Genomic study of the mammary gland in bovines acclimated to a tropical environment

D. Wetzel-Gastal[1], F. Feitor[2], S. van Harten[1], M. Sebastiana[3], L.M.R. Sousa[3] & L.A. Cardoso[4#]

[1] Interdisciplinary Animal Health Research Centre (CIISA), Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal; [2] ISA- Institute of Agronomy, University of Lisbon, Lisbon, Portugal; [3] BIOFIG – Faculty of Science, University of Lisbon, Lisbon, Portugal; [4] FMV– Faculty of Veterinary Medicine, University of Lisbon, Lisbon Portugal

(Received 5 March 2015; Accepted 17 November 2015; First published online 6 February 2016)

Abstract

This study aims at examining the expression of genes on the mammary gland, corresponding to various levels of adaptation or acclimatization to environmental stress. The authors utilized 18 cows from three genetic groups, Holstein Brazil (HB), Gyr (GG) and Gyrolando (GH), all in the same stage of lactation, and subjected them to the same management conditions. Venous and arterial blood were collected to determine the hormonal profile and blood chemistry. Mammary gland tissue was used for transcriptomic studies. Prolactin and GH plasmatic concentrations were higher in Holstein animals. There were no differences in IGF-1 concentrations among the experimental groups. T_3 concentrations were similar among the Holstein and Gyr groups. From the 4608 transcripts in the BLO-Bovine EST (Michigan State University, US) databank that were used in this experiment, 105 differentially expressed genes were identified in at least one of the groups. Among these, the authors highlighted 14 genes that were related to the structure of the mammary gland (CRDGF, CD97, GH, endoglin, LTF, INPP, PTP), to response to thermal stress (Crh_11, v-Fos, Cdc37) and to milk protein (RPL35, κ-casein, β-casein, α-s2-casein). Eight of these were validated through real-time polymerase chain reaction. The HB animals, in comparison with the GH and Gyr groups, presented up-regulated genes associated with epithelium cellular differentiation and proliferation, milk productivity and decreased heat stress tolerance. Gyr animals presented up-regulated transcripts associated with cellular defence, apoptosis processes and increased tolerance to heat stress. The GH group showed intermediary results compared with the other two groups.

Keywords: Dairy cow, mammary gland, transcriptomic, thermic stress
Corresponding author: alfarocardoso@fmv.ulisboa.pt

Introduction

Most of livestock is found within tropical regions where seasonal weather conditions affect productivity, with relevance to dairy production (Steinfield *et al*., 2006, Collier *et al*., 2006; 2012). Global rising temperatures tend to increase the areas in which the effects are felt, particularly within agriculture activities (Aydinalp & Cresser, 2008). Tropical region abiotic stress induced upon livestock leads to genetic expressions including regulation of cellular functions (Renaudeau *et al.,* 2012) and ecosystems adaptation and acclimatization mechanisms that can reduce dairy production (Columbiano, 2007). The identification of genes associated with these mechanisms allow us a deeper perception on the impacts of heat stress within the normal cell metabolism and can participate in mitigating reduced dairy productivity problems faced by producers.

Animal heat stress in tropical regions leads to genetic expression mechanisms that change cellular function regulations. In milk production, the acclimatization process is done through genetically induced systemic metabolic changes affecting in particular the mammary gland, with acute and chronic phases including endocrine and genetic control (Cincovic *et al*., 2011; Collier *et al.,* 2012;). The adaptation process includes hereditable characteristics contributing to the survival of a population within a particular environment (International Commission for Thermal Physiology, 2001).

URL: http://www.sasas.co.za
ISSN 0375-1589 (print), ISSN 2221-4062 (online)
Publisher: South African Society for Animal Science
http://dx.doi.org/10.4314/sajas.v46i1.1

According to Azêvedo & Alves (2009), the thermoregulatory physiology of dairy cattle has changed because of genetic selection in order to increase milk productivity. These authors stressed that although body temperature and production levels are directly related, productivity improved more than tolerance to temperature increases. Ravagnolo *et al.* (2000) concluded that if genetic selection takes place only for milk production, the heat tolerance of dairy cattle is decreased, since the genetic correlation between these parameters is 0.36. Columbiano (2007) found QTLs in chromosome 11 that are associated with heat stress tolerance, such as hair length and sweating index, thus has identified genetic markers that could be used as tools for heat tolerance. Silva (2000) found a positive correlation between sweat glands and milk production.

Despite the interest in improving dairy productivity, and adaptation and acclimatization to environmental conditions, much has still to be learned of the molecular mechanisms that regulate the genetic expression of this adaptation (Collier *et al*., 2006; Bionaz & Loor, 2007; Bernabucci *et al*., 2010). The genetic base that induces energy metabolism efficiency changes and gene-environment interaction is being studied (Sonstegard *et al*., 2002; Suchyta *et al*., 2003a; Karminski *et al*., 2005; Bionaz & Loor, 2007). Heterosis application in dairy cattle production systems is also being examined (Lopez-Villalobos *et al*., 2000; Suchyta *et al*., 2003b). However, little is known about the differences in genetic expression between breeds showing different adaptation or acclimatization capacities to abiotic stress because of tropical environments affecting dairy production.

In a large part of the tropical regions, dairy *Bos indicus* breeds are being used (e.g. Gyr breed adapted to tropical environment) and they are crossbred with Holstein (e.g. Gyrolando, a 5/8 Holstein/Gyr) (Assis *et al*., 2005). In these regions, pure Holstein herds are not frequent, but can be found in intensive management systems and high production levels (World Holstein-Friesian Federation, 2009).

The enhancement of milk production in tropical areas has induced the study of genetic adaptation or acclimatization to abiotic stress of pastures, forages and livestock. Among livestock, genetic and functional mammary gland-changing mechanisms owing to the temperature and humidity conditions of a tropical environment need to be thoroughly examined. Hence, the experimental animals used in this work were located in Brazil. Brazil has a tropical/subtropical climate in which dairy cattle generally are submitted to temperatures and humidity levels above optimal production conditions, which may induce stress levels that affect productivity.

This work aims to contribute to the study of the expression in the mammary gland of genes that are related to milk production in cows, with breeds responding to various levels of heat stress tolerance.

Materials and Methods

Eighteen animals were used from three experimental groups: Holstein Brazil (HB), n = 6, registered in the Associação Brasileira de Criadores de Bovinos da Raça Holandesa (2015), Gyr (GG), n = 6, registered in the Associação Brasileira de Criadores de Gir Leiteiro, (2015) and Gyrolando (GH), n = 6, registered in the Associação Brasileira de Criadores de Girolando (2015). They were all multiparous females, on the third or fourth lactation and in the second lactation month. All animals were pregnant (confirmed through gynaecological examination and blood progesterone RIA levels), with average parturition intervals of 14 months. The average milk production per day recorded for HB was 29.5 kg, 17.0 kg for GG and 25.9 kg for GH. The cows were located in Fazenda Palma, Distrito Federal, Brazil and subjected to confined management.

They were fed maize silage, mineral supplement and concentrate (milled maize and soya bean) throughout the year. The daily concentrate intake for the HB, Gyr and GH were 9.8 kg, 6.7 kg and 7.6 kg dry matter (DM), which contained 220, 180 and 200 g total protein/kg and a total digestible nutrient (TDN) content of 780, 650 and 720 g/kg, respectively. These nutrient levels corresponded to the optimal maintenance and production requirements of the cows. To evaluate mammary gland equity conditions in terms of main nutrients, the total amino acids and fatty acid concentrations of mammary gland arterial affluent blood were determined through GC-MS and HPLC, respectively. Results showed no differences between total (micromol/L) amino acids (HB = 1983.5 ± 233.10; GH = 1691.0 ± 275.48; Gyr = 1811.5 ± 130.06, P >0.05) and total (ng/ µL) fatty acid concentrations (HB = 110.5 ± 31.94; GH = 113.5 ± 12.57; Gyr = 134.8 ± 30.14, P >0.05; Table 1). Furthermore, there was no differences between the experimental groups in the expression of Δ-9-desaturase transcript (present in the BLO library used in the microarray analysis) as an indication of diet equity (Duchemin *et al*., 2013). The milk protein composition was 3.35% for HB, 3.43% for Gyr and 3.75% for GH (P >0.05). However, significant differences (P <0.05) in concentrations were found among the groups for certain amino acids (Table 1) and fatty acids (Table 2).

The experimental protocol followed EU legislation nº86/609/EEC and was approved by Lisbon Technical University Veterinary Faculty Ethical Committee.

Table 1 Mean (± SD) amino acid concentration (micromol/L) of arterial blood according to genetic group (HB, Gyr and Gyrolando)

Amino acids		Experimental groups			*P*
		Holstein Brazil	Gyrolando	Gyr	
Ala	Alanine	240.55 (58.32)	264.63 (50.48)	281.62 (61.09)	ns
Sar	N-methyl-glycine	5.89 (2.41)	4.17 (0.53)	5.35 (1.26)	ns
Gly	Glycine	179.18 (10.50)	245.91 (22.99)	190.23 (10.22)	ns
Val	Valine	278.87[a] (62.41)	144.38[b] (18.77)	216.04[a] (75.00)	*
Leu	Leucine	161.16[a] (25.53)	97.38[b] (13.79)	140.96[a] (39.46)	*
Ile	Isoleucine	129.64[a] (22.09)	68.30[c] (10.09)	90.12[b] (12.97)	*
Thr	Threonine	79.21[a] (11.76)	43.93[b] (17.99)	51.94[b] (6.80)	*
Ser	Serine	57.32 (10.21)	56.40 (37.37)	47.53 (0.75)	ns
Pro	Proline	78.29 (9.09)	53.08 (7.33)	62.72 (3.92)	ns
Asn	Asparagine	11.79[b] (2.48)	17.80[a] (4.87)	20.41[a] (3.65)	*
Met	Methionine	7.79 (2.63)	13.40 (2.27)	8.10 (2.63)	ns
Hyp	Hidroxiprolina	7.04 (3.69)	5.40 (4.11)	12.07 (7.89)	ns
Glu	Glutamate	148.50[a] (41.74)	57.63[b] (7.28)	68.20[b] (5.38)	*
Phe	Phenylalanine	46.23 (7.44)	41.23 (9.78)	43.58 (9.27)	ns
Gln	Glutamine	265.08 (72.03)	187.08 (71.82)	197.62 (62.50)	ns
Orn	Ornithine	44.84 (4.14)	34.49 (4.02)	38.78 (16.74)	ns
Lys	Lysine	85.23 (11.15)	62.18 (7.04)	83.09 (24.88)	ns
His	Histidyne	42.82 (4.04)	38.11 (8.73)	43.55 (8.29)	ns
Tyr	Tyrosine	48.05 (11.14)	34.21 (10.17)	50.25 (16.22)	ns
Trp	Tryptophan	45.75 (45.75)	27.91 (7.17)	32.79 (10.84)	ns

[a,b,c] row means with different superscripts differ significantly at $P < 0.05$ by Tuckey's Studentized Range test.

In Brasília, DF, Brazil, where the experimental animals were located, temperature varies between 19 ºC and 23 ºC, with humidity of 45% (June - July) with the highest temperature in December-February (27 - 30 ºC) with a humidity of 75% - 80%. Sampling was done in April in a thermal comfort index (TCI) of 69, a thermos-neutrality zone (Instituto Nacional de Meteorologia, 2015) so that there would be no short-term interferences in results. A TCI of 72 is considered the upper limit, above which temperature and humidity induce thermal stress in bovine.

Experimental animals were anesthetized locally (epidural) (2% Lidovet® Bravet). Biopsies were performed with the Bard® Monopty disposable biopsy Instrument 14 G/16 cm kit (Buehring, 1990) on the left posterior quarter of the mammary gland, after asepsis (Iodopovidone, Braun®), to collect 100 mg mammary tissue. Samples were kept in RNAlater™ RNA (Ambion, Applied Biosystems, CA, USA) at −80 ºC for transcriptomic analysis. To prevent mastitis, an intra-mammary treatment (Synulox-Phiser) was administered for three days.

A total of 30 mg RNA was extracted from each animal with the TRIzol® Reagent (Invitrogen), and purified through an RNeasy® mini kit (QIAGEN). NanoDrop (Thermo Scientific) was used to study RNA quantity and quality, and RNA integrity was confirmed by 1.2% agar gel electrophoreses.

Table 2 Mean (± SD) fatty acid concentration (ng/µL) of arterial blood according to genetic group (Holstein Brazil, Gyrolando and Gyr)

Fatty acids	Holstein Brazil	Gyrolando	Gyr	*P*
∑SFA	31.92	32.53	28.62	ns
∑MUFA	42.95[a]	27.04[b]	10.11[c]	*
∑PUFA	24.26[c]	39.76[b]	60.92[a]	*
C18:2c9t11 (CLA)	0.22	0.17	0.27	ns
SC (C 12:0)	0.03	0.05	0.04	ns
LC	25.05	19.85	22.98	ns
Isómero C:18	2.07	2.40	2.11	ns

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids;
CLA: conjugated linoleic acid; SC: short-chain fatty acids; LC: long-chain fatty acids.
[a,b,c] row means with different superscripts differ significantly at $P < 0.05$ by Tukey's Studentized Range test.

The mammary differential genomic mammary gland expression profiles were achieved through the bovine cDNA microarray BLO-Bovine EST (Michigan State University, US) databank, (http://nbfgc.msu.edu) and the microarray web resource (http://amadeus.biosc.arizona.edu/bovine), described in the GPL 8564 and stocked in the NCBI Gene Expression Omnibus. These databases were completed through NCBI data (www.ncbi.nlm.nih.go). BLO-Bovine EST is a component of the National Bovine Functional Genomics Consortium (http://nbfgc.msu.edu). The microarrays integrated 4608 *Bos taurus* and other species genes (e.g. *Homo sapiens*) with BLAST sequence information known for *Bos taurus*. Three biological replicates were used for each experimental group and each replicate included a two-animal RNA pool. For each biological replicate, two technical replicates were utilized. The same RNA quantity from each animal was used.

A total of 100 ng RNA from all biological replicates was utilized for antisense RNA (aRNA) synthesis through a MessageAmp™ II aRNA amplification kit (Ambion, Applied Biosystems). From each biological replicate, 2 aRNA µg were utilized to synthesize and mark cDNA through Amersham CySribe Post-Labelling Kit (GE Healthcare). The cDNA synthesis was done through reverse transcriptase with nucleotide incorporation (aminoallyl-dUTPs). HP experimental group cDNA was marked with fluorescent Cy 3-dUTP. Marked cDNA purification was done through CyScribeTM GFXTM purification kit (Amersham, GE Healthcare)

Each microarray slide was hybridized with two marked cDNA representing each of the experimental animal groups, according to the comparisons to be done (HB x GG; HB x GH ; GG x GH). Microarray slides were blocked (pre-hybridization) with a 0.1% SDS and a H_20 MilQ wash, followed by incubation in a BSA SSC 20x and a SDS 10% solution. After blocking, two more washes were performed with SSC 2x e SSC 0.2x to remove residual salts and excess DNA.

The marked DNA hybridization solution – which integrated 40 µL probe mix, 2 µL polyA DNA (Sigma, 10 mg/mL), 2 µL of 50x Denhardt's, 2 µL SS DNA (5 mg/mL), 9.75 µL 20 x SSC and 1.95 µL of 10% SDS and buffer DIG blocking solution (Roche) – was applied to the microarray slide. Hybridization was done according to the BioFIG (ICAT) protocol in a hybridization chamber (ArrayIt Telechem International, Sunnyvale, Calif, USA) at 65 ºC (waterbath) for 17 h. Slides were then washed with 0.5 x SSC/0.1% SDS at 60 rpm for 15 min, 0.5 x SSC/0.01% SDS at 60 rpm for 15 min, 0.06x SSC at 60 rpm for 2 min, and 0.06x SSC at 60 rpm for 1 min, and centrifuged at 1600 rpm for 10 min.

Three hybridizations were done for each microarray study between experimental groups (HB x GG; HB x GH ; GG x GH), with two technical replicates for each, with a total of 18 hybridizations.

Microarray fluorescence images were obtained with VersArray ChipReader ® (Bio-Rad, USA). Spot and background fluorescence intensity were quantified with the VersArray Analyzer (Bio-Rad, USA) software. Background was calculated as the trimmed mean of pixel intensity on local corners of spots. Low intensity spots (trimmed mean of raw intensity/trimmed mean of background <1.4), spots with uneven background (trimmed mean of raw intensity/standard deviation of background <2) and uneven spots (trimmed mean of raw intensity/standard deviation of raw intensity <1) were removed from the data. Saturated spots (median raw intensity above 50000 fluorescent counts) were also flagged. Data were imported to the GEPAS web gene analysis tool for microarrays (Gene Expression Pattern Analysis Suite 3.1, 2015) and analysed with diagnosis and normalization for microarray data (Quackenbush, 2002) for background reduction, log2-transformation and normalization by the print-tip loess method (Yang *et al*., 2002). Normalized data were imported into the pre-processor (http://gepas.org) for filtering, merging replicated spots and imputing missing data. Normalization was done according to Quackenbush (2002) and Yang *et al*. (2002).

The microarrays identified 18 genes with physiological functions associated with mammary gland structure and stress thermic. Eight (CDRGF, CD97, INPP, GH, RPL35, LTF, CDC37, PTP) were for real-time PCR validation. These transcripts showed different genetic expressions in least one genetic group (breed). The real-time PCR was done through a modified Amersham CyScribe Post-Labeling Kit (GE Healthcare) protocol. Hence 500 ng of each sample total RNA was utilized for cDNA synthesis (SuperScript® III First-Strand Synthesis SuperMix commercial kit, Invitrogen). For each sample, a 20 µL solution was prepared with 500 ng RNA, 10 µL 2X RT reaction mix and 2 µL RT enzyme mix. This solution was incubated for 10 min at 25 ºC, 30 min at 50 ºC and 5 min at 85 ºC. To each sample 1 µL (2U) E. coli RNAse H was then added and incubated for 20 min at 37 ºC; cDNA was kept at −20 ºC.

Five housekeeping candidate genes were selected: β-actin, ribosomal protein L0 (RPL0), cyclofillin, PPR11 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Goossens *et al*., 2005; Mustafa *et al*., 2005; Collier *et al*., 2006; Bionaz & Loor, 2007). The results from housekeeping gene expressions were analysed through geNorm (http://medgen.ugert.be/~jrdesomp/genorm) and Norm-Finder (http://www.mdl.dk/publicationsnorm- finder.htm) applications. β-actin was the housekeeping gene with the least variations among the samples (Figure 1).

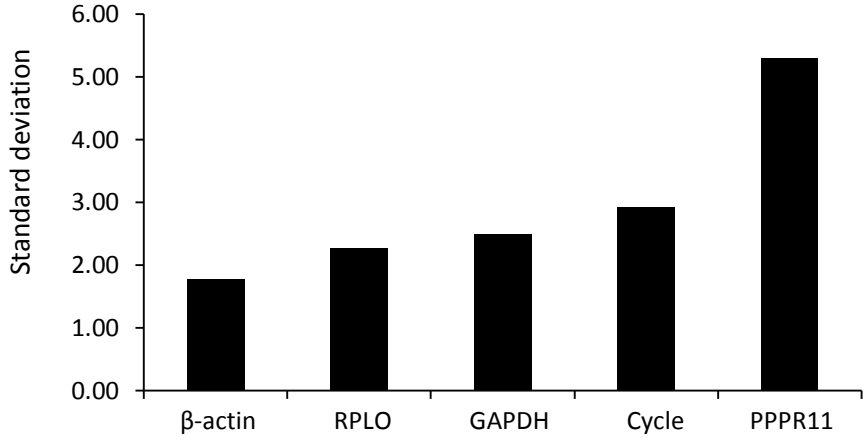

Figure 1 Standard deviation of the expression relative housekeeping candidate genes evaluated (β-actin, ribosomal protein L0 (RPL0), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ciclofillin (Cicle), PPPR11).

The PCR Miner program (Zhao & Fernald, 2005) was used to access the reaction efficiency and the limits of fluorescence reading cycles from individual PCR wells (http://www.miner.ewindup.info/).

Gene expression data were analysed through the non parametric Rank Products (RP) test (Breitling *et al*., 2004). Each gene RP test was compared with RPs from 1000 random permutations with the same repetitions and genes used in the current study. False positive results (*false discovery rate* FDS) less than 10% and fold change higher than 1.5 were considered differentially expressed (Suchyta *et al*., 2003a). Hormonal profiles and gene expressions were studied with ANOVA, and averages were compared through Tukey's studentized range test (P >0.05) using STATISTICA 10.0 (StatSoft).

Results and Discussions

Serum prolactin and growth hormone concentrations were significantly higher (P <0.05) in HB than in the GG and GH groups. IGF-1 concentrations did not vary among the three groups. T_3 serum levels were the same between HB and GG and between GG and GH, but these groups showed smaller T_3 concentrations to HB animals (P <0.05) (Table 3).

Table 3 Mean (± SD) of the serum hormonal profile (prolactin, T_3, IGF-1 and growth hormone) according to breed (Holstein Brazil, Gyrolando and Gyr)

Hormone	Holstein Brazil	Gyrolando	Gyr	*P*
Prolactin (ng/mL)	37.97^{a} ± 6.68	23.82^{b} ± 2.74	28.52^{b} ± 6.64	0.0004
T_3 (ng/mL)	1.07^{a} ± 0.39	0.77^{b} ± 0.10	0.97^{ab} ± 0.15)	0.05
GH (ng/mL)	0.16^{a} ± 0.02	0.13^{b} ± 0,01	0.14^{b} ± 0.02	0.05
IGF-1 (ng/mL)	98.38 ±16.72	112.32 ± 12.67	108.24 ± 16.57	ns

^{a,b,c} row means with different superscripts differ significantly at P <0.05 by Tukey's Studentized Range test.
T_3: triiodothyronin total; GH: growth hormone: IGF: insulin growth factor.

The T_3 and growth hormones showed higher serum values in the most productive animals (Nascimento *et al.*, 2013), which was confirmed by the results of this study of the HB animals. T_3 hormones induce energy production and consumption (Nascimento *et al*., 2006). During the initial heat stress due to high temperatures, T_3 circulating levels tended to decrease with a re-establishment of normal concentrations after a long period of animal exposure to high temperatures and humidity. This reflects physiological adaptations to tropical abiotic stress (Nascimento *et al*., 2013). The results of this study agree with these conclusions, showing that the experimental HB animals were not under heat stress. Gyr and GH groups had the same adaptation to environmental stress as expected.

Growth hormone levels are associated with mammary gland development and productivity (Mullen *et al*., 2011). These hormone concentrations decrease when animals are submitted to heat stress (McGuire *et al*., 1991), which was not observed in the HB group, showing that these animals were in thermo neutral zone. Gyr and GH results agree with their productivity levels against the Holstein animals.

IGF-1 serum levels did not differ among the groups (P >0.05). These results agree with studies by other authors (Knight *et al*., 1994; Gabai, 2003) showing that in spite of IGF-1 levels being associated with growth hormone concentrations, they do not necessarily reflect milk production levels. The results of this study indicate that under the experimental conditions, environmental adaptability mechanisms did not induce changes in IGF-1 serum levels.

From the 4608 transcripts included in the microarrays slides, 105 gene differentially expressed ESTs identified in the mammary gland in at least one of the groups. The ontological categories with wider representation in the differentially expressed transcripts of this study were associated with protein synthesis, metabolism and cellular communication, including the synthesis of milk protein and mammary gland stress adaptation, accordingly with MIPS Functional Catalogue (Munich Information Centre for Protein Sequences (http://mips.helmholtz-muenchen.de/proj/funcatDB) and with the Gene Ontology Consortium (http://www.geneontology.org) (Figure 2).

Of the differentially expressed EST, 35 had greater expression in the HB group mammary gland (FC ≥1.5 and FDR ≤0.1). From these, 22 and 13 were up-regulated in relation to GG and GH groups, respectively. The authors identified 40 transcripts with greater expression (FC ≥1.5 and FDR ≤0.1) in the GH

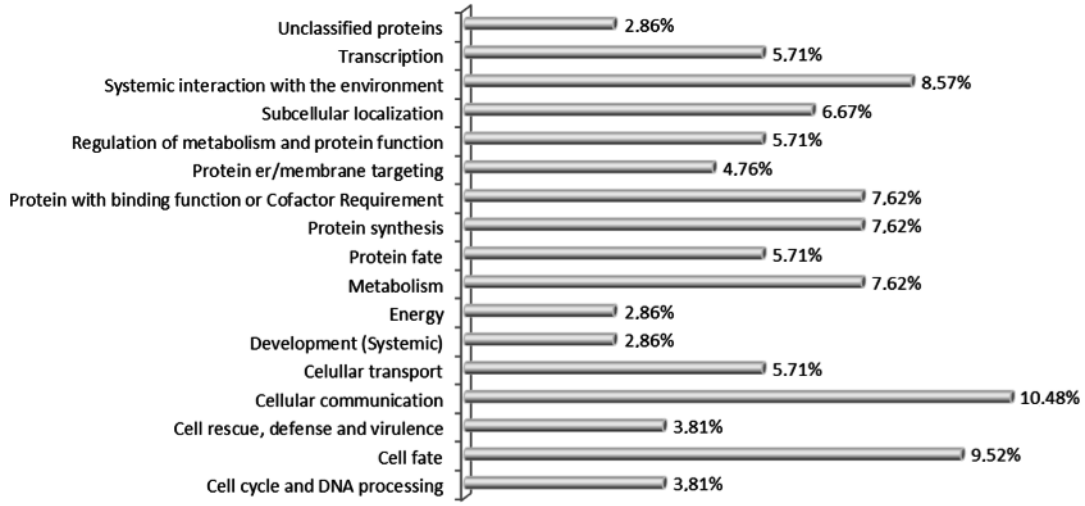

Figure 2 Main gene molecular ontological functions differentially expressed. Each clone was classified according to the BLASTn value of the bovine EST and microarray web resource (http://amadeus.biosci.arizona.edu/bovine) for cDNA sequences present in the library microarrays. The percentage of transcripts classified in each of the functional category is indicated.

Table 4 Fold change of the bovine EST and microarray web resource clones with differential expression on mammary gland tissue in at least one of the experimental groups (HB, GH and Gyr)

Gene identification (GenebBank)	Gene description	Fold change		
		HB/GG*	GG/GH **	HB/GH ***
CRDGF (BC142081)	*Bos taurus* amphiregulin	2.67	-	1.77
GH1 (NM_180996.1)	*Bos taurus* growth hormone 1	3.49	−1.66	2.57
Endoglin (Z23142.1)	S. *scrofa* Endoglin	1.79	-	2.48
CD97 (AJ416058.1)	*Bos taurus* mRNA for CD97 protein	-	2.19	1.59
LTF (NM_180998.2)	*Bos taurus* lactotransferrin (LTF), mRNA	−2.54	1.75	−1.88
PTP (NM_002833.2)	Bos *taurus* protein tyrosine phosphatase, non-receptor type 9	−3.02	2.91	-
INPP5B (XM_002686466.1)	*Bos taurus* inositol polyphosphate-5-phosphatase	−3.98	3.42	−2.29
	Heat stress tolerance			
Chr_11 (NW_001492966)	Bos *taurus* chromossome 11 cdc.	−3.69	-	−2.61
v-Fos (BC118280)	*Bos taurus* murine FBJ osteosarcoma viral (v-fos) oncogene homolog (FOS)	4.75	-	2.31
CDC37L1 (NM_017913.1)	*Homo sapiens* Hsp90-associating relative of Cdc37 (HARC), mRNA	4.06	−2.11	-
	Milk protein			
RPL35 (BC105179)	*Bos taurus* ribosomal protein L35	3.89	−2.44	1.74
κ-casein (X14908.1)	Bovine gene for k-casein exons 3-5	−3.07	-	−1.65
β-casein (AF409096.1)	Bovine β -casein gene	−3.11	-	−1.96
α-s2-like casein (M16644.1)	Bovine alpha-s2-like casein mRNA	4.09	−1.72	-

*HB/GG: ratio between Holstein Brazil and Gyr.
**GG/GL: ratio between Gyr and Gyrolando.
**HB/GL: ratio between HB and Gyrolando.

mammary gland, with 17 and 23 showing up-regulations against HB and GG groups, respectively. A total of 30 transcripts showed increased expression in the GG mammary gland (FC ≥1.5 and FDR ≤0.1), with 16 and 14 being up-regulated against HB and GH animals, respectively. The results of this study did not show differences (FC <1.5 and FDR >0.1) in the three groups regarding transcript expressions related to milk lipid composition (e.g. Δ-9-desaturase transcript AB075020 (GeneBank ID)).

For the discussion section in this study, the researchers selected 14 genes (Table 4), including functions regarding mammary gland structure, reaction to heat stress and milk protein. From these genes, eight were chosen for real-time validation as referred to above (Table 5).

Table 5 Means (± SD) of the relative quantification* of gene expressions determined by real-time PCR according experimental groups (Holstein Brazil, Gyrolando and Gyr)

Gene	Genetic group			*P*
	Holstein Brazil	Gyrolando	Gyr	
CRDGF	0.1004^{a} ± 0.0502	0.0004^{b} ± 0.00001	0.0038^{b} ± 0.0008	0.052
CD97	0.0186 ± 0.0030	0.0062 ± 0.0026	0.0180 ± 0.0041	ns
INPP	0.0266^{c} ± 0,0013	0.1046^{b} ± 0.0180	0.1551^{a} ± 0.0083	0.017
GH	0.351^{a} ± 0.0002	0.180^{b} ± 0.0009	0.152^{b} ± 0.0025	0.023
RPL35	15.8628 ± 9.28	11.9804 ± 1.28	9.3414 ± 7.96	0.05
LTF	5.1109^{b} ± 1.15	6.6045^{b} ± 0.26	9.4350^{a} ± 2.98	0.019
CDc37	1.0354^{a} ± 0.40	0.7002^{a} ± 0.25	5.7744^{a} ± 2.79	ns
PTP	0.0126^{b} ± 0.0035	0.0219^{a} ± 0.0054	0.0140^{b} ± 0.0032	0.041

^{a,b,c} row means with different superscripts differ significantly at P <0.05. Means followed by the same superscript do not differ significantly, by Tuckey's Studentized Range test (P >0.05).
CRDGF: collectorum cell-derived growth factor; CD97: CD97 protein; INPP: inositol polyphosphate-5-phosphatase; GH : growth hormone; RPL35: ribossomal protein L35; LTF: protein tyrosine phosphatase; Cdc37: Hsp90-associating relative of Cdc37; PTP: protein tyrosine phosphatase.

Mammary gland structure related transcripts

Among the mammary gland transcripts that were studied, these were found to be important because of their role in mammary gland structure: CRDGF, GH , endoglin, CD97, INPP, LTF and PTP genes.

CRDGF – *Bos taurus* similar to amphiregulin precursor (AR) *(*Colorectum cell-derived growth factor*)* (CRDGF) (LOC538751), mRNA: The CRDGF transcript is a protein that is similar to amphiregulin (Culouscou *et al.*, 1992) with a growth factor effect similar to EGF activating its receptor (Shoyab *et al*., 1989). It induces mammary gland stromal growth through the action of the enzyme ADAM17, allowing the transcript, once free from the epithelial cells, to activate the receptors (Sternlicht *et al.*, 2005). This transcript was up-regulated in the HB group relative to the other two (GG and Gyrolando; $FC_{HB/GG}$ = 2.67; $FC_{HB/GL}$ = 1.77; FDR <0.1), between which there were no differences in transcript expression (FC< 1.5 e FDR >0.1).

GH – Bos taurus growth hormone, mRNA: The results of this study show an up-regulation in growth hormone gene expression in the HB group compared with the GG and GH animals ($FC_{HB/GG}$ = 3.49; $FC_{HB/GL}$ = 2.57; FDR <0.1). This result is in line with the HB group presenting higher serum concentrations compared with the other experimental groups (P <0.05). GH presented up-regulated in GG bovines, against GL group. ($FC_{GG/GL}$ = −1.66; FDR <0.1). The gene coding for growth hormone is essential in milk production and productivity (Bauman, 1992) and in mammary gland development (Tucker, 2000). Growth hormone increases milk production by aiming nutrients at the mammary gland, decreasing its concentration during caloric stress conditions (McGuire *et al*., 1991) and when the temperature and humidity index is higher than 70 (West, 2003). The HB group's enhanced growth hormone expression could mean that these animals were under environmental adaptation, are not under a short time heat stress and presented increased milk production capacity over the other two groups.

Endoglin – S. scrofa endoglin mRNA, complete CDS: No difference was observed between GG and GH groups on endoglin gene expression (FC <1.5, FDR >0.1). HB animals showed an up-regulated response against the other two groups ($FC_{HB/GL}$ = 1.79; $FC_{HB/GG}$ = 2.48; FDR <0.1). Endoglin gene has a morphologic function associated with mammary gland development regulation through differentiation,

proliferation and apoptosis processes (Matsuo *et al*., 2006). Endoglin is a glycoprotein included in transforming growth factor-beta (TGF-beta receptor) complex (Barbara *et al*., 1999; Guerrero-Esteo *et al*., 2002). The results of this study show a direct proportion between milk productivity and endoglin gene expression in the experimental groups.

CD97 – Bos taurus mRNA for CD97 protein: CD97 transcript is a leukocyte glycoprotein acting as a receptor signalizing an adhesion process after these cells activation (Gray *et al*., 1997; Hamman *et al.*, 1997). This EST is associated with EGF terminal region, which is active in Ca^{+} metabolism regulation intervening in the maintenance of protein-protein interaction in the signal receptor family EGF-TM7 (Eicher *et al*., 2004). This transcript did not present a differential expression between GG and HB groups (FC <1.5, FDR >0.1) and was down regulated in the GH animals when they were compared with the other two genetic groups ($FC_{GG/GL}$ = 2.19; $FC_{HB/GL}$ = 1.59; FDR <0.1). This result might be a consequence of crossbreeding interactions decreasing the transcript expression.

INPP – Bos taurus inositol polyphosphate 5-phosphatase: INPP transcript codes the enzyme inositol poliphosphate-5-phosphatase, related to carbohydrate metabolism and associated with cellular functions such as proliferation, differentiation, apoptosis (Gewinner *et al.*, 2009) and mammary gland glucose transport (Zhao & Keating, 2007). The results of this study show that this gene was up-regulated in the GG animals ($FC_{HB/GG}$ = −3.98; $FC_{GG/GL}$ = 3.42; FDR <0.1) over the other two experimental groups. This transcript presented a down-regulated in the HB group, against GH animals. $FC_{HB/GL}$ = −2.29; FDR <0.1).

LTF – Bos taurus lactotransferrin mRNA: Lactoferrin is a multifunctional glycoprotein present in mammary gland epithelial secretions acting as a defence factor for this gland, associated with the apoptosis process (Legrand *et al.*, 2004). This protein is found in high concentrations during gestation and mammary gland involution (Baumrucker *et al.*, 2006). The GG experimental group showed up-regulated in the expression of this transcript, against HB and GH groups ($FC_{HB/GG}$ = −2.54; $FC_{GG/GL}$ = 1.75; FDR <0.1) and presented down-regulated in HB group compared with GH group ($FC_{HB/GL}$ = −1.88; FDR <0.1).

This result can suggest that GG animals present an increased expression inducing apoptosis related to mammary gland cellular homeostasis and maintenance (Baumrucker *et al*., 2006).

PTP – Homo sapiens protein tyrosine phosphatase, non-receptor type 9 (PTPN9), mRNA: PTP transcript is an apoptosis inductor regulating its activity through growth factors such as Epidermal Growth Factor (Rishi *et al*., 2006; Zhang *et al*., 2007). EGF receptors induce PTP tyrosine phosphorylation promoting apoptosis through caspase-9 activation (Richi *et al*., 2006) and the sequential caspase activation, leading to apoptosis (Thornberry & Lazebnik, 1998; Lavrik *et al*., 2005). Experimental group GG presented an up-regulated PTP transcript, against GH and HB groups ($FC_{HB/GG}$ = −3.02; $FC_{GG/GL}$ = 2.91; FDR <0.1) which between themselves presented the same regulation levels (FC <1.5 e FDR >0.1). This result might show an increased capacity for mammary cell renovation in the GG (Rishi *et al*., 2006).

In relation to mammary gland structure, the results of this study showed that the genes associated with epithelium cellular differentiation, proliferation and milk productivity are up-regulated in the HB animals against the other two experimental groups.

The GG breed group showed increased expression in transcripts related to cellular defence and apoptosis process. Regarding these transcripts, with the exception for Endoglin and CD97, folds changes between HB and GG groups were higher than folds changes between HB and GL for transcripts related to epithelium cellular differentiation, proliferation, milk productivity, apoptosis process and cellular defence. These results can indicate that for these transcripts, the GH breed is nearer to the HB breed than to GG animals.

Heat stress-related transcripts

The genes associated with heat stress that were identified in this study were CDc37, v-FOS and CHr11 genes.

Cdc37 – Homo sapiens Hsp90-associating relative of Cdc37 (HARC), mRNA: This transcript is associated with the expression of a 90-kDa protein (HSP90) from the heat shock proteins group involved in cellular growth and differentiation, associated to cellular heat stress tolerance (Collier *et al*., 2006). Animals submitted to acute heat stress tend to increase the synthesis of this protein, similarly to the increase of HSP 90 synthesis during prolonged acclimatization process (Collier *et al*., 2012). The microarray results of this study show that in the HB group, this transcript was up-regulated in comparison to the GG breed group ($FC_{HB/GG}$ = 3.06; FDR <0.1); however, it did not present a differential expression between the HB and GH breed groups (FC <1.5; FDR >0.1).

The high expression of this transcript in the HB and GH groups indicates that these animals whilst not yet adapted to tropical environments, can still be in the acclimatization process.

*v-FOS – Bos taurus murine FBJ osteosarcoma viral (v-fos) oncogene homolog (FOS), mRNA, complete cds (*coding sequence portion): The murine FBJ viral osteosarcoma or v-FOS is a protein inducing

changes in cell survival and in the expression of stress-related proteins (Fabre-Jonca *et al*., 1995). This author shows that this transcript is associated with small tolerance to heat stress. Thus, an increased v-FOS EST expression is associated with a decreased adaptation to high temperatures. This study´s results show that this gene was up-regulated ($FC_{HB/GG}$ = 3.75; $FC_{HB/GH}$ = 2.31; FDR <0.1) in the HB group over GG and GH animals which did not present different expressions between themselves (FC <1.5 and FDR >0.1). These data suggest that these two breeds are more tolerant of heat stress. The most useful transcript sequences are derived by complete insert sequencing of clones containing the entire length, or at least the full protein, of the source mRNA

Chr_11 – Bos taurus chromosome 11: The transcript Chr11 is related to a chromosome where QTL associated with sweating were mapped (Columbiano, 2007). According to this author, animals that are adapted to a tropical climate, such as *Bos indicus*, have increased numbers and size of sweat glands and the climate adaptation process can induce changes in the structure and density of sweat glands. The results of this study show that Chr11 was down-regulated in the HB animals compared with GG and GH groups ($FC_{HB/GG}$ = −3.69; $FC_{HB/GH}$ = −2.61; FDR <0.1), which, between themselves, presented the same regulation levels. These results agree with authors stating that *Bos indicus* have a greater density of sweat glands than *Bos taurus* cattle (Hansen, 2004; Columbiano, 2007).

Concerning the analysed heat stress-related transcripts, according to the results of the current study, HB animals showed up-regulation in HSP genes and decreased heat stress tolerance gene expression. HB/GG fold changes were higher than HB/GL ones. This is in line with the heat stress acclimatization effort differences expected between the groups.

Milk protein transcripts

These genes that are associated with milk protein were identified in this study: RPL35, k-casein, β-casein and α-s2-casein.

RPL35 – Bos taurus similar to ribosomal protein *L35, mRNA (cDNA clone MGC:128342 IMAGE:7960639), complete cds~*.

The results show that RPL 35 gene was up-regulated in the HB group over the other two breeds ($FC_{HB/GG}$ = 3.89; $FC_{HB/GL}$ = 1.74; FDR <0.1). GH animals showed up-regulated results over the GG group ($FC_{GG/GL}$ = −2.54; FDR <0.1), indicating a crossbreed effect between *Bos taurus* and *Bos indicus* for this transcript.

κ-casein – *b-ovine* gene for *kappa-casein exons 3-5*; *β-casein - Capra hircus beta-casein precursor (csn2) gene, complete cds*; *α-s2-casein - Bovine alpha-s2-like casein mRNA, complete cds*:

κ-casein, β casein and α-s2-casein genes were considered in this study. HB presented down-regulated for the κ-casein ($FC_{HB/GG}$ = −3.07; $FC_{HB/GL}$ = −1.65; FDR <0.1) and β-casein transcripts ($FC_{HB/GG}$ = −3.11; $FC_{HB/GL}$ = −1.96; FDR < 0.1) versus GG and GH animals. No differences were observed between GG and GH groups (FDR >0.1; FC <1.5) for these gene expressions.

Bionaz *et al*. (2012) pointed out that milk quantity and protein composition are highly dependent on the animal genetic profile, with *Bos indicus* presenting higher protein contents than *Bos taurus* cattle (Fonseca & Santos, 2000). In bovines, casein genes are localized in chromosome 6, occupying a 200 kb region (Olsen *et al*., 2004). These genes have been associated with differences in milk production, composition and industrialization (Ron *et al*., 2001). K-casein genotype variant B is associated with increased heat stress tolerance and smaller milk coagulation time (Pacheco-Contreras *et al*., 2011).

These results agree with those of Kemenes *et al.* (1999), which showed an increased allele frequency for these genes in *Bos indicus* than in *Bos taurus*. Arcaro *et al.* (2003) found that animals in heat comfort tend to have increased milk protein. K-casein represents about 12% of this protein.

α-s2-casein transcript was down-regulated in GG animals against HB and GH groups ($FC_{HB/GG}$ = 3.09; $FC_{GG/GL}$ = −1.72; FDR < 0.1). Further studies have to be implemented to understand the regulation of this transcript.

These results show that GG and GH groups, with the exception of α-s2-casein transcript, presented a similar casein associated gene expression, which is higher than that verified for the HB group, in line with milk composition results published for *Bos indicus* and *Bos taurus*.

Conclusion

This study revealed that tropical conditions to which dairy cattle are subjected show different effects on expression of transcripts in the mammary gland according to the experimental genetic groups.

In mammary gland structure, the current study showed that HB animals, as opposed to the GG group, presented up-regulated genes associated with epithelium cellular differentiation and proliferation, milk productivity and decreased tolerance to heat stress.

GG animals presented up-regulated transcripts associated with cellular defence, apoptosis regulation and increased tolerance to heat stress.

The GH group showed intermediary results compared with the other two groups, though showing a closer distance to the HB breed in heat stress, cellular defence and mammary gland structure associated transcripts.

The hormonal and transcriptomic study suggests that under these experimental conditions the HB group presents an ongoing tropical environment acclimatization process maintaining dairy productivity.

Acknowledgements

The authors are grateful for the excellent support of Faculty of the Science, University of Lisbon, S. Paes and D. Dias) and of the Animal Laboratory of Centro Nacional de Recursos Genéticos (CENARGEM), Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), Brasília, DF, Brazil.

This work was supported by the grant SFRH/BD/48549/2008 (Daniela Wetzel-Gastal) and SFRH/BPD/34751/2007 (Sofia van Harten) from Fundação para a Ciência e Tecnologia (FCT), Portugal. The support of CIISA through the project UID/CVT/00276/2013 and the Faculty of Veterinary Medicine of the University of Lisbon is also acknowledged.

Authors' contributions

LAC coordinated the project design and implementation. DW-G was in charge of sample collecting (Brazil) and, with MS, FF and LMS, was responsible for RIA and micro-assay procedures. SvH was in charge of RT-PCR studies. All co-authors participated in results, statistics and interpretation. LAC and DW-G were in charge of writing the manuscript.

References

Arcaro Jr, I., Arcaro, J.R.P., Pozzi, C.R., Fagundes, H., Matarazzo, S.V. & Oliveira, C.A., 2003. Teores plasmáticos de hormônios, produção e composição do leite em sala de espera climatizada. Revista Brasileira de Engenharia Agrícola e Ambiental 7, 2-8. (in Portuguese)

Assis, A.G., Stock, L.A., Campos, O.F., Zoccal, R. & Silva, M.R., 2005. Sistema de produção de leite no Brasil. (Ed. Embrapa) Juiz de Fora, Minas Gerais, Brazil. 85 p. (in Portuguese)

Associação Brasileira de Criadores de Bovinos de raça Holandesa. 2014. Available at: www.gadoholandes.com.br (in Portuguese).

Associação Portuguesa de Produtores de raça Frízia. 2015. Available at: http://www.apcrf.pt (in Portuguese)

Ayadi, M., Caja, G., Such, X., Knight, C.H., 2003. Use of ultrasonography to estimate cistern size and milk storage at different milking intervals in the udder of dairy cows. J. Dairy Sci. 70, 1-7.

Aydinalp, C. & Cresser, M.S., 2008. Red soils under Mediterranean type of climate: their properties use and productivity. Bulg. J. Agric. Sci. 14, 576-582.

Azevêdo, D.M.M.R. & Alves, A.A., 2009. Bioclimatologia aplicada à produção de bovinos leiteiros nos trópicos. (Ed. Embrapa), Brasília, Distrito Federal, Brazil. 83 p. (in Portuguese)

Barbara, N.P., Wrana, J.L. & Letarte, M., 1999. Endoglin is an accessory protein that interacts with the signaling receptor complex of multiple members of the transforming growth factor-beta superfamily. J. Biol. Chem. 274, 584-94.

Bauman, D.E., 1992. Bovine somatotropin: Review of an emerging animal technology. J. Dairy Sci. 75, 3432-3451.

Baumrucker, C.R., Schanbacher, F., Shang, Y. & Green, M.H., 2006. Lactoferrin interaction with retinoid signalling: Cell growth and apoptosis in mammary cells. Domest. Anim. Endocrin. 30, 289-303.

Bernabucci, U., Lacetera, N., Baumgard, L.H., Rhoads, R.P., Ronchi, B. & Nardone, A., 2010. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. Animal 4, 1167-1183.

Bionaz, M. & Loor, J.J., 2007. Identification of reference genes for quantitative real-time PCR in the bovine mammary gland during the lactation cycle. Physiol. Genomics 29, 312-319.

Bionaz, M., Hurley, W. & Loor, J.J., 2012. Milk Protein Synthesis in the Lactating Mammary Gland: Insights from Transcriptomics Analyses. Ed: Hurley, W., Department of Animal Sciences, University of Illinois, Urbana, Illinois, USA. pp. 285-324.

BLO microarray EST database, 2015. Available at: http://nbfgc.msu.edu

Breitling, R., Armengaud, P., Amtmann, A. & Herzyk, P., 2004. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Letters 573, 83-92.

Buehring, G.C., 1990. Culture of mammary epithelial cells from milk. J. Dairy Sci. 73, 956-963.

Cincovic, M.R., Belic, B., Toholj, B., Potkonjak, A., Stevancevic, B., Lako, B. & Radovic, I., 2011. Metabolic acclimation to heat stress in farm housed Holstein cows with different body condition scores. Afr. J. Biotechnol. 10, 10293-10303.

Collier, RJ., Rungruang, S., Zimbleman, R.B. & Hall, L.W., 2012. Metabolic implications of heat stress. Proceedings of the 27th Annual Southwest Nutrition and Management Conference, Tempe, Arizona. pp. 57-64.

Collier, R.J., Stiening, C.M., Pollard, B.C., VanBaale, M.J., Baumgard, L.H., Gentry, P.C. & Coussens, P.M., 2006. Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. J. Anim. Sci. 84, E1.

Columbiano, V.S., 2007. Identificação de QTL's nos cromossomas 10, 11 e 12 associados ao stress calórico em bovinos. Thesis Masters Science. University Federal of Viçosa, Viçosa, Minas Gerais, Brazil. 60 p. (in Portuguese)

Culouscou, J.M., Remacle-Bonnet, M., Carlton, G.W., Plowman, G.D. & Shoyab, M., 1992. Colorectum cell-derived growth factor (CRDGF) is homologous to amphiregulin, a member of the epidermal growth factor family. Growth Factors 7, 195-205.

Duchemin, S., Bovenhuis, H., Stoop, W.M., Bouwman, A.C., Van Arendonk, J.A.M. & Visker, M.H.P.W., 2013. Genetic correlation between composition of bovine milk fat in winter and summer, and DGAT1 and SCD1 by season interactions. J. Dairy Sci. 96, 592-604.

Eicher, S.D., McMunn, K.A., Hammon, H.M. & Donkin, S.S., 2004. Toll-like receptors 2 and 4, and acute phase cytokine gene expression in dexamethasone and growth hormone treated dairy calves. Vet. Immunol. Immunop. 98, 115-125.

Fabre-Jonca, N., Gonin, S., Diaz-Latoud, C., Rouault, J.P. & Arrigo, A.P., 1995. Thermal sensitivity in NIH 3T3 fibroblasts transformed by the v-Fos oncogene. Eur. J. Biochem. 232, 118-128.

Fonseca, L.F.L. & Santos, M.V., 2000. Qualidade do leite e controle de mastite. (Ed Lemos),. São Paulo, São Paulo, Brazil. pp. 39-141. (in Portuguese)

Gabai, G., 2003. Physiological and metabolic adaptations in the mammary gland and consequences for the dairy cow. Vet. Res. Comm. 27 (Suppl. 1), 129-136.

Gene Expression Pattern Analysis Suite 3.1, GEPAS. 2015. Available at: http://gepas.bioinfo.cipf.es

Gene Expression Pattern Analysis Suite. 2015. Available at: http://gepas.org

Gene Ontology Consortium. 2015. Available at: http://www.geneontology.org

Gewinner, C., Wang, Z.C., Richardson, A., Teruya-Feldstein, J., Etemadmoghadam, D., Bowtell, D. & Cantley, L.C., 2009. Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling. Cancer cell 16, 115-125.

Goossens, K., Van Poucke, M., Van Soom, A., Vandesompele, J., Van Zeveren, A. & Peelman, L.J., 2005. Selection of reference genes for quantitative real-time PCR in bovine preimplantation embryos. BMC Develop. Biol. 5: 27 doi: 10.1186/1471-213X-5-27

Gray, J.X., Haino, M. & Roth, M.J., 1997. CD97 is a processed, seven-transmembrane, heterodimeric receptor associated with inflammation. J. Immunol. 157, 5438-5447.

Guerrero-Esteo, M., Sanchez-Elsner, T., Letamendia, A. & Bernabeu, C., 2002. Extracellular and cytoplasmic domains of endoglin interact with the transforming growth factor-beta receptors I and II. J. Biol. Chem. 277, 197-209.

Hamann, J., Vogel, B., Van Schijndel, G.M. & Van Lier, R.A., 1997.The seven-span transmembrane receptor CD97 has a cellular ligand (CD55, AF). J. Exp. Med. 184 (3), 1185-1189. doi:10.1084/jem.184.3.1185.PMID 9064337.

Hansen, P.J., 2004. Physiological and cellular adaptations of zebu cattle to thermal stress. Anim. Repr. Sci. 82-83, 349-360.

Instituto Nacional de Meterologia. 2015. http://www.inmet.gov.br (in Portuguese)

International Commission for Thermal Physiology (ICTP) 2001. Glossary of terms for thermal physiology, 3rd ed. JPN. J. Physiol. 51, 245-280.

Kaminski, S., Ahman, A., Ruse, A., Wojcik, E. & Malewski, T., 2005. Milk ProtChip – a microarray of SNP's in candidate genes associated with milk protein biosynthesis. J. Appl. Genet. 46, 45-58.

Kemenes, P.A., Regitano, L.C.A. & Rosa, A.J.M., 1999. K-casein, B-lactoglobulin and growth hormone allele genetic distances in Nelore, Gyr, Guzerá, Caracu, Charolais, Canchim and Santa Gertrudis cattle. Genet. Mol. Biol. 22, 539-541.

Knight, C.H., France, J. & Beever, D.E., 1994. Nutrient metabolism and utilization in the mammary gland. Livest. Prod. Sci. 39, 129-137.

Lavrik, I.N., Golks, A. & Krammer, P.H., 2005. Caspases: pharmacological manipulation of cell death. J. Clin. Invest. 115, 2665-2672.

Legrand, D., Elass, E., Pierce, A. & Mazurier, J., 2004. Lactoferrin and host defence: an overview of its immuno-modulating and anti-inflammatory properties. Biometals 17, 225-229.

Lopez-Villalobos, N., Garrick, D.J., Holmes, C.W., Blair, H.T. & Spelman, R.J., 2000. Profitabilities of some mating systems for dairy herds in New Zealand. J. Dairy Sci. 83, 144-53.

Matsuo, S.E., Leoni, S.G., Colquhoun, A. & Kimura, E.T., 2006. Transforming growth factor-b1 and activin A generate antiproliferative signaling in thyroid cancer cells. J. Endocrinol. 190, 141-150.

McGuire, M.A., Beede, D.K., Collier, R.J., Buonomo, F.C., DeLorenzo, M.A., Wilcox, C.J., Huntington, G.B. & Reynolds, C.K., 1991. Effects of acute thermal stress and amount of feed intake on concentrations of somatotropin, insulin-like growth factor (IGF)-I and IGF-II, and thyroid hormones in plasma of lactating Holstein cows. J. Anim. Sci. 69, 2050-2056.

Microarray WEB Resource database. 2015. Available at: http://amadeus.biosc.arizona.edu/bovine.

Mullen, M.P., Berry, D.P., Howard, D.J., Diskin, M.G., Lynch, C.O., Giblin, L., Kenny, D.A., Magee, D.A., Meade, K.G. & Waters, S.M., 2011. Single nucleotide polymorphisms in the insulin-like growth factor 1 (IGF-1) gene are associated with performance in Holstein-Friesian dairy cattle. Front. Gen. 2: 3 doi:10.3389/fgene.2011.00003

Munich Information Centre for Protein Sequences (MIPS) Functional Catalogue. 2015. Available at: http://mips.helmholtz-muenchen.de/proj/funcatDB

Mustafa, T., Eckert, A. & Klonisch, T., 2005. Expression of the epidermal growth factor seven-transmembrane member CD97 correlates with grading and staging in human oral squamous cell carcinomas. Cancer Epidem., Biomar. 14, 108-119.

Nascimento, M.R.B.M., Vieira, R.C. & Silva, G.C., 2006. Effects of month, order and lactation stage on the thyroid hormones of heifers and cows of the Holstein breed. Archiv. Vet. Sci. 11, 55-60.

Nascimento, M.R.B.M., Storti, A.A., Guimarães, E.C. & Simioni, V.M., 2013. Thyroid hormone profile of Holstein and Guzera dairy cattle in a tropical environment. Biosci. J. 29, 179-184.
Olsen, H.G., Lien, S., Gautier, M., Nilsen, H., Roseth, A., Berg, P.R. & Meuwissen, T.H., 2005. Mapping of a milk production quantitative trait locus to a 420-kb region on bovine chromosome 6. Genetics 169, 275-283.
Pacheco-Contreras, V.I., Lourenco-Jaramillo, D.L., Parra-Bracamonte, G.M., Martínez-González, J.C. & Sifuentes-Rincón, A.M., 2011. Convenient genotyping of nine bovine K-casein variants. Electronic J. Biotechnol. 14, 12-12.
PCR Miner, 2015. Available at: http://www.miner.ewindup.info/
Quackenbush, J., 2002. Microarray data normalization and transformation. Nature Genetics 32, 496-501.
Ravagnolo, O., Misztal, I. & Hoogenboom, G., 2000. Genetic component of heat stress in dairy cattle, development of Heat Index Function. J. Dairy Sci. 83, 2120-2125.
Renaudeau, D., Collin, A., Yahav, S., Basilio, V., Gourdine, J.L. & Collier, R.J., 2012. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6, 707-728.
Rishi, A.K., Zhang, L., Yu, Y., Jiang, Y., Nautiyal, J., Wali, A., Fontana, J.A., Levi, E. & Majumdar, A.P.N., 2006. Cell Cycle- and Apoptosis-regulatory Protein-1 is involved in apoptosis signalling by Epidermal Growth Factor Receptor. J. Biol. Chem. 281, 13188-13198.
Ron, M., Kliger, D., Feldmesser, E., Seroussi, E., Ezra, E. & Weller, J.I., 2001. Multiple quantitative trait locus analysis of bovine chromosome 6 in the Israeli Holstein population by a daughter design. Genetics 159, 727-735.
Shoyab, M., Plowman, G.D., McDonald, V.L., Bradley, J.G. & Todaro, G.J., 1989. Structure and function of human amphiregulin: A member of the epidermal growth factor family. Science 243, 1074-1076.
Silva, R.G., 2000. Introdução à bioclimatologia animal. (Ed. Nobel) São Paulo, São Paulo, Brazil. 286 p. (in Portuguese)
Sonstegard, T.S., Capuco, A.V., White, J., Van Tessel, C.P., Connor, E.E., Cho, J., Sultana, R., Shade, L., Wray, J.E., Wells, K.D. & Quackenbush, J., 2002 .Analysis of bovine mammary gland EST and functional annotation of the *Bos Taurus* gene Index. Mamm. Genome 13, 373-379.
Steinfield, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M. & de Haan, C., 2006. Livestocks' Long Shadow. Environmental issues and options. Ed FAO. Rome. 267 p.
Sternlicht, M.D., Sunnarborg, S.W., Kouros-Mehr, H., Yu, Y., Lee, D.C. & Werb, Z., 2005. Mammary ductal morphogenesis requires paracrine activation of stromal EGFR via ADAM17-dependent shedding of epithelial amphiregulin. Development 132, 3923-3933.
Suchyta, S.P., Sipkovsky, S., Kruska, R., Jeffers, A., McNulty, A., Coussens, M.J., Tempelman, R.J., Halgren, R.G., Saama, P.M., Bauman, D.E., Boisclair, Y.R., Burton, J.L., Collier, R.J., DePeters, E.J., Ferris, T.A., Lucy, M.C., McGuire, M.A., Medrano, J.F., Overton, T.R., Smith, T.P., Smith, G.W., Sonstegard, T.S., Spain, J.N., Spiers, D.E., Yao, I. & Coussens, P.M., 2003a .Development and testing of a high-density cDNA microarray resource for cattle. Physiol. Genomics 15, 158-164.
Suchyta, S.P., Sipkovsky, S., Halgren, R.G., Kruska, R., Elftman, M., Weber-Nielsen, M., Vandehaar, M.J., Xiao, L., Tempelman, R.J. & Coussens, P.M., 2003b. Bovine mammary gene expression profiling using a cDNA microarray enhanced for mammary-specific transcripts. Physiol. Genomics 16, 8-18.
Thornberry, N.A. & Lazebnik, Y., 1998. Caspases: enemies in. Science 281, 1312-1316.
Tucker, H.A., 2000. Symposium: Hormonal regulation of milk synthesis: Hormones, mammary growth, and lactation: A 41-Year Perspective. J. Dairy Sci. 83, 874-884.
West, J.W., 2003. Effects of heat-stress on production in dairy cattle. J. Dairy Sci. 86, 2131-2144.
Yang, Y.H., Dudoit, S., Luu, P., Lin, D.M., Peng, V., Ngai, J. & Speed, T.P., 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systemic variation. Nucleic Acid Res. 30, (4): e15. doi: 10.1093/nar/30.4.e15
Zhang, L., Levi, E., Majumder, P., Yu, Y., Aboukameel, A., Du, J., Xu, H., Mohammad, R., Hatfield, J.S., Wali, A., Adsay, V., Majumdar, A.P.N. & Rishi, A.K., 2007. Transactivator of transcription–tagged cell cycle and apoptosis regulatory protein-1 peptides suppress the growth of human breast cancer cells in vitro and in vivo. Mol. Cancer Ther. 6, 1661-1672.
Zhao, S. & Fernald, R.D., 2005. Comprehensive algorithm for quantitative real-time polymerase chain reaction. J. Comput. Biol. 12, 1045-1062.
Zhao, F. & Keating, A.F., 2007. Functional properties and genomics of glucose transporters. Current Genomics 8, 113-128.