrix-assisted laser desorption–ionization time-of-flight mass spectrometry (Abel *et al.* 2006). The region of DNA containing the SNP was amplified by PCR, a primer extension reaction was performed to generate allele-specific DNA products, and the size and amount of each allele-specific product was determined using chip-based mass spectrometry. The average call rate was 96.3%. A random sample of 10 SNPs was genotyped in duplicate for each animal, and the agreement between duplicates was 97.0%. Animals in which the call rate was <70% ($n = 1$) were removed from the analysis. Five animals were genotyped twice – agreement between genotypes was 98.5%. When the genotype did not match between duplicated samples, both observations were deleted and treated as no call.

Statistical analysis

Statistical analysis was performed using software packages of the Statistical Analysis System (SAS, version 9.4; SAS Institute, Inc., Cary, NC, USA). An initial analysis was performed with Proc GLM to identify the dry-bulb temperature classes associated with increased rectal temperature, respiration rate and sweating rate. Dry-bulb temperatures were classified as less than 28.0°C, 28.0–28.9°C, 29.0–29.9°C, 30.0–30.9°C, 31.0–31.9°C, 32.0–32.9°C, 33.0–33.9°C, 34.0–34.9°C and ≥35.0°C, as illustrated in Figure 1. The model included fixed effects of dry-bulb temperature class and cow. Subsequently, analyses of SNP effects for rectal temperature, respiration rate and sweating rate were conducted using data collected on days where environmental temperature exceeded that causing a deviation in thermal regulation. Thus, analysis of SNPs associated with rectal temperature was based on measurements when the dry-bulb temperature was ≥31°C, that is, when cows were exposed to heat stress sufficient to induce hyperthermia. The SNP

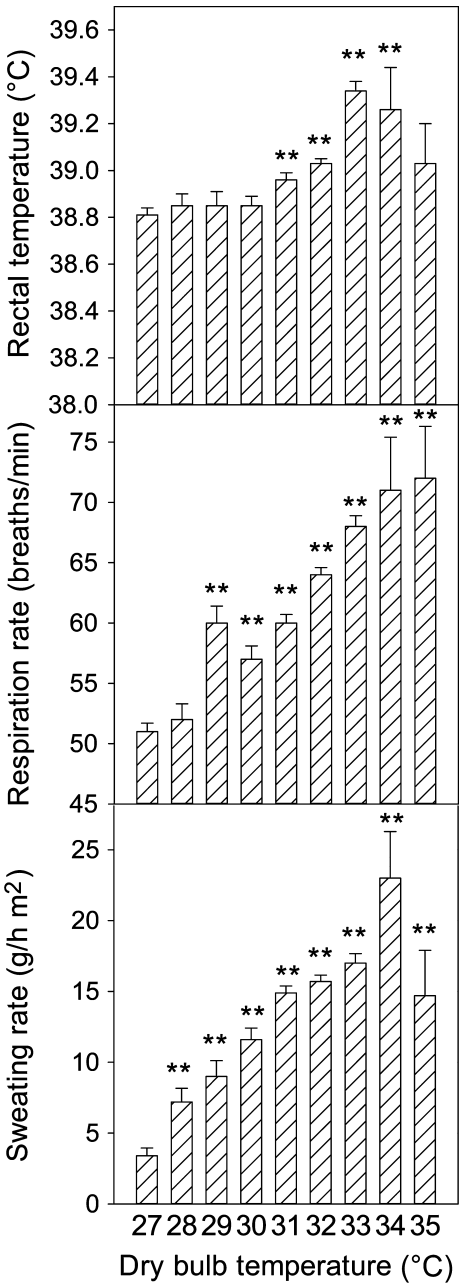

Figure 1 Relationship between dry-bulb temperature and physiological variables. Dry-bulb temperatures at the time of measurement were classified as being <28°C (27), 28–28.99°C (28), 29–29.99°C (29), 30–30.99°C (30), 31–31.99°C (31), 32–32.99°C (32), 33–33.99°C (33), 34–34.99°C (34) or ≥35°C. Data are least-squares means ± SEM. Means that differ from 27°C are indicated by asterisks (*, $p < 0.05$ **; $p < 0.01$).

effects on respiration rate involved measurements at ≥29°C, and SNP effects on sweating rate involved measurements at ≥28°C. Data were analysed for 435 cows (rectal temperature), 450 cows (respiration rate) and 455 cows (sweating rate). Summary statistics for cows used in genetic analyses for each trait are presented in Table 1.

Data analysed for SNP effects were the least-squares means of values for individual cows after adjusting values for dry-bulb temperature at the time of measurement. Initial analyses also included effect of parity (primiparous versus multiparous) and continuous effect of days in milk and milk yield on the day of body temperature measurement. These effects were not significant for any traits and were not included in the analysis to determine the least-squares means of individual cows.

The association of genetic variants for each SNP was estimated individually using the Mixed procedure of SAS. Genotype was considered a categorical variable, and the analysis was weighted for the number of observations per cow using the weight option of Proc Mixed. The full model was as follows:

$$Y_{ij} = \mu + a_i + g_j + e_{ij},$$

where Y_i is the least-squares mean of the trait of interest for the ith cow ($i = 1, 2, \ldots, n$), a_i is the random polygenic effect of the ith cow, g_j is the fixed effect of SNP genotype j, and e_{ij} is the random residual effect. The $a_i \sim A\sigma_a^2$ and $e_{ij} \sim W\sigma_e^2$, where A is the numerator relationship matrix, W is an diagonal matrix of weights, σ_a^2 is the additive genetic variance of the trait of interest, and σ_e^2 is the residual error variance. The elements of W are proportional to the number of observations for each cow in the dataset. All of the available pedigree information for each cow was used when modelling the covariance among the polygenic effects.

The additive effect at locus j was estimated as $(g_{j3}–g_{j1})/2$ and the dominance effect at locus j was estimated as $g_{j2} - (g_{j3} + g_{j1})/2$ where g_{j2} refers to the heterogygous genotype. The SNPs in which the additive or dominance effect was significant ($p < 0.05$) were noted.

Distributions of genotypes of SNPs were tested for deviation from Hardy–Weinberg equilibrium using a chi-square test and the ALLELE procedure of SAS. Pearson correlation coefficients between rectal temperature, respiration rate and sweating rate were calculated for all observations at dry-bulb temperatures ≥28°C using the CORR procedure of SAS.

Results

Environmental effects on thermoregulation

Rectal temperature, respiration rate and sweating rate were affected by dry-bulb temperature at the time of

Table 1 Summary statistics for cows used in genetic analyses[a]

Trait under analysis	Variable	Number of cows	Mean	SD	Minimum–maximum
Rectal temperature	Measurements per cow	435[b]	2.7	1.4	1–10
Rectal temperature	Rectal temperature, °C	435	39.03	0.54	37.8–41.4
	Adjusted rectal temperature, °C	435	39.08	0.44	38.0–41.3
	Milk yield, kg/day	400	33.8	8.8	4.1–62.7
	Days in milk	434	214.1	19.4	10–547
	Parity	434	2.0	1.1	1–6
	Dry-bulb temperature, °C	435	32.29	0.76	31.1–38.4
Respiration rate	Measurements per cow	450[b]	3.1	1.5	1–11
	Respiration rate, breaths per minute	450	57.4	13.2	30–114
	Adjusted respiration rate, breaths per minute	450	61.8	10.9	38–96
	Milk yield, kg/day	413	33.9	8.8	4–62.7
	Days in milk	449	214.0	106.4	10–547
	Parity	449	2.1	1.2	1–6
	Dry-bulb temperature, °C	450	31.99	1.05	29.1–38.4
Sweating rate	Measurements per cow	455[b]	3.4	1.6	1–11
	Sweating rate, g/h m^2	455	15.79	11.32	0.1–66.2
	Adjusted sweating rate, g/h m^2	455	15.09	7.71	–4.3–45.0
	Milk yield, kg/day	418	33.9	8.8	4.1–62.7
	Days in milk	454	213.3	106.0	10–547
	Parity	454	2.0	1.2	1–6
	Dry-bulb temperature, °C	455	31.70	1.34	28.1–38.4

[a]Data represent statistics regarding the mean and variation of values calculated on a per cow basis (i.e. the average of multiple measurements per cow) or, for the case of adjusted measurements, the mean and variation of values of cow least-squares means after adjusting for dry-bulb temperature on the day of measurement.
[b]Cows were sired by 94 sires.

measurement (Figure 1). As compared to cows in cool conditions (dry-bulb temperature < 28°C), rectal temperature was increased when dry-bulb temperature at the time of measurement was ≥31°C. Thermoregulatory responses were increased at lower dry-bulb temperatures than for rectal temperature. Sweating rate increased at dry-bulb temperatures ≥28°C and respiration rate increased at dry-bulb temperatures ≥ 29°C.

Further analyses on physiological and genetic effects on rectal temperature and other traits associated with thermotolerance were performed using data at dry-bulb temperatures above those causing a deviation in the trait. After screening data according to this criterion, neither rectal temperature, respiration rate nor sweating rate was affected by parity, milk yield or days in milk on the day of measurement.

Among cows at dry-bulb temperatures ≥28°C, there were significant Pearson correlations among physiological traits. Cows with higher rectal temperature had increased respiration rate ($r = 0.53$, $p < 0.001$). There were also positive but smaller relationships between sweating rate and rectal temperature ($r = 0.10$; $p < 0.001$) and sweating rate and respiration rate ($r = 0.06$; $p = 0.009$).

Effects of SNPs previously associated with rectal temperature using GWAS

Genotype effects on physiological variables related to heat tolerance were determined for 19 SNP previously associated with genetic variation in rectal temperature during heat stress (Dikmen *et al.* 2013). Of these, four SNPs were significantly associated with rectal temperature (Table 2). Two of the significant SNPs, Hapmap30420-BTC-039335 and Hapmap47403-BTA-76048, are located close to each other on BTA6. For both SNPs, there were significant additive and dominance effects. Cows that were homozygous for the minor allele had lower rectal temperature than the homozygotes for the major allele or the heterozygotes. The latter two groups had similar rectal temperatures. In the previous experiment by Dikmen *et al.* (2013), the minor allele was associated with lower rectal temperature for one SNP (Hapmap30420-BTC-039335) but higher rectal temperature for the other (Hapmap47403-BTA-76048). There was also a dominance effect for the other two SNP associated with rectal temperature. For ARS-BFGL-NGS-458 on BTA4, heterozygotes had higher rectal temperature than either homozygote. In the experiment by Dikmen *et al.* (2013), the minor allele was associated

Table 2 Significant relationships between genotype and physiological characteristics for SNPs previously associated with thermotolerance using GWAS[a]

SNP	Chr.	Location (bp)	*n*	Number of copies of minor allele[b]			p Value		r^2
				0	1	2	Additive	Dominance	
Rectal temperature (°C)									
ARS-BFGL-NGS-458	4	64 351 574	422	39.06 ± 0.048	39.17 ± 0.040	39.02 ± 0.054	0.6262	0.0064	2.4
Hapmap30420-BTC-039335	6	45 175 137	434	39.14 ± 0.045	39.12 ± 0.037	38.87 ± 0.060	0.0001	0.0239	5.0
Hapmap47403-BTA-76048	6	45 153 190	435	39.14 ± 0.044	39.11 ± 0.038	38.86 ± 0.061	0.0001	0.0276	4.4
Hapmap58887-rs29013502	24	28 907 154	434	39.20 ± 0.044	39.03 ± 0.038	39.13 ± 0.056	0.2964	0.0021	2.1
Respiration rate (breaths per minute)									
Hapmap47403-BTA-76048	6	45 153 190	450	63.0 ± 1.00	61.0 ± 0.88	59.6 ± 1.41	0.0423	0.8065	−1.2
Hapmap58887-rs29013502	24	28 907 154	449	62.3 ± 1.00	60.8 ± 0.90	64.1 ± 1.30	0.2207	0.0254	−0.1
Sweating rate (g/h m^2)									
ARS-BFGL-NGS-10307	26	20 259 486	454	15.6 ± 0.62	15.8 ± 0.70	8.4 ± 2.20	0.0014	0.0023	0.7
BFGL-NGS-30169	29	48 329 079	455	13.9 ± 0.59	16.3 ± 0.76	19.0 ± 1.82	0.0062	0.9236	−0.6
BTB-1267098	5	89 545 151	455	14.0 ± 0.69	15.1 ± 0.66	16.7 ± 1.18	0.0303	0.7557	−2.4
Hapmap47861-BTA-120563	5	89 472 174	455	13.9 ± 0.70	15.2 ± 0.66	16.5 ± 1.07	0.0237	0.9728	0.3

SNPs, single nucleotide polymorphisms; Chr., chromosome; GWAS, genomewide analysis study.

[a]Among cows exposed to dry-bulb temperatures ≥31°C (rectal temperature), ≥29°C (respiration rate) and ≥28°C (sweating rate).

[b]Values are least-squares means ± SEM.

with lower rectal temperature. For Hapmap58887-rs29013502 on BTA24, cows that were heterozygous had lower rectal temperature than cows that were homozygous for either allele (dominance effect, p = 0.0021). In the previous study, the minor allele was associated with lower rectal temperature (Dikmen *et al.* 2013).

Several SNPs previously associated with genetic variation in rectal temperature during heat stress (Dikmen *et al.* 2013) were significantly associated with respiration rate (n = 2) and sweating rate (n = 3) (Table 2). For both of the two SNP associated with respiration rate, the genotypes associated with low respiration rate were also associated with low rectal temperature. The proportion of variance in respiration rate explained by the SNPs was very low, however (i.e. estimates of r^2 were negative). None of the three SNPs associated with sweating rate were also associated with rectal temperature or respiration rate. Two of the SNPs were located close to each other on BTA5. The other, ARS-BFGL-NGS-10307, was on BTA26.

Examination of SNPs previously associated with milk yield differences caused by heat stress

In a GWAS, Hayes *et al.* (2009) identified four SNPs associated with the heat stress-induced change in milk yield of a bull's daughters: ARS-BFGL-NGS-139, ARS-BFGL-BAC-38208, ARS-BFGL-NGS-89500 and BFGL-NGS-30169. In the current experiment, each of these four SNPs was examined for relationship to rectal temperature, respiration rate and sweating rate. There were no significant effects of ARS-BFGL-NGS-139, ARS-BFGL-BAC-38208 or ARS-BFGL-NGS-89500 on any trait. There was, however, a significant effect of BFGL-NGS-30169 on sweating rate (Table 2). The minor allele was associated with an increase in sweating rate (additive effect, p = 0.0062).

Candidate gene SNPs previously associated with thermotolerance

Two candidate gene SNPs associated with heat tolerance were also evaluated for association with rectal temperature, respiration rate and sweating rate. One of these is a deletion in the AP box of the promoter region of *HSP70A* (Rosenkrans *et al.* 2010) that has been associated with superior thermoregulation (rectal temperature and respiration rate) in Frieswal cattle (Deb *et al.* 2013). The other is a C/A silent mutation in the coding region of *ATPA1A* where the A allele was associated with higher rectal temperature in Chinese Holsteins (Liu *et al.* 2011). In contrast to these studies, there was no association between either SNP and rectal temperature, respiration rate or sweating rate (Table 3).

SNPs previously associated with reproduction, production or health traits

Relationships between thermotolerance traits and 66 SNPs previously associated with reproduction,

Table 3 Relationship between genotype and physiological variables associated with regulation of body temperature during heat stress for candidate gene SNPs previously associated with thermotolerance[a]

Gene	Chr.	Location (bp)	Variable	*n*	Number of copies of minor allele[b]			p Value	
					0	1	2	Additive	Dominance
HSP70A (HSP70C895D)[c]	23		Rectal temperature	387	39.11 ± 0.042	39.10 ± 0.044	38.98 ± 0.080	0.1036	0.2623
			Respiration rate	402	62.6 ± 0.94	61.9 ± 1.00	59.2 ± 1.94	0.1008	0.4444
			Sweating rate	406	15.0 ± 0.65	15.6 ± 0.69	15.3 ± 1.29	0.7903	0.6018
ATPA1A (ss159831435)[d]	3	27 007 790	Rectal temperature	435	39.08 ± 0.035	39.10 ± 0.049	38.77 ± 0.208	0.1491	0.1237
			Respiration rate	450	61.3 ± 0.76	61.8 ± 1.17	62.4 ± 4.43	0.8066	0.9892
			Sweating rate	455	15.2 ± 0.57	14.2 ± 0.83	11.0 ± 3.08	0.1806	0.5325

SNPs, single nucleotide polymorphisms; Chr., chromosome.

[a]Values represent analyses of measurements taken at dry-bulb temperatures ≥31°C (rectal temperature), ≥29°C (respiration rate) and ≥28°C (sweating rate).

[b]Values are least-squares means ± SEM. Units are °C (rectal temperature), breaths per minute (respiration rate) and g/h m^2 (sweating rate).

[c]Rosenkrans *et al.* (2010).

[d]Liu *et al.* (2011).

production or health traits in Holsteins (Cochran *et al.* 2013a,b) were determined. There were 12 SNPs significantly associated with rectal temperature, eight SNPs significantly associated with respiration rate and three SNPs significantly associated with sweating rate (Table 4). All significant SNPs were in Hardy–Weinberg equilibrium except for *DEPDC7* ($p = 0.009$). In this case, the frequency of cows that were homozygous for the minor allele (6.8%) was higher than expected (4.6%) and the frequency of heterozygotes was lower than expected (29.4 versus 33.8%). In no case was a significant SNP located physically close to significant SNP from previous GWAS studies.

The SNPs that explained the most variation were *PGR* ($r^2 = 3.8\%$), *ASL* ($r^2 = 3.7\%$) and *CAST* ($r^2 = 3.2\%$) for rectal temperature; *ACAT2* ($r^2 = 7.4\%$) for respiration rate; and *ARL6IP1* ($r^2 = 4.8\%$) and *SERPINE2* ($r^2 = 3.0\%$) for sweating rate. The other SNPs explained a smaller proportion of variance ($r^2 < 3.0\%$).

One SNP, *ARL6IP1*, was associated with all three thermotolerance traits. The allele substitution effect was additive for rectal temperature and sweating rate (lowest rectal temperature and highest sweating rate for the homozygote of the minor allele) but was characterized by dominance for respiration rate (lowest respiration rate was for the heterozygote).

Discussion

Results indicate specific genetic markers responsible for genetic variation in thermoregulation during heat stress in Holsteins. This is so despite the fact that only a small proportion of the SNPs identified earlier as associated with resistance to heat stress in GWAS experiments (Hayes *et al.* 2009; Dikmen *et al.* 2013) was associated with physiological determinants of heat tolerance in the present experiment. In part, lack of repeatability could reflect the poor reliability of many GWAS studies (Ioannidis *et al.* 2011) and the relatively small sample sizes of the present study and that of Dikmen *et al.* (2013). In addition, the heritability of rectal temperature is not large (Dikmen *et al.* 2012) so most phenotypic variation in the study was the result of non-genetic causes. Nonetheless, six possible loci associated with thermoregulation were identified from the group of markers previously associated with thermoregulation by GWAS and a total of 20 other SNPs were identified that had significant effects on one or more traits associated with thermotolerance.

Of the markers associated with genetic control of rectal temperature in the study of Dikmen *et al.* (2013), there were five possible loci associated with thermotolerance in the present study. These included a SNP in BTA4 associated with rectal temperature (ARS-BFGL-NGS-458), two closely located SNP in BTA5 associated with sweating rate (BTB-1267098 and Hapmap47861-BTA-120563), two closely related SNP in BTA6 associated with rectal temperature (Hapmap30420-BTC-039335 and Hapmap47403-BTA-76048) and respiration rate (Hapmap47403-BTA-76048), a SNP on BTA24 (Hapmap58887-rs29013502) associated with rectal temperature and respiration rate and a SNP on BTA26 (ARS-BFGL-NGS-10307) associated with sweating rate.

In a GWAS, Hayes *et al.* (2009) identified SNPs associated with the heat stress-induced change in

Table 4 Relationship between genotype and physiological traits associated with thermotolerance for SNPs located in coding regions of genes previously associated with reproduction, health or production traits[a]

SNP	Chr.	Location (bp)	*n*	Number of copies of minor allele[b]			p Value		r^2
				0	1	2	Additive	Dominance	
Rectal temperature (°C)									
ACAT2 (rs109967779)	9	97 478 396	362	39.00 ± 0.051	39.12 ± 0.044	39.17 ± 0.061	0.0160	0.3887	−0.4
ARL6IP1 (rs110541595)	25	16 544 291	341	39.20 ± 0.058	39.07 ± 0.045	39.02 ± 0.066	0.0285	0.4690	2.3
ASL (rs110127056)	25	28 248 091	360	39.11 ± 0.050	39.06 ± 0.042	39.27 ± 0.068	0.0475	0.0107	3.7
BDH2 (rs133674837)	6	23 051 485	362	39.06 ± 0.047	39.08 ± 0.045	39.22 ± 0.066	0.0214	0.2437	0.3
CAST (rs137601357)	7	98 485 273	358	39.06 ± 0.051	39.19 ± 0.044	38.97 ± 0.067	0.2545	0.0018	3.2
DEPDC7 (rs110270752)	15	64 476 283	359	39.12 ± 0.041	38.97 ± 0.053	39.29 ± 0.097	0.0924	0.0004	−1.9
EPAS1 (rs43676052)	11	28 650 973	342	39.05 ± 0.043	39.14 ± 0.049	38.92 ± 0.109	0.2375	0.0238	0.6
FGF2[c]	17	35 247 491	362	39.00 ± 0.048	39.12 ± 0.044	39.24 ± 0.067	0.0014	0.8823	0.9
GCNT3 (rs109830880)	10	50 709 147	362	39.10 ± 0.039	39.11 ± 0.052	38.39 ± 0.200	0.0005	0.0013	−0.3
GOLGA4 (rs42339105)	22	10 887 536	357	39.13 ± 0.037	38.94 ± 0.071	39.66 ± 0.340	0.1207	0.0140	1.6
NLRP9 (rs109383758)	18	62 241 722	362	39.12 ± 0.055	39.04 ± 0.044	39.16 ± 0.057	0.6128	0.0448	−0.1
PGR (rs109506766)	15	8 158 458	361	39.00 ± 0.045	39.17 ± 0.043	39.17 ± 0.079	0.0365	0.1388	3.8
Respiration rate (breaths per minute)									
ACAT2 (rs109967779)	9	97 478 396	377	59.3 ± 1.07	63.9 ± 0.94	64.2 ± 1.31	0.0016	0.0607	7.4
*ARL6IP1 (*rs110541595)	25	16 544 291	355	62.9 ± 1.25	60.9 ± 0.95	64.2 ± 1.45	0.4689	0.0282	0.6
CACNA1D (rs135744058)	22	47 726 446	373	61.6 ± 0.88	63.6 ± 0.98	58.3 ± 2.42	0.1842	0.0139	1.1
DYRK3 (rs109561866)	16	4 284 409	371	61.9 ± 0.82	62.3 ± 1.35	45.1 ± 8.43	0.0480	0.0472	0.5
FYB (rs109262355)	20	35 249 040	373	59.7 ± 1.08	62.6 ± 0.96	63.7 ± 1.50	0.0239	0.4625	1.1
HSD17B7 (rs110828053)	3	6 630 548	373	62.7 ± 0.86	60.8 ± 1.12	66.0 ± 2.67	0.2257	0.0358	1.3
LDB3 (rs111015912)	28	41 679 976	373	61.2 ± 0.87	61.9 ± 1.11	66.8 ± 2.59	0.0349	0.2151	−0.2
TSHB (rs132789482)	3	28 420 362	342	61.2 ± 0.89	63.2 ± 1.17	71.3 ± 4.67	0.0323	0.2318	1.1
Sweating rate g/h m^2									
ARL6IP1 (rs110541595)	25	16 544 291	358	14.1 ± 0.82	15.6 ± 0.65	18.1 ± 0.99	0.0006	0.4955	4.8
SERPINE2 (rs43321188)	2	112 900 094	380	15.3 ± 0.63	15.0 ± 0.72	19.4 ± 1.64	0.0166	0.0327	3.0
SLC18A2 (rs110365063)	26	37 898 131	372	16.5 ± 0.59	14.1 ± 0.89	6.7 ± 3.67	0.008	0.2177	−0.8

SNPs, single nucleotide polymorphisms; Chr., chromosome.

[a]Among cows exposed to dry-bulb temperatures ≥31°C (rectal temperature), ≥29°C (respiration rate) and ≥28°C (sweating rate).

[b]Least-squares means ± SEM (°C).

[c]FGF11646 (Wang *et al.* 2008).

milk yield of a bull's daughters. A total of four SNPs were identified in a discovery population of 62 343 Holsteins sired by 798 genotyped bulls: ARS-BFGL-NGS-139, ARS-BFGL-BAC-38208, ARS-BFGL-NGS-89500 and BFGL-NGS-30169. One of these, BFGL-NGS-30169, was subsequently found to explain variation in heat tolerance in two separate populations (one Holstein and one Jersey). Of these four SNPs, one (BFGL-NGS-30169 on BTA29) was associated with sweating rate in the present study. In the study of Dikmen *et al.* (2013), BFGL-NGS-30169 was not one of the 30 most explanatory SNP for rectal temperature but genetic merit for low rectal temperature was associated with the minor allele at this locus. Moreover, a nearby SNP (RS-BFGL-NGS-107395) was one of the 30 most explanatory SNP for rectal temperature. The fact that BFGL-NGS-30169 has repeatedly been shown to be associated with heat tolerance adds confidence that the relationship is a real one. Perhaps, the physiology behind the genetic variation in thermotolerance at this locus relates to differences in capacity for thermal sweating. At high air temperatures, sweating is the largest source of heat transfer from a cow to its environment (McLean 1963).

BFGL-NGS-30169 was identified by Hayes *et al.* (2009) as close to *FGF4*. This growth factor could play a role in thermotolerance because it has been implicated in protection of cells from elevated temperature (Hirai *et al.* 2004). However, the SNP is actually located in *SHANK2*, a gene involved in adherens junction formation (Du *et al.* 1998), and very close to another adherens junction gene (*CTTN;* Han *et al.* 2014). Adherens junctions are formed on myoepithelial cells lining apocrine sweat glands in cattle (Uematsu *et al.* 2005).

Neither of the two candidate genes previously found to be associated with rectal temperature and

respiration rate during heat stress, *ATPA1A* (Liu *et al.* 2011) and *HSP70A* (Deb *et al.* 2013), had significant associations with traits related to thermoregulation in the current study. Both of the previous experiments involved small numbers of animals of different genetic backgrounds than the cows used here, and for *HSP70A*, not all genotypes were assessed in the earlier study (there were no cows homozygous for the minor allele).

Other SNPs were identified in specific genes that explained a significant amount of variation in rectal temperature, respiration rate and sweating rate. These SNPs had previously been associated with one or more production, reproduction or health traits in Holsteins (Cochran *et al.* 2013a). It will be important to validate these SNPs using other populations of cows. Nonetheless, it is likely that several contribute to genetic variation in thermotolerance. Most of these SNPs cause an amino acid change in the coding region of the gene (Cochran *et al.* 2013a). Moreover, several of the genes in which the SNPs are located exert functions that are potentially important for physiological regulation of body temperature during heat stress. *PGR* was the SNP that explained the largest proportion of variation in rectal temperature and is one of the few SNP from the Cochran *et al.* (2013a) study that is located in an intron. *PGR* encodes for the progesterone receptor that mediates actions of progesterone on blood flow to the skin during local heating of the skin (Brunt *et al.* 2011). Another SNP explaining a large proportion of variation in rectal temperature was in *CAST*. This gene has been associated with genetic variation in feed efficiency in cattle (Karisa *et al.* 2013) and might affect thermoregulation through changes in efficiency of energy utilization and heat production. *SERPINE2,* which explained 3.0% of the variation in sweating rate, encodes for a proteinase inhibitor that inhibits thrombin and urokinase-type plasminogen activator. Thrombin can induce changes in epithelial cells from human eccrine sweat glands (Bovell *et al.* 2009). The one SNP associated with all three measures of thermotolerance was *ARL6IP1*. This gene encodes for a transmembrane protein in the endoplasmic reticulum that functions to block apoptosis (Lui *et al.* 2003). Additional experimentation could resolve how mutations in these and other genes affect physiological mechanisms important for body temperature regulation during heat stress.

Upper critical temperature is defined as the environmental temperature at which a homoeotherm can no longer regulate body temperature under hot conditions. Using this definition, the upper critical temperature was approximately 31°C. This value is similar to an earlier estimate of 29.7°C for lactating dairy cows managed in similar housing in Florida (Dikmen & Hansen 2009). In contrast, upper critical temperature for lactating dairy cows in Israel was estimated at 25–26°C (Berman *et al.* 1985). The higher estimate of upper critical temperature for cows in Florida may represent adaptation to heat stress or better housing that minimized effective heat stress. Respiration rate first increased at dry-bulb temperatures of approximately 29°C and sweating rate at temperatures of approximately 28°C. Thus, the rise in rectal temperature due to heat stress occurs after activation of physiological mechanisms to cool cows become inadequate to prevent hyperthermia. The fact that sweating increased at lower dry-bulb temperatures than respiration rate is consistent with sweating being a more important mode of evaporative heat loss in cattle than respiration (McLean 1963; Da Silva *et al.* 2015).

Heat stress is one of the most deleterious environmental factors affecting dairy production because it can reduce milk yield (Keister *et al.* 2002), depress fertility (Flamenbaum & Galon 2010) and disrupt immune function (Elvinger *et al.* 1992). Selection for cattle that are genetically resistance to heat stress is complicated by the negative genetic correlation between heat tolerance and milk yield (Dikmen *et al.* 2012). Identification of genetic markers could allow selection for heat tolerance independent of selection against milk yield provided the markers are not negatively associated with milk yield. The markers identified here may prove useful in executing this strategy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Allele frequency of SNPs previously related to thermoregulation in cattle.

Table S2. Allele frequency of SNPs in candidate genes previously related to production, reproduction and health traits in cattle.