

BREEDING AND GENETICS SYMPOSIUM: Breeding for resilience to heat stress effects in dairy ruminants. A comprehensive review¹

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ABSTRACT: Selection for heat tolerant (HT) animals in dairy production has been so far linked to estimation of declines in production using milk recording and meteorological information on the day of control using reaction norms. Results from these models show that there is a reasonable amount of genetic variability in the individual response to high heat loads, which makes feasible selection of HT animals at low costs. However, the antagonistic relationship between level of production and response to heat stress (HS) implies that selection for HT animals under this approach must be done with caution so that productivity is not damaged. Decomposition of the genetic variability in principal components (PC) can provide selection criteria independent of milk production level although biological interpretation of PC is difficult. Moreover, given that response to heat stress for each animal is estimated with very sparse information collected under different physiological and management circumstances, biased (normally underestimation) and lack of accuracy may be expected. Alternative phenotypic characterization of HT can come from the use of physiological traits, which have also shown moderate heritability. However,

costs of a large scale implementation based on physiological characteristics has precluded its use. Another alternative is the use of biomarkers that define heat tolerance. A review of biomarkers of HS from more recent studies is provided. Of particular interest are milk biomarkers, which together with infrared spectra prediction equations can provide useful tools for genetic selection. In the ‘omics’ era, genomics, transcriptomics, proteomics and metabolomics have been already used to detect genes affecting HT. A review of findings in these areas is also provided. Except for the slick hair gene, there are no other genes for which variants have been clearly associated with HT. However, integration of omics information could help in pointing at knots of the HS control network and, in the end, to a panel of markers to be used in the selection of HT animals. Overall, HT is a complex phenomenon that requires integration of fine phenotypes and omics information to provide accurate tools for selection without damaging productivity. Technological developments to make on-farm implementation feasible and with greater insight into the key biomarkers and genes involved in HT are needed.

Key words: dairy ruminants, genetic selection, heat stress

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INTRODUCTION

It is widely accepted that heat stress (**HS**) has detrimental effects on livestock production. Changes in climatic conditions are expected to worsen this problem in the next decades (IPCC, 2014). Dairy animals are more susceptible to HS because milk production results in a large metabolic heat strain. As a consequence, selection for increased milk production will tend to make animals more susceptible to increasing heat loads. Thus, selection for highly-productive and heat tolerant (**HT**) dairy animals is a major challenge.

The effect of high heat loads on dairy animals is a complex phenomenon (Collier et al., 2008, Baumgard et al., 2016). Consequences of the exposure to high temperatures are observed at the intracellular, metabolic, hormonal and physiological levels with visible effects on decreasing feed intake, increased body temperature, and respiration and heart rates, decreased production and reproduction and altered immunological traits (Silanikove, 2000; West, 2003). Thus, identifying criteria that define heat tolerance and quantifying individual levels for the chosen trait to establish a selection program is not an easy task. A HT animal would be that which is able to maintain homeostasis under high heat loads, what is translated into maintenance or little damage of productivity. However, homeostasis depends on many aspects, such as ability to protect the cells from heat damage, regulate internal heat production by modulating metabolism, and dissipate internal heat to the environment through sweating and panting, so that body temperature stays below critical temperatures. In this paper, results concerning selection for heat tolerance are reviewed and discussed. More precisely, up to date findings aiming at characterizing HT animals from a phenotypic and a genetic perspective to develop breeding tools oriented toward selection for heat tolerance without damaging productivity are considered.

QUANTIFICATION AND SELECTION OF HEAT TOLERANCE FROM MILK PRODUCTION OR REPRODUCTIVE PERFORMANCE AND METEOROLOGICAL INFORMATION

The first and most followed approach to selection for HT animals was suggested by Misztal (1999) based on the estimation of the individual productive response to increasing heat loads (norm of reaction to changes in the environmental temperature). This approach has the advantage of relying on easily-accessible production or reproduction records (available from current selection schemes) and meteorological information from weather stations close to the farms (available from meteorological agencies). Its implementation within current selection

schemes in dairy populations would thus be straightforward and would require no additional costs. Review and discussion of results concerning the implementation of the reaction norm models to select HT animals follow.

Reaction Norm Models

Reaction norm models describe the pattern of phenotypic expression of a genotype, for example, individual milk (or milk components) production across a range of environmental conditions, in our case, a range of heat loads. Heat load is measured in most studies as a combination of the ambient temperature and relative humidity, using the so called temperature and humidity index (**THI**). The weights on temperature and relative humidity are calculated to reflect the comfort of the animal. In cattle, the most common THI index is:

$$\text{THI} = (1.8 \times T_{\text{db}} + 32) - (0.55 - 0.0055 \times \text{RH}) \times (1.8 \times T_{\text{db}} - 26) \text{ (National Research Council, 1971),}$$

where T_{db} = dry bulb temperature ($^{\circ}\text{C}$) and RH = relative humidity of the air (%).

Alternative indices for dairy cattle are compared in Bohmanova et al. (2007) and Hammami et al. (2013). For sheep, the same index, expressed in the Celsius degrees scale, has been frequently used:

$$\text{THI} = T_{\text{db}} - (0.55 - 0.0055 \times \text{RH}) \times (T_{\text{db}} - 14.4) \text{ (Finocchiaro et al., 2005).}$$

Using the THI as a continuous measure of environmental conditions, Misztal (1999) proposed a norm of reaction model for milk production traits showing a comfort zone with no effect of temperature on production (thermoneutral region) followed by a linear decay in production. This model, which can be named as broken line (**BL**) model, is based on 2 parameters that can be used to quantify individual heat tolerance: the thermotolerance threshold (the environmental heat load after which production starts to decay) and the subsequent slope of decay. Both parameters are indicators of tolerance and have a direct biological interpretation. A HT animal will have a high threshold and a small slope of decay. However, estimation of individual thresholds for heat stress is cumbersome, providing slow mixing and convergence rates of the estimation algorithms (Sánchez et al., 2009). Thus, in most applications, a common threshold is assumed for all animals and only slopes of decays after the given threshold are used to quantify HT. Because thresholds are likely to vary across animals, this assumption may cause bias in the estimated slopes of decay. The BL model relies on other strong assumptions, such as that production remains constant at temperatures before the

threshold and starts to decay abruptly afterward, that the decay is then linear, and, in its simplest version, that all animals share the same comfort threshold. Overall, the BL model, which is theoretically attractive and follows the models previously used to describe the physiological response to temperatures (e.g., Kadzere et al., 2002), may be a too-constrained model to describe the expected smoothness and graduality of a biological process.

Other functions such as orthogonal polynomials, have been used in animal breeding to describe norms of reaction for different traits. Orthogonal polynomial bases can be used to approximate complex patterns. However, the use of polynomials also has disadvantages when fitting biological processes, such as not having asymptotes, which leads to unrealistic fittings in the extremes of the X-axis scale when information is scarce for these extreme values, and their ‘wiggly’ nature. Brügemann et al. (2011) and Carabaño et al. (2014) have used cubic Legendre polynomials (LP) to describe the effect of heat load on individual production using random regression models. Individual random regression parameters (intercept, linear, quadratic, and cubic coefficients) can be used to assess HT but these parameters lack a per se biological interpretation, except for the intercept. Nevertheless, functions of them can be used to quantify individual HT in several ways, for example by calculating the slope of decay at a given high heat load, providing the same type of HT criterion as the BL model. Moreover, by making use of the ideas of Kirkpatrick et al. (1990), the decomposition of covariance functions that describe changes of a trait (e.g., milk production) across an environmental (e.g., heat load) scale into orthogonal components can provide selection criteria to change the trajectory of the trait without changing its level. In other words, it provides a theoretical framework to find selection criteria that improve HT with no effect on production level. Thus, overall, polynomials can provide similar selection criteria as BL models and additional information about components of response to environmental temperature independent from production level. However, care should be taken when fitting high-degree polynomials and little information is available for uncommonly high heat loads in the case of HS. Fig. 1 illustrates estimation of breeding values across a heat load scale from both types of reaction norms (BL and LP).

Most of the applications of reaction norm models to produce breeding values for heat tolerance in dairy production have been performed in highly-selected international dairy cattle breeds, which lack adaptation to high thermal loads (Ravagnolo and Misztal, 2002; Bohmanova et al., 2005; Aguilar et al., 2009; Sánchez et al., 2009; Brügemann et al., 2011; Bernabucci et al., 2014; Carabaño et al., 2014), but only a few in local breeds of small dairy ruminants that are not in-

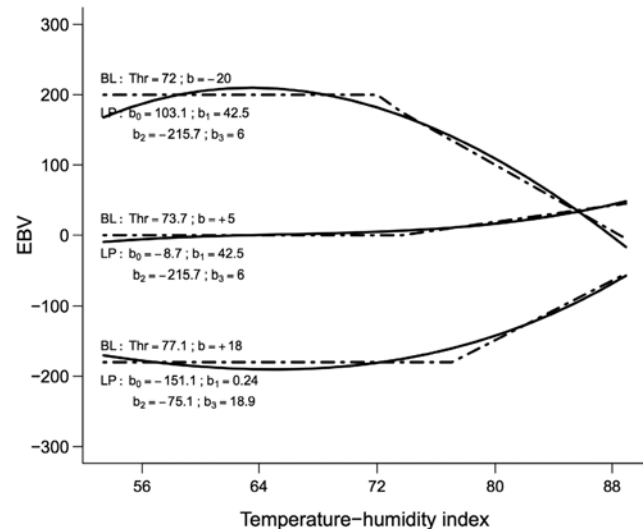


Figure 1. Individual genetic responses to HS obtained from a broken-line (BL; solid line) and a Legendre Polynomial (LP; dot-dashed line) reaction norm approaches. For each approach, parameters obtained by the model are presented (threshold of HT and slope of decay for BL and intercept (b_0), linear (b_1), quadratic (b_2) and cubic (b_3) coefficients for LP).

tensively selected and are expectedly better adapted to the environment (Finocchiaro et al., 2005; Menéndez-Buxadera et al., 2012). Comparison across populations with differing production environments and selection programs can shed light in the characterization of the genetics of HT.

Thresholds for Heat Tolerance and Production Loss under Heat Stress

Application of reaction norms at the population level provides estimates of heat tolerance thresholds and of the slopes of decay after the threshold. For dairy cattle, heat stress thresholds have been estimated for a THI over 69 to 72 for milk traits (Ravagnolo et al., 2000; Freitas et al., 2006; Bohmanova et al., 2007; Sánchez et al., 2009) and over 70 for reproductive traits (Ravagnolo and Misztal, 2002). However, estimates of comfort thresholds can vary across climatic regions because of the different effect of summer heat waves followed by milder temperatures in more temperate climates or steadily hot summer season in warm regions. As an example, the upper limit of the comfort region for production traits was around 6 to 7 units of THI greater in the warm Mediterranean regions of Spain than in the more temperate climate of Belgium-Luxembourg (Carabaño et al., 2016a). Milk production level can also condition the threshold for heat tolerance. Zimelman et al. (2009) re-evaluated the validity of the THI 72 threshold and established a more realistic value of 68 for the increasingly-productive Holstein cow. For dairy sheep, a decay on milk production has been reported for average test-day temperatures above 20 to 24°C (Peana et al., 2007; Ramón et al., 2016), over 30 to

35°C of maximum temperature (Caroprese et al., 2011; Ramón et al., 2016) and over a daily THI of 18 to 23 (Finocchiaro et al., 2005; Ramón et al., 2016), with some differences among breeds and production environments. The decay in milk production above those thresholds has been reported of -0.15 and up to -0.6 kg per unit of THI in dairy cattle (Bohmanova et al., 2007; Sánchez et al., 2009). For dairy sheep, production losses from 3 and up to 9 g/unit of THI for milk yield, and from 0.4 and up to 8.6 g per THI unit for fat and protein yields were reported (Finocchiaro et al., 2005; Ramón et al., 2016). Finally, these studies have also addressed the estimation of annual production losses derived from heat load. In dairy cattle, Bohmanova et al. (2007) reported annual milk yield losses of 1 to 2%, and Hammami et al. (2013), in a study conducted in Holsteins under a continental climate in Luxembourg, reported maximum production decays due to heat load of 0.6, 0.9, and 0.7% of total annual production for milk, fat, and protein, respectively. In dairy sheep, Ramón et al. (2016) reported annual production losses up to 2.4% in Manchega sheep; Finocchiaro et al. (2005) reported losses from 4.2 to 4.9% in the Valle del Belice breed; and Peana et al. (2007) reported a decay of milk production from 15% and up to 20% due to high heat loads in the Sarda sheep.

Genetic Variability for Heat Tolerance and Genotype by Environment Interaction

The use of reaction norm models at the individual level has allowed quantifying genetic variability for heat tolerance, measured as the slope of the production response curve at high heat loads. Variability in the genetic component of productive or reproductive response to increasing temperatures has been found in a number of studies in cattle [Ravagnolo and Misztal (2002), Bohmanova et al. (2005), Aguilar et al. (2009), and Sánchez et al. (2009) for production traits in US Holstein; Boonkum and Duangjinda (2015) in Thai cattle; Brügemann et al. (2011) in German Holstein; Bohlouli et al. (2013) in Iranian Holstein; Bernabucci et al. (2014) in Italian Holstein; Carabaño et al. (2014) in Spanish Holstein; Hammami et al. (2015) for production traits and fatty acids in Belgian Holstein] and a few studies involving small ruminants (Finocchiaro et al., 2005; Menéndez-Buxadera et al., 2012; Carabaño et al., 2015) or from the reproductive performance under HS in Holstein cattle (Ravagnolo and Misztal, 2002; Oseni et al., 2004; Brügemann et al., 2013) and in Holstein crosses (Boonkum et al., 2011). Thus, there is a genetic background that determines the differences in reaction to HS in productive and reproductive traits and the possibility of selecting animals that show more steady pro-

duction and good reproductive performance under high heat loads.

Associated with genetic differences in heat tolerance (defined as the slope of the decay in production under HS) is the non-unity genetic correlation between performance under cold or under comfort conditions and performance under high heat loads. This is a form of genotype-by-environment interaction ($G \times E$) implying that animals that perform best under cold or under thermal neutrality may not be best under HS. A review of estimates in the literature can be found in Table 1. Estimated genetic correlations from the studies using the BL model approach were obtained from the estimated genetic (co) variances for intercept and slope and the corresponding matrix of THI covariates. Estimated genetic correlations vary across traits, lactations, breeds, climates and statistical models. Overall, $G \times E$ (genetic correlations < 0.8 are considered to indicate $G \times E$) was observed in all species, except for reports by Brügemann et al. (2012) in German Holstein, where the THI threshold for HT was 60, and by Finocchiaro et al. (2005) for the Valle de Belice sheep. In cattle, $G \times E$ seems to increase from first to third parity, while no evident pattern was observed for sheep and goats across lactations. The increasing production level for multiparous cows vs. primiparous might explain the greater $G \times E$ with parity number in cattle. Differences in the reaction norm model used had also an effect on the estimated genetic correlations of production across temperatures. Estimated correlations between comfort and HS were greater when quadratic polynomials were used compared with estimates under cubic polynomials or BL models. The $G \times E$ was more severe when colder temperatures were considered, showing negative correlations in the case of one local breed of goat in Spain (Carabaño et al., 2015). Thus, selecting for HT may have no impact or decrease cold tolerance because both processes seem to be regulated by different genes or genetic mechanisms.

Another important result of reaction norm models is the estimated genetic correlations between the general level of production and the slope of decay under heat stress. A summary of results found in the literature is shown in Table 2. In all studies, estimates were negative, ranging from -0.3 for fat yield in second and third lactation in the US Holstein study (Aguilar et al., 2009) to -0.8 for the Valle de Belice sheep breed in Italy (Finocchiaro et al., 2005). These estimates would allow quantification of the expected loss in HT under the current selection for improved yield. Figure 2 shows the estimated trend for the general level of production and HT measured by the slopes of decay in the BL model for three dairy ruminant populations in Spain. These trends were obtained by from data described in Carabaño et al. (2016b) using a BL reaction norm. The estimated genetic progress in productive traits is accompanied by a detrimental effect on

Table 1. Estimated genetic correlations between production levels under comfort or cold loads and production level under heat stress using Broken line or Legendre polynomial reaction norms in different studies. Measures of heat load are in parentheses

Breed	Heat load		Milk yield			Protein yield			Fat yield		
			Parity 1	Parity 2	Parity 3	Parity 1	Parity 2	Parity 3	Parity 1	Parity 2	Parity 3
	Comfort	HS ¹	Correlation between comfort and heat stress from BL ² models								
US Holstein ³ (THI _{max}) ⁴	72	84	0.65	0.59	0.43	0.73	0.63	0.52	0.74	0.67	0.56
Italian Holstein ⁵ (THI _{max})	Milk/Fat/Protein 4/72/71	84	0.75	0.75	0.64	0.89	0.69	0.52	0.86	0.63	0.73
Spanish Holstein ⁶ (T _{avg})	Milk/Fat/Protein 29/15/18	33	0.98	–	–	0.66	–	–	0.66	–	–
Valle de Belice sheep ⁷ (THI _{max})	23	30	0.90			0.89					
	Comfort or cold	HS	Correlation between comfort or cold and heat stress from LP ² models								
German Holstein ⁸ (THI _{avg}) ⁴ LP3 ²	54	72	–	–	–	0.93	–	–	–	–	–
	10	33	0.89/0.86	–	–	0.83/0.73	–	–	0.85/0.75	–	–
Spanish Holstein ⁶ LP2/LP3 (T _{avg})	0	33	0.82/0.82	–	–	0.74/0.41	–	–	0.81/0.40	–	–
	15	29	0.70	0.77	0.58	0.73	0.62	0.72	0.46	0.74	0.80
Manchega sheep ⁹ (THI _{avg}) LP3	6	29	0.37	0.26	0.25	0.42	0.56	0.36	0.43	0.46	0.35
	15	30	0.80	0.82	0.71	0.85	0.72	0.71	0.76	0.73	0.71
Florida goats ⁹ (THI _{avg}) LP3	6	30	-0.21	0.29	0.26	-0.10	-0.02	0.03	-0.40	0.18	0.09

¹HS = Heat stress.

²BL = Broken line; LP = Legendre polynomial; LP2/LP3 = Quadratic/Cubic LP.

³From Aguilar et al. (2009).

⁴T_{avg} = Daily average temperature; THI_{avg}/THI_{max} = Daily average/maximum temperature and humidity index.

⁵From Bernabucci et al. (2014)

⁶From Carabaño et al. (2014).

⁷From Finocchiaro et al. (2005).

⁸From Brügemann et al. (2011).

⁹From Carabaño et al. (2015).

heat tolerance, not only in the highly-selected Holstein population but also in the local breeds of small dairy ruminants for which the selection intensity is much lower than in the Holstein. This implies that even though these breeds are selected in their productive environment, increasing deterioration of heat tolerance is expected as the production level increases.

Selection Criteria under Reaction Norm Models

Two selection criteria, the tolerance threshold (animals with higher thresholds would be desirable) and the slope of decay after the threshold, can be used under the BL models. Estimation of the genetic value for the tolerance threshold for each animal is not easy because of the difficulty in finding the breaking point given the relatively scarce and noisy information available for each animal (an animal would normally have 10 to 14

Table 2. Review of estimated genetic correlations between intercept (general level of production) and slope of decay after the heat tolerance threshold from Broken Line reaction norms in different studies

Breed	Milk yield			Protein yield			Fat yield		
	Parity 1	Parity 2	Parity 3	Parity 1	Parity 2	Parity 3	Parity 1	Parity 2	Parity 3
US Holstein ¹	-0.46	-0.38	-0.47	-0.43	-0.36	-0.48	-0.39	-0.29	-0.30
Italian Holstein ²	-0.51	-0.33	-0.36	-0.49	-0.53	-0.48	-0.31	-0.52	-0.56
Spanish Holstein ³	-0.34	–	–	-0.31	–	–	-0.31	–	–
Manchega sheep ⁴		-0.45			-0.77			-0.77	
Valle de Belice sheep ⁵		-0.81					-0.83		
Payoya goats ⁶		-0.36					-0.37		

¹From Aguilar et al. (2009).

²From Bernabucci et al. (2014).

³From Carabaño et al. (2014).

⁴From Ramón, M. (CERSYRA-IRIAF, Valdepeñas, Spain, personal communication).

⁵From Finocchiaro et al. (2005).

⁶From Menéndez-Buxadera et al. (2012).

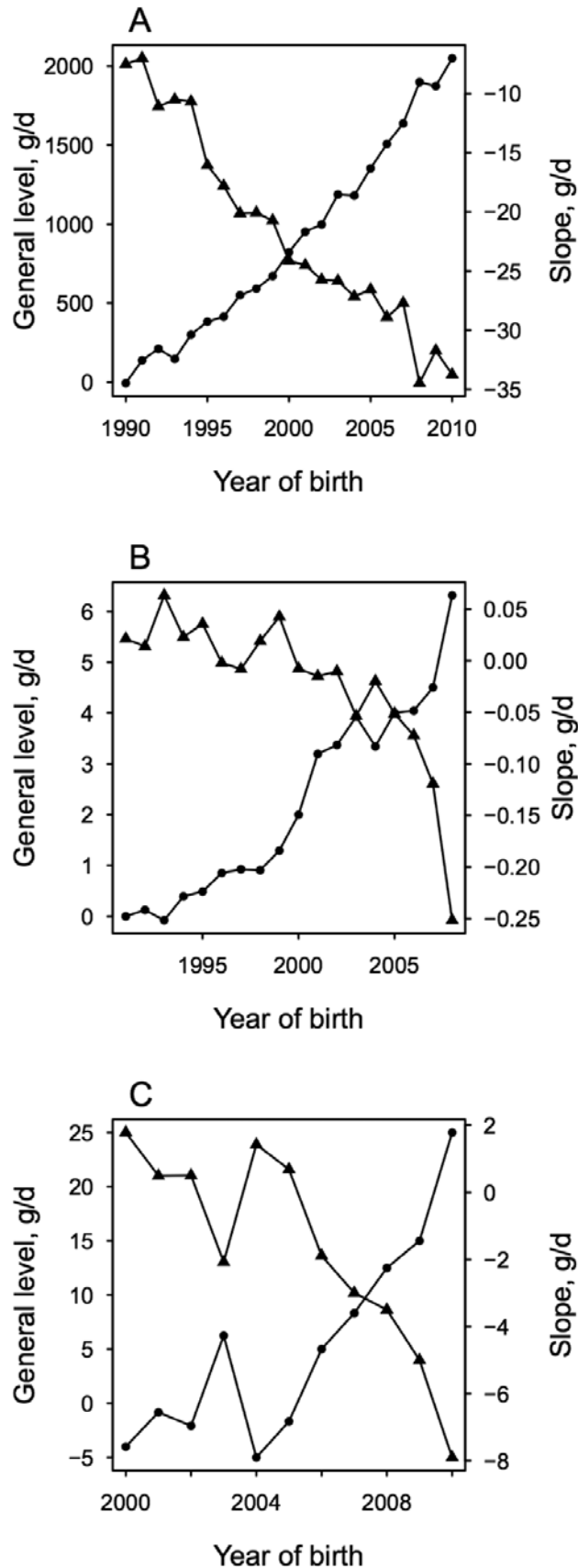


Figure 2. Genetic trends for the level of production (line with circles) and slope of decay under HT (line with triangles) for milk yield in Holstein cattle (Panel A), fat plus protein yield in Manchega sheep (Panel B) and fat plus protein percent in Florida goat (Panel B) breeds.

milk records per lactation in the case of cattle, 4 to 5 in sheep, and 6 to 8 in goats and only 1 to 3 of those would be taken under HS in each lactation). Thus, the slope of decay would be the selection criteria for HT under this type of model. However, because of the antagonistic relationship between general level and slope found in nearly all populations studied, as shown in Table 2, selection emphasis on heat tolerance is likely to lead to losses in the genetic progress for production. The use of polynomial models allow a greater flexibility (no need to specify or estimate the tolerance threshold) and provide the possibility of decomposing the variability in shapes of response to temperature in independent components by making use of the eigen-decomposition of the matrix of (co)variances among the random regression coefficients defining the reaction norm. On the other hand, HT characterization is not unique as in BL reaction norms and biological interpretation of regression coefficients and principal components (PC) obtained from them is not obvious. Fig. 3 shows the eigen-functions found by Carabaño et al. (2014) for Holstein data in first lactation under a cubic polynomial norm of reaction to increasing heat loads. The first PC gathers near 80% of the genetic variability in fat or protein yield observed and is mainly associated with the overall level of production (intercept of the random regression). The other 3 PC which are independent from the first, (i.e., from the level of production), represent variability associated to changes in the heat load, and could be used to select for HT individuals without damaging the production level. For example, the second variable is a combination of the linear quadratic and cubic coefficients with a close to zero weight on the intercept and is associated with a trend to decrease production at extreme temperatures while maintaining the level for intermediate heat loads. Selection of animals with negative or low values for this variable would be expected to be HT. This appealing approach needs further investigation because of the difficulty of a biological interpretation of the canonical variables and because the consistency of the decomposition has not been checked across populations, lactations, or across time.

Gene Discovery Using Reaction Norms

The use of measures of individual HT derived from reaction norm models to detect genomic regions associated with this trait is enhanced by the increasing availability of genomic information from selection programs, mainly in dairy cattle but also in sheep and goats (Rupp et al., 2016). A genomic selection approach for HT has recently been explored by Nguyen et al. (2016) for Holstein cattle in Australia. Genomic information may help in the separation of the productive and non-productive components of HT.

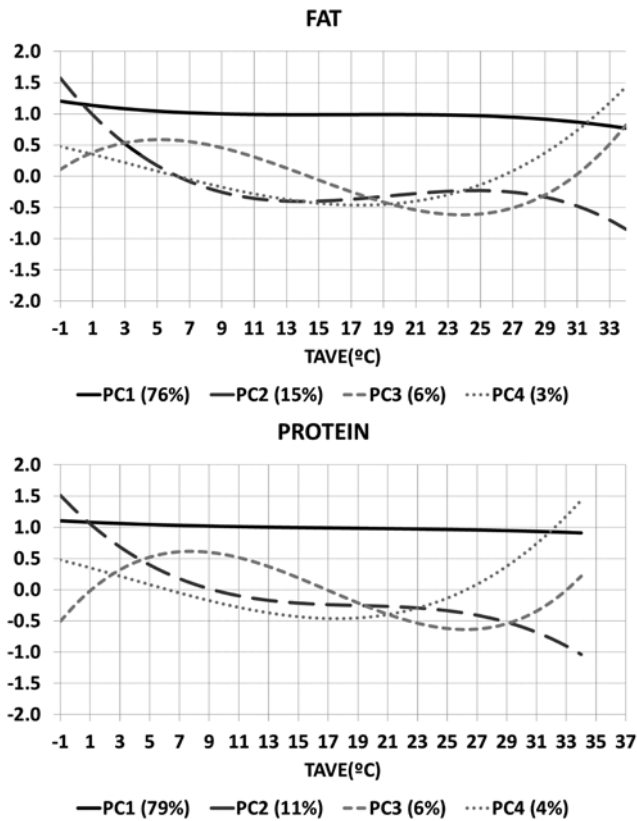


Figure 3. Eigenfunctions and percentage of variability explained by the principal components (PC, bottom in parenthesis) obtained from the matrix of (co)variance components of the additive genetic regression coefficients for a model adjusting a cubic Legendre polynomial on daily average temperatures. Modified from Carabaño et al. (2014).

Definition of heat tolerance is an issue for conventional selection and for the introduction of genomic information to improve accuracy of selection for HT. In this regard, one of the controversial issues in genome wide association studies (GWAS) for HT using estimates of individual slopes of decay in production is what measure of HT should be used in these studies. Hayes et al. (2009), used a BL approach with the estimated slopes after a fixed threshold as pseudo-phenotype for the GWAS but to estimate individual slopes of decays after the HT threshold, found one SNP in BTA29 associated with slope of production decay associated to heat load that was validated in the Australian Holstein and Jersey population. From the genes in the region containing the SNP, the strongest candidate for harboring a mutation affecting the trait was fibroblast growth factor (**FGF**)4, a gene with effect on the remodeling of mammary tissue at the end of lactation (Coleman-Krnacik and Rosen, 1994) in the mouse. Biffani et al. (2015) also did a GWAS analysis using genotypes from Italian Holstein bulls and 2 types of pseudo-phenotypes, the slope from the BL model (called by the authors THI EBV), as in Hayes et al. (2009), and the components from PC analysis performed on milk test-day records corrected for environmental effects except for THI. No significant associations were detected for milk

or protein THI EBV. Significant signals were detected for the second PC in BTA 6, 16, and 26, for milk and BTA20, 14, and 8 for protein. The positional candidate genes associated were *FGF8*, as in Hayes et al. (2009), the dehydrogenase-reductase member 3, involved in the embryonic development in humans, and the junctophilin 1 gene, whose expression has been found to be upregulated in the hypothalamus of chickens subjected to heat stress according to Biffani et al. (2015).

Genome wide association studies have been performed in our group using the reaction norm approach for the Holstein cattle population raised in the hottest regions of Spain and for 2 local breeds, Manchega sheep and Florida goats (see description of data sets in Carabaño et al., 2016b). Six pseudo-phenotypes per trait (protein and fat test day yields) for the GWAS were obtained from fitting quadratic reaction norms to animal effects ignoring relationships among animals. Those were the intercept and linear regression coefficient, the slope under a moderate and a high heat load, and the second and third PC (PC2, PC3) obtained from the eigenvalue decomposition of the estimated (co)variances for the random regression coefficients for animal effects. Our first approach allowed us to validate in Manchega Sheep and Spanish Holstein, a number of genes and gene families that have been identified previously as functional or positional candidate genes by other authors. This is the case of the heat shock proteins (**HSP**) and J-domain cochaperone (**DNAJ**) families that we could validate in both cattle and sheep. Chaperone DNAJ proteins seem to be crucial partners of the HSP70 (Qiu et al., 2006) and they are important for protein translation, folding, unfolding, translocation, and degradation. In addition, genes from the interleukin, cluster of differentiation (**CD**), and NADH:ubiquinone oxidoreductase core families could also be validated in both species. These families are mostly involved in immunological and inflammatory processes that appear to be one of the consequences for animals being exposed to environments with high heat loads (Baumgard and Rhoads, 2013). These families of genes have been identified in studies searching for selection footprint (Chan et al., 2010) that evidence their interest as genes involved in adaptive events. On the other hand, *DGATI*, coding for a protein involved in the formation of triglycerides and, *PDL5*, coding for Phospholipase D5 are genes specifically validated in Holstein and Machega, respectively. Both genes are involved in lipid metabolism that is known to be particularly affected under heat stress periods because adipose tissue mobilization diminishes. Although our results have been obtained modeling the effect of heat stress on production traits, some of the genes and families validated here are clearly highlighting mechanisms involved in the hyperthermia processes and, therefore, adaptation.

OTHER TOOLS FOR SELECTION OF HEAT TOLERANT ANIMALS WITHOUT DAMAGING PRODUCTIVITY

Fine Phenotypes

As mentioned previously, selecting animals for heat tolerance by using milk recording information together with weather station data has several advantages. It does not require additional costs once milk recording and weather information from close public weather stations are available. Moreover, genetic variability in the parameters that define the response curve of production to increasing heat loads has been found in the 3 main dairy ruminant species, making selection to decrease the decline in production under high temperatures possible. However, estimation of individual decays in production due to heat stress from monthly information may be inaccurate because this decay has to be determined from 1 or 2 records taken under heat stress and under varying physiological stages (lactation number-stage of lactation combinations or pregnancy status) and other circumstances affecting an individual's production that may vary along time (herd management, immunological status of the animal, etc.) and that are difficult to correct for in the estimation process. This may result in biased estimates of the response to HS in certain animals compared with others depending on the type of information available for each animal, that is, more or less information under HS and more or less influence of other nuisance effects. Misztal et al. (2006), in a review of issues in genetic evaluation of dairy cattle for heat tolerance, concluded that "Only a fraction of response to heat stress is captured with test days as opposed to more frequent measurements, especially if cows are in different lactation stages." Lactation number and lactation stage have been found to have a significant effect on productive response to increasing heat loads. Multiparous cows or cows in the peak of lactation have greater productive strain and are expected to be more susceptible to suffer HS (Brügemann et al., 2011, Bernabucci et al., 2014, Carabaño et al., 2014). As a consequence, not accounting for these factors may bias the estimate of individual heat tolerance from milk recording and weather data. In addition to inaccurate estimation, the selection criteria for heat tolerance derived from the use of production records have been found to be antagonistically related with the level of production since reducing milk production is a way to diminish climate-induced strain (Berman, 2011). Thus, selection of animals with reduced slopes of decay may result in selection of less productive breeding stock. Moreover, the use of monthly milk recording does not allow to account for differences in response to steady heat periods that allow for animal's acclimation or isolated heat waves along

summer. There is thus the need to use other than milk recording measurements to accurately select HT animals without damaging productivity.

Physiological Traits

Probably, the gold standard measure of tolerance to heat is the body temperature. Animals that are able to maintain their body temperature below critical values should be those that possess metabolic, physiological, and morphological characteristics that enable them to cope with high environmental temperatures and, thus, maintain productivity or minimize the losses in production. Some evidence for this hypothesis was provided by Olson et al. (2003) who found that cows carrying a favorable mutation for a hair type associated with heat tolerance showed lower body temperature and greater milk production under hot conditions than cows not carrying the mutation. This gene has been located in the BTA20 (Mariasegaram et al., 2007) and finally mutations of the prolactin signaling pathway that define hairy and slick cattle have been described by Littlejohn et al. (2014) and Sonstegard et al. (2014). Effects of introgression in the US- and New Zealand-selected dairy cattle breeds to produce heat tolerant animals have been shown by Dikmen et al. (2008, 2014) and Davis et al. (2016).

Several authors have found that body temperature is heritable, with estimates ranging from moderately low values of 0.10 for broilers cloacal temperature by Van Goor et al. (2015) and 0.17 for rectal temperature in dairy cattle by Dikmen et al. (2012) to 0.35 in Gourdine et al. (2016) in lactating sows. Howard et al. (2014) obtained a 0.68 heritability estimate (percentage of variation accounted for by marker genotypes) for tympanic temperature in beef heifers using genomic information. Estimates of the genetic correlation between body temperature and productive performance range from slightly negative to slightly positive values, with large standard errors (Renaudeau et al., 2004; Prayaga et al., 2009; Dikmen et al., 2012). Thus, body temperature may be a measure of heat tolerance less dependent on the productive component than measurements derived from production decays under HS and a useful tool to assess HT levels without damaging productivity. However, measuring body temperature may be costly because of the high labor costs when using conventional thermometers or the high cost of using automated devices in a large number of animals. Respiration or sweating rates are traits related to body temperature that have also been used to measure HT but are also labor intensive traits and not fully correlated with body temperature (Gourdine et al., 2016).

Genomic analyses associating SNP markers panels with physiological traits have been performed in several studies. Dikmen et al. (2013, 2015) performed GWAS

for rectal temperature and respiration and sweating rate in Holstein cattle. Dikmen et al. (2013) found that of all the candidate genes proposed in the GWAS, only *SLC01C1*, which is involved in regulation of metabolic rate through transport of thyroxine, plays a known role in processes controlling body temperature. Other candidate genes played roles that are important for stabilizing cellular function during stress (*GOT1*, *KBTBD2*, and *RFWD12*) or were involved in RNA metabolism (*LSM5*, *SCARNA3*, *SNORA19*, and *UI*). Interestingly, these authors mention that none of the 10 largest explanatory loci for rectal temperature were in common with 1586 SNP markers related to 31 other traits in dairy cattle previously identified in the US Holstein population. Thus, it might be possible to use SNP associated with rectal temperature to select for thermotolerance without damaging selection for other traits. Dikmen et al. (2015) tested the association of 19 SNP that had been previously identified as being involved in heat tolerance (Deb et al., 2013; Hayes et al., 2009; Dikmen et al., 2013). Two genes, *PGR* and *ASL*, were proposed as candidate genes that explained the most variation for rectal temperature, *ACAT2* and *HSD17B7* genes were similarly identified for respiration rate, and *ARL6IP1* and *SERPINE2* for sweating rate. Only *ARL6IP1*, involved in apoptosis blocking, was associated with all three thermotolerance traits. Howard et al. (2014), in a GWAS for body temperature in winter and summer in beef cattle, found that only 9% of the top windows showing significant signals for body temperature were common between the 2 seasons. The correlation between estimated genomic values for both seasons was moderate, 0.4, which agrees with previous results from quantitative studies shown in Table 1. For the summer season data, significant signals for SNP close to *TUBB2A* and *TUBB2B* genes, involved in gap junction, cellular response to stress (*STAC*, *WRNIP1*, *MLHI*, *RIPK1*, *SMC6*, and *GENI*), response to heat (*STAC*), apoptosis (*MLHI*, *RIPK1*, and *SERPINB9*), and ion transport (*KCNS3*, *SLC22A23*, and *TRPC4*) were identified.

Biomarkers

Heat stress conditions a series of physiological, endocrine, metabolic, and cellular responses that result in up- and downregulation of metabolites and other molecules that could help in a more accurate identification of HT animals. Singularities in metabolic shifts under HS have been extensively studied and a HS-specific metabolic response that cannot be explained by the decrease in DM intake has been found (Rhoads et al., 2009; Wheelock et al., 2010). Under HS, the metabolic routes involving carbohydrates, amino acids and lipids are modified to change the pathways of energy acquisition. Glycolysis and protein degradation are the main sources

of energy as opposed to fat mobilization. Lipolysis is the common way to obtain energy under thermoneutrality in situations of negative energy balance, such as those resulting from the reduction in DM intake coupled with high metabolic demands for intensive production around the lactation peak. The immunological and endocrine systems are also altered. At the hormonal level, thyroid hormones are downregulated. Changes at the cellular level are also produced to deal with cell stress due to hyperthermia, oxidation, and hypoxia. Modification of rumen conditions has also been observed (Bernabucci et al., 2009; Tian et al., 2015). As a consequence of all these changes, a large number of molecules have been found to be down- or upregulated in HS conditions compared with their production under thermoneutrality or by comparing heat tolerant vs. heat sensitive breeds under HS (Scharf et al., 2010). Examples can be found in Table 3. However, although a number of candidate biomarkers that are altered by HS have been already proposed, it is not clear yet which ones could be used to identify thermotolerant animals. For example, there is not much information about the relationship between the level of these biomarkers under HS and changes in body temperature or productivity, or about the correlations among them (Gaughan et al., 2013). In addition, biomarker levels can change in the acute response resulting from a heat wave or at the beginning of the summer from the phase when animals are becoming acclimated after persistent heat. For example, Hamzaoui et al. (2013) found that in heat-stressed goats, the levels of circulating haptoglobin and NEFA increased at d 7 of the HS period but after this day, goats seemed to be more adapted to HS conditions and haptoglobin and NEFA levels returned to values similar to those of goats under thermoneutrality. Gaughan et al. (2013) found an increase in HSP70 level in plasma after acute HS that lowered after this initial phase. This may be due to HSP only being associated with an initial stress challenge, which together with HSP responsiveness to other types of stress, would question the use of HSP as biomarkers. Similar results have been found for cortisol (Christison and Johnson, 1972). Finally, biomarkers identified in high-producing dairy cattle may not be useful for the sheep and goat populations, which have undergone less intensive selection and can show species-specific mechanisms to deal with HS. For example, sheep and goats have been found to be more HT than cattle (Silanikove, 2000; Marai et al., 2007) and have shown different metabolic pathways of response to HS than dairy cattle. Increased levels of plasma insulin have been observed in dairy cows as a consequence of HS (Rhoads et al., 2009; Baumgard and Rhoads, 2013), but this increase has not been observed in goats (Salama et al., 2014).

Table 3. Examples of candidate heat stress biomarkers analyzed in ruminants in recent conventional and metabolomics studies

Authors	Biomarkers	Species-breed
Bernabucci et al. (2002)	Erythrocytes: glutathione peroxidase, thiols, superoxide dismutase, thiobarbituric acid reactive substances	Holstein cows
Pereira et al. (2007)	Plasma: Cortisol, T3, T4	Portuguese cattle vs. Limousine & Holstein
Wheelock et al. (2010)	Plasma: Urea, Insulin, glucose	Holstein cattle
Scharf et al. (2010)	Plasma: Leptin, Creatinine, Prolactin and Cholesterol	Romosinuano vs. Angus Steers
O'Brien et al. (2010)	Plasma: Glucose, Insulin, Urea Nitrogen and NEFA	Holstein calves
Alhidary et al. (2012)	Plasma: Creatine, Glucose, total protein, Cholesterol, NEFA, Ca, Na, K	Australian Merino Sheep
Gaughan et al. (2013)	Plasma: HSP70	Angus cattle
Salama et al. (2014)	Plasma: β -hydroxybutyrate	Murciano-Granadina goats
Mahjoubi et al. (2014)	Plasma: Glucose, Urea Nitrogen, NEFA, Insulin	Sheep
Chauhan et al. (2014)	Plasma: Cortisol, Se, ROM, Biological antioxidant potential, T3, T4	Merino x Poll Dorset ewes
Ippolito et al. (2014)	Plasma: Ornithine, 3-(4-Hydroxyphenyl), N1-Methyladenosine, Xylolate, 1-Stearoylglycerol, Carnitine, Taurodeoxycholate, Deoxycholate, Trigonelline, Diisopropanolamine	Rats (Metabolomic)
Bernabucci et al. (2015)	Milk: caseins	Holstein cows
Hammami et al. (2015)	Milk: Oleic acid	Holstein cows
Tian et al. (2015, 2016)	Plasma and milk: Lactate, pyruvate, β -hydroxybutyrate, creatine, acetone, trimethylamine, oleic and linoleic acids, lysophosphatidylcholine, phosphatidylcholine	Holstein cows (metabolomics)
El-Tarabany et al. (2016)	Plasma: cholesterol, glucose, TAC, catalase, T3	Baladi goats

An important issue would be to propose biomarkers that could be determined in milk instead of blood, so that on-farm large-scale determinations can be performed for management or selection purposes. In this regard, Hammami et al. (2015) found that among all fatty acids in milk measured by mid-infrared technology, C18:1 cis-9 was the most sensitive to heat stress and might be a candidate biomarker for HT. Moreover, Tian et al. (2016) found 10 biomarkers that can be used to diagnose HS in dairy cattle that are present both in plasma and milk: lactate, pyruvate, creatine, acetone, β -hydroxybutyrate, trimethylamine, oleic acid, linoleic acid, lysophosphatidylcholine 16:0, and phosphatidylcholine 42:2, indicating

that the blood-milk barrier became leaky and the levels of these 10 biomarkers in milk can reflect HS-induced metabolomic alterations in blood.

In addition to this type of metabolites, packed cell volume (PCV) of blood, which represents the total volume of blood occupied by cells, could be used as a candidate indicator of dehydration associated with heat stress. Some authors have proposed this type of indicator to monitor the welfare of animals that are affected by water deprivation during for example the transport (MacManus et al., 2008). In this regard, PCV could be a sensible blood variable to monitor susceptibility to heat stress. Animals that are able to maintain PCV values under stressful condition may be animals less susceptible to high temperatures.

Omics

As mentioned previously, the advances in precision phenotyping can enhance the quantification of the observable degree of heat tolerance. These advances ought to be coupled with the advances in the techniques that allow discovering the genetic mechanisms that determine HT to provide an accurate identification of the genetically superior individuals for this trait. Genomics, transcriptomics, proteomics and metabolomics are fields that have already been explored in relation to the genetic control of HT.

At the genomic level, the already mentioned wide use of genotyping in genomic selection schemes has allowed the development of GWAS, although with a relatively low discovery profile of genes involved in heat tolerance. Apart from GWAS, comparison of genotypes or complete DNA sequences of animals from heat adapted and unadapted breeds can shed light in the discovery of genomic regions, genes or markers of interest in the selection for HT. Determination of markers specific to one or other group, fixation of alternate alleles in one of the groups, regions with significantly different allele frequencies, differential extended haplotype homozygosity or other indicators of selection fingerprints followed by a functional genomics analysis of the discovered differential regions can provide functional candidate genes, regions or markers associated with HT. By comparing genotypes of SNP markers distributed genome-wide of taurine and zebu cattle, Chan et al. (2010) discovered genes involved in tropical attributes including keratins, HSP, and genes involved in immunological response linked to environmental stress (*IL33*, *IL16*, *IL17RB*, *IL17RA*, *CD9*, *CD38*, *CD44*, *CD59*, *CD274*, *IL2RG*) or tick resistance (*NDUFA12*, *NDUFA9*, *NDUFAF1*, and *NDUFV2*). Similarly, Gautier et al. (2009) found candidate genes whose protein is involved in physiological functions mainly related to immune response (MHC region, *CD79A*, *CXCR4*, *DLK1*, *RFX3*, *SEMA4A*,

TICAM1, and *TRIM21*), nervous system (*NEUROD6*, *OLFM2*, *MAG11*, *SEMA4A*, and *HTR4*), and skin and hair properties (*EDNRB*, *TRSP1*, and *KRTAP8-1*). Kim et al. (2016) compared 50K genotypes of local Egyptian sheep and goats with exotic breeds and found thermo-tolerance (melanogenesis) genes (*FGF2*, *GNAI3*, *PLCB1*), apart from other genes involved in adaptation to extensive grazing in hot and arid regions. These studies were performed using SNP marker arrays of low and medium density. Comparative sequencing of genes previously associated with heat tolerance (e.g., HSP from animals of adapted and non-adapted breeds) was also used to detect polymorphisms as a tool to provide genetic markers for selection of HT animals (Charoensook et al., 2012; Kumar et al., 2016). Another line of search for heat adaptation genes is the use of genome-wide SNP data to study the geographic distributions of putatively-selected alleles at a range of geographic scales that include a wide thermal gradient (Joost et al., 2008). Using this type of approach, Salces-Ortiz et al. (2015) studied the role that climatic factors played in determining species distribution. For that purpose, they examined the distribution and frequencies of 11 polymorphisms at the *HSP90AA1* gene promoter in 31 sheep breeds and 9 species of the Caprinae subfamily distributed in a wide range of geographical zones and climates, finding evidence of the existence of genetic footprints related to climatic variables.

Transcriptomics under HS vs. normothermia [Sørensen et al. (2005) in flies; Salama et al. (2014) in milk of Murciano-Granadina goats, Coble et al. (2014) in broilers, Mehla et al. (2014) in blood of Sahiwal cattle, Li et al. (2015) in mammary gland, and Shahzad et al. (2015) in liver of Holstein cattle], in adapted vs. unadapted breeds, or in tolerant vs. intolerant individuals (Quinn et al. [2011] in arctic fish) is an area that has also been explored to determine the genetic mechanisms of HT. Results from these studies are difficult to summarize since most studies find differential expression for thousands or at least hundreds of genes, involved in a large number of functions. Of particular interest in the discovery of on-farm useful markers is the use of noninvasive and easy-to-obtain milk transcriptomes, which have been shown to have high correlation with the blood transcriptome (Cánovas et al., 2010, 2014).

Metabolomics is also a new discipline that can help in the discovery of genes and gene products that can help in selection of HT animals. Tian et al. (2015, 2016) proposed a list of candidate biomarkers for HS in dairy cows that are present in blood and also in milk (shown in Table 2), which may provide useful information for large scale application in dairy farm management and selection. Ippolito et al. (2014) selected a list of 10 candidate biomarkers in plasma (Table 2), predictive of cardiac injury and up- or downregulated in heat stressed rats under

experimental conditions. Both studies followed previous transcriptomic (and proteomic in the case of Ippolito et al., 2014) studies by the same authors, providing an integrative approach to establish the foundation for network analysis and computational-based experiments to develop models of thermoregulation.

SUMMARY AND CONCLUSIONS

Selection to improve heat tolerance in dairy ruminants, as well as other domestic species appears to be a complex task. On one hand, there is a limitation with the trait definition and measurement; on the other hand, there is the complexity of mechanisms underlying thermoregulation. However, there is a wealth of accumulated knowledge and a number of new tools that could help to understand the underlying mechanisms of heat tolerance and to develop accurate selection tools to improve heat tolerance without damaging productivity.

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