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1 # Transcriptome profiling in Indian Cattle revealed novel insights
2 # into response to heat stress

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18 ## Abstract
19

20 Heat stress induced by high environmental temperature and humidity affects livestock
21 production and health. With global warming on the uprise, indigenous cattle known for their heat
22 tolerance are gaining importance than the Crossbreds. However, systems biology behind this
23 phenotype in indigenous cattle is less known. This study, revealed novel insights in response to
24 heat stress in Indian cattle (Tharparkar breed – Indigenous breed vs Vrindhavani – Crossbred
25).The number of DEGs in Crossbred were found to be more, than in Tharparkar, suggesting a
26 greater dysregulation in systems biology in Crossbred. A contrast in gene expression was
27 observed with 18.5 % of upregulated genes in Crossbred (Vrindavani cattle) were downregulated
28 in Tharparkar and 17.5% upregulated genes in Tharparkar were downregulated in Crossbred. The
29 increased HSPs levels have been found positively correlated with tolerance in many species.
30 Upregulation of *HSF, HSP70, HSP90*, and activation of EIF2 signaling pathway in Tharparkar
31 and vice-versa in Crossbred delineates how Tharparkar, withstands heat stress. Unlike Crossbred,
32 Tharparkar is not only endowed with higher expression of the scavengers (*UBE2G1, UBE2S*, and
33 *UBE2H*) of misfolded proteins but also with protectors (*VCP, Serp1*, and *CALR*) of naïve
34 unfolded proteins. Further, the apoptotic genes that were dysregulated in both genetic groups
35 indicated a relatively higher probability of apoptosis in Crossbred than in Tharparkar. Also,
36 higher expression of the antioxidants in Tharparkar enables it to cope up with higher levels of

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37 free radicals generated as a result of heat stress. In this study we found relevant molecules/genes
38 dysregulated in Tharparkar in the direction that can counter heat stress. To best of our knowledge
39 this is a comprehensive comparison between Tharparkar and Crossbred under heat stress using
40 transcriptome analysis.
41

42 ## Introduction
43
44 Cattle being homoeothermic, modulate their internal body temperature in sync to
45 environmental temperature by equilibrating the amount of heat produced within the body and
46 dissipating it to the ambient environment. The stress that arises due to disproportionate
47 thermodynamic behavior between cattle and its surrounding environment is termed as heat stress
48 [1]. Environmental induced hyperthermic stress lowers feed intake, which in turn reduces growth,
49 milk production and reproductive efficiency, thereby negatively affecting the economics of
50 livestock keepers [2-4]. Heat stress has been associated with reduced fertility through its deleterious
51 impact on oocyte maturation and early embryonic development [5]. Increased morbidity and
52 mortality was observed in animals due to immune depressive effect of heat stress [6].

53 India has a wide variety of indigenous cattle breeds distributed throughout its agro-
54 climatic zones. These are known for their natural tolerance to tropical heat [7,8].To meet the
55 growing demand for milk and to combine the heat tolerance and tick resistance of zebu with the
56 productivity of temperate dairy breeds [9] several crossbreeding programs were taken up in India.
57 Every state had its own crossbreeding policy, which is agro-climatic and breed-specific. Though
58 the zebu crosses with European breeds produced more milk than zebu, they were found not
59 withstanding heat/solar radiation [10]. Crossbreds are susceptible to tropical diseases and require a
60 constant input of good management conditions [8]. Antagonism exists between heat tolerance and
61 milk productivity [11]. The adaptive capacity to heat stress varies between species and genetic
62 groups within species. Among various adaptive mechanisms, physiological adaptability seems to
63 be the primary step in cattle. Sahiwal cows better regulate body temperature in response to heat
64 stress than Karan Fries [8]. It was observed that Ongole cattle rely on the respiration rate to
65 maintain thermal balance, while, Bali cattle rely on rectal temperature [12]. In Brazil, Sindhi and
66 Girolando breeds showed better physiological response to thermal stress than Gir cattle [13].
67 Increase in respiration rate was reported in Nellore breed when exposed to heat load [14].

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68 In India, Tharparkar is one among the best dairy breeds. It is adapted to the Indian states
69 of Punjab and Haryana. [15,16]. It is considered to be the hardiest, disease resistant, heat tolerant
70 and tick resistant indigenous cattle breed of the country [17]. This breed has also been used in
71 several crossbreeding programs. Currently, percentage of purebreds is exceptionally low in India
72 (Department of Animal Husbandry & Dairying, Govt. of India). Most of the farmer's in India
73 have Crossbreds and the percentage of exotic inheritance in these Crossbreds is unknown.
74 Vrindavani, a Crossbred (synthetic), with 27% of Indigenous blood and 73% of exotic
75 inheritance [18] is a representation of the kind of admixture that prevails in Indian cattle. Therefore,
76 comparing Tharparkar with Vrindavani may establish the lost importance of Indigenous cattle
77 and would further emphasize the need of conserving our indigenous purebreds because of
78 advantageous traits like Heat tolerance.

79 Studies explaining the difference between the genetic groups (Crossbreds and Indigenous
80 cattle) have been done mainly to address the physiological responses vis – a – vis heat stress and
81 very few studies at the genomic level have been taken up [19,20].Also, there is an increasing need to
82 develop methods by combining the knowledge from -omics technologies to identify heat tolerant
83 animals [11]. Transcriptome profiling / RNA-Sequencing (RNA-seq) is a high throughput omics
84 approach to measure relative global changes in the transcripts under specific condition(s) [21-23] to
85 study the systems biology behind a phenotype [23,24]. RNA - seq allows for analysis of
86 transcriptome in an unbiased way, with, a tremendous dynamic detection range (>8,000 fold),
87 and low background signals [25]. It has been used as an investigating tool in understanding disease
88 pathogenesis [26,27] and differential physiological response to various biotic and abiotic factors
89 [28,29].

90 In this study, Tharparkar and Crossbred - Vrindavani cattle were subjected to heat stress
91 and blood samples were collected on 0th day and 7th day, as it is known that short term
92 acclimation occurs around 5 - 6 days [30,31]. The transcriptome of 7th day was compared and with
93 0th day in both the genetic groups to understand their differential response to heat stress.

94 ## Results

95 ### Physiological Parameters

96 The overview of the analysis is given in Figure 1. Respiration rate (RR), rectal
97 temperature (RT) and T3 level increased significantly ($p<0.05$) on 7th - day post heat stress in

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98 both the genetic groups (n=5) (Figure 2). However, the increase in RR, RT and T3 level, was
99 found significantly ($P<0.05$) higher in Crossbred than in Tharparkar.

100 **Comparison of DEGs of Crossbred and Tharparkar under heat stress**

101 The differentially expressed genes for each genetic group were obtained on comparing
102 the 0 day and 7th day RNA-seq data using EdgeR after obtaining the gene counts from RSEM.
103 Under heat stress, global expression profiles of Crossbred and Tharparkar were identified with
104 6042 and 4718 differentially expressed genes (DEGs), respectively (Supplementary Table 1).
105 Among these, 3481 DEGs were found common between the two genetic groups, while 2561 and
106 1238 DEGs were uniquely found in Crossbred and Tharparkar, respectively (Figure 3a).
107 Additionally, 3132 and 2924 genes were upregulated and downregulated in Crossbred,
108 respectively, while 2367 and 2358 genes were upregulated and downregulated in Tharparkar,
109 respectively (Figure 3b). On comparison of upregulated and downregulated genes, 724 and 1416
110 genes were found uniquely upregulated and 514 and 1145 genes were found uniquely
111 downregulated in Tharparkar and Crossbred, respectively. The comparison also revealed that
112 17.5% of upregulated genes (1278) in Tharparkar were downregulated in Crossbred and 18.5%
113 downregulated genes (1344) in Tharparkar were upregulated in Crossbred. However, the number
114 of common upregulated and downregulated genes in both the genetic groups were 357 (4.9%)
115 and 498 (6.8%), respectively (Figure 3c).

116 **Analysis of knowledge-based genes**

117 Under heat stress four major physiological processes are found to be usually associated
118 with heat stress viz. elicitation of unfolded protein response (UPR) in cells; Induction of
119 apoptosis; Ubiquitination and; Imbalance in production of ROS and antioxidants (refer to
120 materials and methods for details). Heat shock genes and its associated genes are involved in
121 elicitation of unfolded protein response (UPR) in cells. Heat shock genes have been found
122 dysregulated under heat stress in both the genetic groups. Most of the genes encoding Heat
123 shock proteins (HSPs) - Heat shock 70 kDa protein 4 (*HSPA4),* Heat shock cognate 71 kDa
124 protein *(HSPB8),* Heat shock 70 kDa protein 1A *(HSPA1A)* ,Heat shock cognate 71 kDa protein
125 *(HSPA8),* Heat shock protein HSP 90-beta *(HSP90AB1)* and Heat shock protein HSP 90-alpha
126 (*HSP90AA1*) and heat shock protein regulating factors- Heat shock factor 1 *(HSF1)* and
127 Eukaryotic Translation Elongation Factor 1 Alpha 1 *(EEF1A1)* have been found to be

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128 downregulated/not-differentially expressed in Crossbred but upregulated in Tharparkar.
129 However, Calcium/Calmodulin Dependent Protein Kinase II Delta *(CAMK2D) that* is involved
130 in the regulation of expression of heat shock genes was upregulated in Crossbred and
131 downregulated in Tharparkar.

132 Among the apoptotic genes, genes encoding Bcl-2-like protein 11 (*BCL2L11)* , Tumor
133 necrosis factor ligand superfamily member 6 *(FASLG)* , TIR domain-containing adapter
134 molecule 2 *(TICAM2)* , Toll-like receptor 4 *(TLR4),* Adenomatous polyposis coli protein
135 *(APC),* Caspase-3 *(CASP3),* Mitogen-activated protein kinase 8 *(MAPK8),* Mixed lineage
136 kinase domain-like protein *(MLKL),* Late endosomal/lysosomal adaptor and MAPK and MTOR
137 activator 5 *(XIP),* Vimentin *(VIM),* and High mobility group protein B2 *(HMGB2)* were found to
138 be upregulated in Crossbred and downregulated in Tharparkar. The number of upregulated genes
139 involved in achieving the balance of ROS production and antioxidants, were found to be more in
140 Tharparkar than in Crossbred. Among these, genes encoding Glutathione peroxidase 3
141 (*GPX3),* Nudix Hydrolase 2 *(NUDT2),* Catalase *(CAT),* Cytochrome c *(CYCS),* Copper
142 chaperone for superoxide dismutase *(CCS),* Peroxiredoxin-5 *(PRDX5),* Peroxiredoxin-6
143 *(PRDX6),* Peroxiredoxin-1 *(PRDX1),* Superoxide dismutase *(SOD1),* and Cytochrome b-245
144 heavy chain *(CYBB*) were found either downregulated/not-differentially expressed in Crossbred
145 and upregulated in Tharparkar. More number of genes involved in Ubiquitination were
146 differentially expressed in Crossbred than in the Tharparkar. Genes encoding Ubiquitin-
147 conjugating enzyme E2 G1 (*UBE2G1),* Ubiquitin-conjugating enzyme E2 *(UBE2S),* Ubiquitin-
148 conjugating enzyme E2 H *(UBE2H),* Ubiquitin A-52 residue ribosomal protein fusion product 1
149 *(UBA52),* and Ubiquitin-activating enzyme E1 *(UBA1)* have been found downregulated/not-
150 differentially expressed in Crossbred and upregulated in Tharparkar. However, Valosin-
151 containing protein *(VCP),* RING finger protein 40 *(RNF40)*, and Ubiquitin-conjugating enzyme
152 E2 L3 *(UBE2L3)* have been found downregulated in Crossbred but not-differentially expressed
153 in Tharparkar. Among the genes involved in Unfolded Protein folding response (UPR), genes
154 encoding Membrane-bound transcription factor site-1 protease (*MBTPS1),* Cyclic AMP-
155 responsive element-binding protein 3-like protein 1 *(CREB3L1),* Stress-associated endoplasmic
156 reticulum protein 1 *(SERP1)* , Glycogen synthase kinase-3 alpha *(GSK3A),* Eukaryotic
157 translation initiation factor 2 subunit 3 *(EIF2S3),* Calreticulin *(CALR)* , and Stress-associated

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158 endoplasmic reticulum protein 1 (*SERP1)* have been found downregulated in Crossbred and
159 upregulated in Tharparkar (Figure 4).

160 Protein - protein interaction (PPI) network revealed functional importance of HSP70
161 (*HSPA8* and *HSPA1A)* and ubiquitin (UBB, UBA52), in coordinating genes involved in heat
162 stress

163 A total of 246 knowledge-based genes were identified from the reactome database. Out of
164 these 177 and 194 genes were found to be differentially expressed in Tharparkar and Crossbred,
165 respectively. Among these 126 genes were found to be commonly differentially expressed in
166 Tharparkar and Crossbred. PPI network for these common knowledge-based genes between
167 Tharparkar and Crossbred was constructed (Supplementary Figure 1). In PPI networks, hubs
168 define the functional and structural importance of a network. The genes, which act as hubs in PPI
169 networks were found to be *UBB, UBA52, HSPA8*, and *HSPA1A* (Supplementary Figure 1).
170 Among the 4 hubs, *UBB* was downregulated in both genetic groups and the rest were
171 downregulated in Crossbred and upregulated in Tharparkar.

172 A change in the expression of the hub protein will have a larger effect than change in
173 expression of non-hub proteins [32]. Therefore, UBB, UBA52, HSPA8, and HSPA1A are taken to
174 be critical for coordinating the changes in systems biology under heat stress. The hubs HSPA8
175 and HSPA1A are connected to genes that are associated with regulation of stress viz.
176 nucleoporins genes - *NUP188, NUP155, NUP210* & *NUP214*; BAG family molecular chaperone
177 regulators - *BAG1, BAG3* & *BAG4*; Heat Shock Protein Family A - *HSPA5, HSPA4, HSPA12B*
178 & *HSPA9*; DnaJ Heat Shock Protein Family i.e. HSP40 - *DNAJA1, DNAJC2* & *DNAJB6*; Heat
179 shock factor - *HSF1*; Ubiquitin - *UBB* & *UBA52* and; Sirtuin - *SIRT1*. The hubs - UBB and
180 UBA52 are connected to molecules of different proteasome subunits viz. ⍰ type subunits -
181 *PSMA1* & *PSMA2*; β type subunits - *PSMB4* & *PSMB8*; ATPase subunits - *PSMC2* & *PSMC5*
182 and non-ATPase subunits - *PSMD2* & *PSMD13*. These hubs were also found connected to
183 ubiquitin specific peptidases - *USP9X* and *USP7* and Ubiquitin-conjugating enzyme - *UBE2B,*
184 *UBE2G1*, *UBE2Z*, *UBE2H, UBE2J2, UBE2S* & *UBE2D2*.

185 Canonical pathway analysis by Ingenuity Pathway Analysis (IPA) revealed contrast in
186 signaling pathways in Crossbred and Tharparkar

187 Canonical pathways associated with Crossbred and Tharparkar are represented in Figure
188 5a and 5b. In Crossbred, Oncostatin M Signaling, Phospholipase C Signaling, EIF2 Signaling,

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189 Integrin Signaling, IL-3 Signaling, and CXCR4 Signaling were found to be highly inactivated (Z
190 – score > 2.0) and PTEN signaling was found to be highly activated (Z – score < 2.0). In
191 Tharparkar, EIF2 Signaling, Androgen Signaling, Oncostatin M Signaling, α-Adrenergic
192 Signaling, BMP signaling pathway, and UVC-Induced MAPK Signaling were found to be highly
193 activated and PTEN signaling was found to be inactivated. The canonical pathway Oncostatin M
194 Signaling and EIF2 Signaling were found to have the highest ratio of genes involved vis-a-vis
195 the genes in the database in Crossbred and Tharparkar, respectively.

196 While carrying out comparative analysis through IPA, Calcium-induced T Lymphocyte
197 Apoptosis, BMP signaling pathway, UVC-Induced MAPK Signaling, Regulation of Cellular
198 Mechanics by Calpain Protease, fMLP Signaling in Neutrophils, Melatonin Signaling, and
199 Leukocyte Extravasation Signaling, were found inactivated in Crossbred and activated in
200 Tharparkar (Supplementary Figure 2). Genes involved in Oncostatin M Signaling- Growth
201 factor receptor-bound protein 2 *(GRB2),* GTPase HRas *(HRAS),* Janus kinase 1 *(JAK1),* Janus
202 kinase 3 *(JAK3),* Mitogen-activated protein kinase kinase 1 *(MAP2K1),* Mitogen-activated
203 protein kinase 1 *(MAPK1),* Oncostatin-M *(OSM),* Ras-related protein Rap-1b *(RAP1B),* Ras-
204 related protein Rap-2a *(RAP2A),* Signal transducer and activator of transcription 1-alpha/beta
205 *(STAT1),* Signal transducer and activator of transcription 5B *(STAT5B),* Non-receptor tyrosine-
206 protein kinase *(TYK2)*, and Ras-related protein *(RRAS)* were found downregulated in Crossbred
207 and upregulated in Tharparkar (Figure 6a, b). While the key genes involved in PTEN Signaling
208 pathway – Fas Ligand (*FASLG),* member of RAS oncogene family *(RAP2A),* Bcl-2-like protein
209 11 *(BIM),*Caspase-3 *(CASP3)* and microspherule protein 1 (*MSP58)* were found upregulated in
210 Crossbred and downregulated in Tharparkar as well (Figure 7a, b).

211 **Variation in microRNAs and Transcription factors**

212 IPA, on evaluating the differentially expression genes predicts miRNAs and
213 Transcription Factors (upstream regulators). In Crossbred, 111 miRNAs were found to be
214 inactivated and 37 activated. In Tharparkar, 205 miRNAs were found to be inactivated and 272
215 activated. Among them, 52 microRNAs were found common between the two genetic groups.
216 Most of the common miRNAs were found activated in Crossbred and inactivated in Tharparkar
217 (Supplementary Figure 3). miR-4779, miR-4651, miR-1207-5p, miR-6967-5p and miR-504-3p
218 are the top 5 miRNAs that were activated in Crossbred and inactivated in Tharparkar.

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219 Various Transcription factors were found to regulate the expression of the identified
220 DEGs. Transcription factors, 19 in Tharparkar (11 activated and 8 inactivated) and 26 in
221 Crossbred (8 activated and 18 inactivated) were identified in IPA that regulate the expression of
222 DEGs. Among them, *PAX5, MTA3, MYC, PROX1* and *SMAD7* in Crossbred and, *HMGA1, MAF,*
223 *MAX NOTCH22* and *NCOR1* in Tharparkar are the top 5 upregulated and activated TFs. On
224 comparing the TFs of Tharparkar and Crossbred, it was found that *BHLHE40, HMGA1,*
225 *HMGB1, IKZF1,* and *TCF7* were found to be common. *BHLHE40, HMGA1,* and *TCF7* were
226 found to be activated in Tharparkar and inactivated in Crossbred and it was vice - versa with
227 *HMGB1* and *IKZF1* (Supplementary Figure 4)

228 **Real-time validation.**

229 Six genes (*HSF1, SOD1, CALR, GSK3A, CAT* & *GPX3*) that were upregulated in
230 Tharparkar but downregulated/not expressed in crossbred and four genes (*CASP3, FASLG,*
231 *BCL2L11* & *APC*) that were upregulated in crossbred but downregulated in Tharparkar were
232 considered for Real time PCR based on their role in heat stress. Briefly, CALR is involved in
233 unfolded protein response upon Endoplasmic reticulum stress; HSF1 in trimer induces the
234 expression of HSPs; GSK3A, kinase inhibits trimerization of HSF1 regulating the expression of
235 HSPs; SOD1 converts free radical into hydrogen peroxide, which is further converted by GPX3A
236 and/or CAT into water and oxygen molecules in regulatory pathway of ROS scavenging and ;
237 BCL2L11, FASLG, APC and CASP3 induce apoptosis in stressed cell. The expression of genes
238 was in concordance with the RNA- Seq results (Supplementary Figure 5 and Supplementary
239 table 2).

240 Discussion

241 Heat stress is a natural phenomenon that affects domestic animals in tropical, sub-tropical
242 and often in temperate regions of the world during summer months. Heat and humidity during
243 the summer months combine to make an uncomfortable environment for dairy cattle. Heat stress
244 negatively impacts a variety of dairy parameters resulting in economic losses [33]. Response to
245 heat stress varies with species and genetic groups within species [5,34,35]. In this study,
246 transcriptome of genetic groups – Crossbred and Tharparkar cattle under heat stress was
247 evaluated to understand their differential response to heat stress.

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248 Animals (n=5) of both the genetic groups were exposed to a temperature of 42 °C for 7
249 days. Around 5th- 6th day, short term heat acclimation occurs [30,31]. This time point was selected
250 to understand the differences in systems biology to heat stress in the two genetic groups.
251 Initially, heat stress indicators - RR, RT, and T3 level were evaluated. RR was found to increase
252 in both genetic groups under heat treatment and the increase in Crossbred was found to be
253 significantly ($P<0.05$) different from that in Tharparkar. A positive correlation exists between
254 RR and heat treatment [36-38]. This increase is an attempt to dissipate excess body heat by
255 vaporizing more moisture in expired air or response to a greater requirement of oxygen by
256 tissues under heat stress. Also, the physiological response to heat stress includes reduced heat
257 production, which is achieved by lowering feed intake and thyroid hormone secretion [39]. T3 level
258 increases under heat stress [40,41]. A significant increase in T3 level in Crossbred as compared to
259 Tharparkar indicates an effective regulatory mechanism in modulating T3 levels in Tharparkar in
260 response to heat stress. The T3 triggered metabolism may be one of the reasons that increases
261 heat production resulting in high rectal temperature in Crossbred in comparison to Tharparkar as
262 was found in our study. The significant increase in RR, RT and T3 level in Crossbreed than in
263 Tharparkar, suggests the inability of Crossbred to cope up with heat stress in comparison to
264 Tharparkar.

265 A phenotype is defined by the changes in systems biology. Transcriptome profiling by
266 RNA-seq is the most common methodology to study the changes in systems biology. RNA
267 profiling based on next-generation sequencing enables to measure and compare gene expression
268 patterns[21]. The transcriptome of Tharparkar and Crossbred indicated differential response to heat
269 stress as evident from the DEGs, which were either distinct to both or have a difference in
270 expression. The number of DEGs were higher in Crossbred than in Tharparkar, suggesting a
271 greater dysregulation in systems biology in Crossbred. Among the dysregulated genes, the
272 number of upregulated genes were more than the downregulated genes in both genetic groups.
273 However, a contrast in expression was observed with 18.5 % of upregulated genes in Crossbred,
274 were downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar
275 weredownregulated in Crossbred. Some of the genes were confirmed for their expression by
276 Realtime PCR. The genes selected for Realtime PCR were selected based on their role in the
277 major physiological processes usually associated with heat stress.

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278 IPA revealed activation or inactivation of several pathways in both the genetic groups. It
279 is known that - EIF2 signalling, helps in initiation of global protein translation [42]; MAPK-
280 signalling pathway, induces cell proliferation [43]; androgen signalling, enhances pro-survival and
281 anti-apoptotic activity in cell [44]; a-Adrenergic signalling, maintains immune defence mechanism
282 [45] and, helps in tissue repair upon stress [46] and increases angiogenesis [47]; integrin pathway, resists
283 the cell against apoptosis and other environmental insults [48]; IL-3 signalling, aids in cell survival
284 and haematopoiesis [49]; CXCR4 signalling modulates cell survival and cell motility [50] and ;
285 Phospholipase C signalling aids in cell survival in stress through protein kinase C dependent
286 phosphorylation of BCL-2 [51]. Inactivation of these pathways except MAPK-signalling pathway
287 in Crossbred and activation of α-Adrenergic signalling, Androgen signalling, EIF2 signalling
288 and MAPK signalling in Tharparkar indicates that the systems biology in Tharparkar is moving
289 towards countering the effects due to heat stress.

290 While exploring the DEGs at a functional level we considered a knowledge-based
291 approach. Under this, four major physiological processes are found to be usually associated with
292 heat stress viz. elicitation of unfolded protein response (UPR) in cells; Ubiquitination; Induction
293 of apoptosis and; Imbalance in production of ROS and antioxidants (Figure 13). Heat shock and
294 its associated genes are involved in elicitation of unfolded protein response (UPR) in cells. Most
295 of the heat shock genes were found upregulated in Tharparkar and downregulated in Crossbred.
296 The increased HSP levels have been found positively correlated with tolerance in many
297 species[52,53]. HSF1, that positively regulates the transcription of *HSP70* and *HSP90* [54,55] was
298 found upregulated in Tharparkar and downregulated in Crossbred. Upregulation of *HSF1,*
299 *HSP70 and HSP90* in Tharparkar and vice-versa in Crossbred corroborates to state that
300 Tharparkar is better equipped to counter heat stress than Crossbred. Further, to ensure that the
301 HSP70 in Tharparkar is maintained at an optimum level, dysregulation of *CAMK2D* and *GSK3A*
302 seems to act as negative feedback. CAMK2D that induces the transcription of HSP70 via HSF1
303 [56] was found downregulated in Tharparkar. GSK3A that inhibits the trimerization of HSF1 that is
304 needed for the induction of HSP70 [57] was found upregulated in Tharparkar. The decreased level
305 of HSP70 in Crossbred makes it inevitable that such negative feedbacks would further reduce its
306 level and hence, *GSK3A* was found downregulated and *CAMK*, upregulated (Figure.13).

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307 Ubiquitination is positively correlated with heat tolerance [58,59]. Ubiquitin-Proteasome
308 System (UPS) regulates the levels of proteins and acts by removing the misfolded or damaged
309 proteins that may accumulate as a result of exposure to abiotic stress. Malfunctioning of
310 ubiquitin-proteasome system (UPS) could have negative consequences for protein regulation,
311 including loss of function [60]. In Tharparkar after heat acclimation, HSP70 tends to activate the
312 ubiquitination pathway to minimize the accumulation of the unfolded proteins that can't be
313 refolded by it [61]. This pathway activation is supported by upregulation of E2 ligases - *UBE2G1,*
314 *UBE2S*, and *UBE2H* that catalyze covalent attachment of E2 to E3 [62-65] in Tharparkar. USP7
315 that deubiquitinates target proteins [66,67] was found upregulated in Crossbred and downregulated
316 in Tharparkar. Further, a group of molecules – *VCP, SERP1*, and *CALR* that ensure the
317 protection of naïve proteins during their transport within the cell [68-70] were found upregulated in
318 Tharparkar and downregulated in Crossbred. Unlike Crossbred, Tharparkar is not only endowed
319 with higher expression of the scavengers of misfolded proteins but also with protectors of naïve
320 unfolded proteins.

321 Activation of apoptosis pathway is one of the major physiological processes linked with
322 heat stress. Among the apoptotic genes, *BCL2L11, FASLG, MLKL, CASP3, MAPK8,* and *VIM*
323 have been found upregulated in Crossbred and downregulated in Tharparkar under heat stress.
324 BCL2L11 induces apoptosis by neutralizing key molecules of pro-survival BCL2 sub-family
325 [71,72], FASLG transduces the apoptotic signal into cells[73,74], CASP3 activates caspases and
326 executes apoptosis [75], and MAPK8, MLKL, and VIM also induce apoptosis [76,77]. PTEN
327 signaling pathway that drives apoptosis [78,79] was found inactivated in Tharparkar and activated in
328 Crossbred. This indicates a relatively higher probability of apoptosis in Crossbred than in
329 Tharparkar.

330 The ability to balance the ROS and antioxidant level, is one of the key factors that would
331 determine the tolerance of an individual to heat stress. The antioxidant triad of GPX, SOD, and
332 CAT that forms the first line of defense against reactive oxygen species [80-82], was found
333 upregulated in Tharparkar and downregulated in Crossbred. Additionally, genes belonging to
334 Peroxiredoxins - *PRDX3, PRDX5* and *PRDX6* that catalyze the reduction of hydrogen peroxide
335 and organic hydroperoxides [83-87],were also found upregulated in Tharparkar and were either
336 downregulated or not-differentially expressed in Crossbred. Higher expression of the

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337 antioxidants in Tharparkar enables it to cope up with higher levels of free radicals generated as a
338 result of heat stress while Crossbred is unable to do so.

339 Conclusion

340 A contrast in expression was observed with 18.5 % of upregulated genes in Crossbred
341 were downregulated in Tharparkar and 17.5% upregulated genes in Tharparkar were
342 downregulated in Crossbred. Transcripts of molecules that stimulate heat shock response,
343 Ubiquitination, unfolded protein response and antioxidant level were found upregulated in
344 Tharparkar and downregulated in Crossbred. EIF2 Signaling that promotes protein translation
345 and PTEN signaling that drives apoptosis were found activated and inactivated in Tharparkar,
346 respectively and vice-versa in Crossbred. We found relevant molecules/genes dysregulated in
347 Tharparkar in the direction that counters heat stress. A proposed contrasting interplay of
348 molecules in both the two groups is shown in Figure 8. To the best of our knowledge this is a
349 comprehensive comparison between Tharparkar and Crossbred at a global level using
350 transcriptome analysis.

351 Methods

352 Experimental condition and Ethical Statement

353 The animals used for the study were from the Indian Veterinary Research Institute. The
354 permission to conduct the study was granted by Indian Veterinary Research Institutional Animal
355 Ethics Committee (IVRI-IAEC) under the Committee for Control and Supervision of
356 Experiments on Animals (CPCSEA), India, vide letter no 387/CPSCEA. Genetic groups -
357 Tharparkar (Indigenous breeds) and Vrindavani (synthetic Crossbred) were considered in this
358 study. Prior to experiment, the animals – 05 Tharparkar and 05 Crossbred (Vrindavani) cattle,
359 were acclimatized for 15 days outside the Psychometric chamber. The experiment was conducted
360 during October when the environmental Temperature Humidity Index (THI) was 73.0242. These
361 animals were exposed in Psychometric chamber at 42 °C for six hours for 7 days (THI
362 =78.5489). All the animals were fed with wheat straw and concentrate mixture in 60:40 ratios.
363 Respiration rate (RR) and rectal temperature (RT) of animals from each genetic group were
364 measured on 0 day (Control, n=5) before exposure to Psychometric chamber and on 7th day of
365 heat exposure (Treated, n=5). Blood samples were collected from the animals at the mentioned

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366 time points and serum concentration of Triiodothyronine (T3) was estimated by RIA technique
367 using T_3 ^{125}I (Immunotech) as per the manufacturer's instructions.

368 **RNA sequencing** (RNA-seq)

369 PBMCs were collected from the blood samples using Ficol histopaque gradient method**.**
370 Total RNA from each of the collected samples (PBMCs) was isolated using the RNeasy Mini kit
371 (Qiagen GmbH, Germany) according to the manufacturer's protocol. The integrity and quantity
372 of isolated RNA were assessed on a Bioanalyzer 2100 (Agilent Technologies, Inc). The library
373 was prepared using NEBNext Ultra RNA Library Prep Kit for Illumina (NewEngland Biolabs
374 Inc.) following the manufacturer's protocol. Approximately, 100ng of RNA from each sample
375 was used for RNA library preparation. The quality of the libraries was assessed on Bioanalyzer.
376 Libraries were quantified using a Qubit 2.0 Fluorometer (Life technologies) and by qPCR.
377 Library (1.3ml, 1.8pM) was denatured, diluted and loaded onto a flow cell for sequencing.
378 cDNA library preparation and Illumina Sequencing was performed at Sandor Life Sciences Pvt.
379 (Hyderabad, India). Finally, the RNA-seq data were provided in FASTQ format.

380 **Data processing**

381 The sequenced reads were paired-end and 150bp in length. Quality control checks on raw
382 sequence data from each sample were performed using FastQC (Babraham Bioinformatics).
383 Processing of the data was performed using prinseq-lite software [88] to remove reads of low
384 quality (mean phred score 25) and short length (< 50) for downstream analysis. The data was
385 submitted to the GEO database with accession number GSE136652.

386 **Identification of Differentially Expressed Genes (DEGs)**

387 *Bos taurus* reference genome (release 94) and its associated gene transfer file (GTF) were
388 downloaded from Ensembl FTP genome browser [89]. The reference genome was prepared and
389 indexed by RNA-Seq by expectation maximization (RSEM) [90] by rsem-prepare-reference
390 command. Further, the clean reads obtained from filtering of raw data were aligned to the
391 indexed reference genome by Bowtie2 [91] to estimate gene abundance in counts by rsem-
392 calculate-expression command. To compare the gene expression levels among different samples,
393 the aligned reads were used to generate a data matrix by rsem-generate-data-matrix command.
394 In each genetic group, all the samples of day 7 (treated) were compared with the day 0 (Control)

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395 for the calculation of differential gene expression by edgeR [92] package. The Ensemble IDs of the
396 differentially expressed genes (DEGs) were converted to the respective gene ID by g: Convert of
397 g: Profiler [93,94].

398 **Functional Analysis of DEGs**

399 Under heat stress four major physiological processes are found to be usually associated -
400 Induction of apoptosis [95,96]; Ubiquitination [97,98]; elicitation of unfolded protein response (UPR)
401 in cells [99] and ; Imbalance in production of ROS and antioxidants [100,101]. The genes involved in
402 these processes were retrieved from Reactome database [102]. From these genes that are involved
403 in the four major physiological processes, the genes that were differentially expressed in both the
404 genetic groups were extracted to compare and contrast their expression between genetic groups.
405 These genes were referred to as knowledge-based genes. Their protein-protein interaction
406 network was also studied in both the genetic groups.

407 **Predicted protein-protein interaction of the knowledge-based genes**

408 Protein-protein interaction (PPI) network among the knowledge-based DEGs that were
409 found common to both Tharparkar and Crossbred, was retrieved using interactions available in
410 the String database [103]. The degree was calculated using igraph package (https://cran.r-
411 project.org/web/packages/igraph/ index.html). The PPI network was then visualized using
412 Cytoscape software V. 3.7 [104]

413 **Ingenuity Pathway Analysis (IPA) Analysis**

414 QIAGEN's IPA (QIAGEN, Redwood City, USA) [105] is used to quickly visualize and
415 understand complex omics data and perform insightful data analysis and interpretation by
416 placing experimental results within the context of biological systems. Here, IPA was used to
417 analyze the identified DEGs of Crossbred and Tharparkar.The list of DEGs from each genetic
418 group was used to identify the canonical pathways and the most significant biological processes
419 against Ingenuity Pathways Knowledge Base (IKB). Core analysis for each dataset was
420 performed to know activated (Z score > 2) or inactivated (Z score < -2) canonical pathways.
421 Upstream regulators - Transcription factors and microRNAs were also identified.

422 **Validation of reference genes identified**

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423 Genes - *BCL2L11, FASLG, CASP3, CAT, SOD1, GSK3A, CALR, HSF1, APC,* and *GPX3*
424 were selected based on their role in heat stress and qRT-PCR was performed on Applied
425 Biosystems 7500 Fast system. *GAPDH* was taken as the internal control. Each of the samples
426 was run in triplicates and relative expression of each gene was calculated using the
427 $2^{-\Delta\Delta CT}$ method with control as the calibrator [106].

428 **Statistical Analysis**

429 Respiration rate, Rectal temperature and T3 level were compared using student's *t*-test in
430 JMP9 (SAS Institute Inc., Cary, USA) to test the significance of the difference between the
431 control (0 day) and treated (7th day). This comparison was done within and between genetic
432 groups. Differences within/between groups were considered significant at $P \leq 0.05$.

433 ## Declarations

434 **Ethics approval and consent to participate**

435 The permission to conduct the study was granted by Indian Veterinary Research Institutional
436 Animal Ethics Committee (IVRI-IAEC) under the Committee for Control and Supervision of
437 Experiments on Animals (CPCSEA), India, vide letter no 387/CPSCEA.

438 **Consent for publication**

439 Not applicable.

440 **Availability of data and materials**

441 The data was submitted to the GEO database with accession number GSE136652

442 **Competing interests**

443 None of the authors had a conflict of interest to declare

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446 Identification of unique factors in indigenous livestock making them resilient to climate change

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448 procurement of License of IPA.

449 **Authors' contributions**

450 AKT and RG conceived and designed the research. SG, SmS, AS,AV, VV , PK , ShS and GS
451 conducted the wet lab work. RINK, ARS, NH, WAM, MRP, SK , AP and RG analyzed the data.
452 RINK, ARS, MRP, RG , AS and GS helped in manuscript drafting and editing. AKT and RG
453 proofread the manuscript

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700 **Legends**

701 **Figure 1**: Overview of the work done : Two genetic groups (Tharparkar and Crossbred) of cattle
702 were exposed to a temperature of 42 °C for 7 days. Heat stress indicators - Respiration rate (RR),
703 Rectal temperature and T3 level before exposure to heat (0day – control group) and at 7th day of
704 exposure (treated) were measured to evaluate heat stress. At these time points, RNA was isolated
705 from PBMCs for high throughput sequencing. Transcriptome analysis was done to identify
706 differentially expressed genes (DEGs) under heat treatment in both genetic groups. Genes
707 involved in physiological processes (heat stress response, apoptosis, ubiquitination, unfolded
708 protein response and antioxidant level) that are commonly associated with heat stress were

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709 compared between the two genetic groups. Further, functional annotation of DEGs was done
710 using IPA.

711 **Figure 2**: Respiration rate, Rectal Temperature and T3 level measured at 0 day (control) and 7
712 day post-heat exposure (treated) in Crossbred and Tharparkar (n=5) Levels sharing the same
713 superscript are not significantly ($P > 0.05$) different from each other.

714 **Figure 3**: Expression of DEGs in Crossbred and Tharparkar under heat stress: (a) Venn diagrams
715 showing unique/common DEGs between Crossbred and Tharparkar (b) Number of upregulated
716 and downregulated in both genetic groups (c) Contrast in the expression of common DEGs

717 **Figure 4:** Contrast in the expression of genes involved in heat stress response, apoptosis,
718 ubiquitination, unfolded protein response and balance in the production of ROS and antioxidants
719 between two genetic groups.

720 **Figure 5 :** Canonical pathways activated/inactivated in (a) Crossbred (b) Tharparkar under heat
721 stress generated in the core analysis of Ingenuity pathway analysis tool. Orange color pathways
722 are activated ($Z > 2$) and blue color pathways are inactivated ($Z< -2$). Height of the bar graphs
723 indicates -log (p-value) and line graph showing the ratio of list genes found in each pathway over
724 the total number of genes in that pathway.

725 **Figure 6:** Canonical pathways generated in Ingenuity Pathway Analysis of Oncostatin M
726 signaling pathway of DEGs in (A) Crossbred, (B) Tharparkar. Genes that were upregulated are
727 shown in red and downregulated in green. The intensity of red and green corresponds to an
728 increase and decrease, respectively, in Log2 fold change. Genes in grey were not significantly
729 dysregulated and those in white are not present in the dataset but have been incorporated in the
730 network through the relationship with other molecules by IPA.

731 **Figure 7:** Canonical pathways generated in Ingenuity Pathway Analysis of PTEN signaling
732 pathway of DEGs in (A) Crossbred, (B) Tharparkar. Genes that were upregulated are shown in
733 red and downregulated in green. The intensity of red and green corresponds to an increase and
734 decrease, respectively, in Log2 fold change. Genes in grey were not significantly dysregulated
735 and those in white are not present in the dataset but have been incorporated in the network
736 through the relationship with other molecules by IPA.

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737 **Figure 8: Predicted interplay of molecules that is underway during heat stress in both**
738 **groups :** Heat stress causes unfolding of native proteins. HSP70 acts as a chaperone to facilitate
739 refolding to restore the structure of unfolded proteins. Under normal condition, HSP70 is bound
740 to HSF1 thereby preventing HSF1 to promote transcription of HSP70. Under heat stress ATP
741 binds to the HSP70 and HSF1 complex to release HSF1, promoting the binding of the unfolded
742 protein to HSP70 and ATP. CAMK2D that induces the transcription of HSP70 via HSF1 was
743 found downregulated in Tharparkar. GSK3A that inhibits the trimerization of HSF1 that is
744 needed for the induction of HSP70 expression was found upregulated in Tharparkar. The
745 decreased level of HSP70 in Crossbred makes it inevitable that such negative feedbacks would
746 further reduce its level and GSK3A was found downregulated and CAMK2D, upregulated.
747 Further, in Tharparkar, HSP70 tends to activate ubiquitination pathway to decrease the
748 accumulation of unfolded proteins that can't be refolded by it. This pathway activation is
749 supported by upregulation of E3 ligases (UBE2G1, UBE2S, and UBE2H) in Tharparkar.
750 However, the E3 ligase in Crossbred was found downregulated. With HSP70 being upregulated
751 and having cytoprotection activity, Tharparkar shows the decline in apoptosis as compared to
752 Crossbred. This is supported by downregulation of BCL2L11, FASLG, MLKL, CASP3,
753 MAPK8 and VIM in Tharparkar and vice-versa. Besides, higher expression of the antioxidants
754 (SOD, CAT, GPX) in Tharparkar enables it to cope up with higher levels of free radicals
755 generated as a result of heat stress while Crossbred is unable to do so. Green arrow indicates
756 downregulation and Maroon arrow indicates upregulation.

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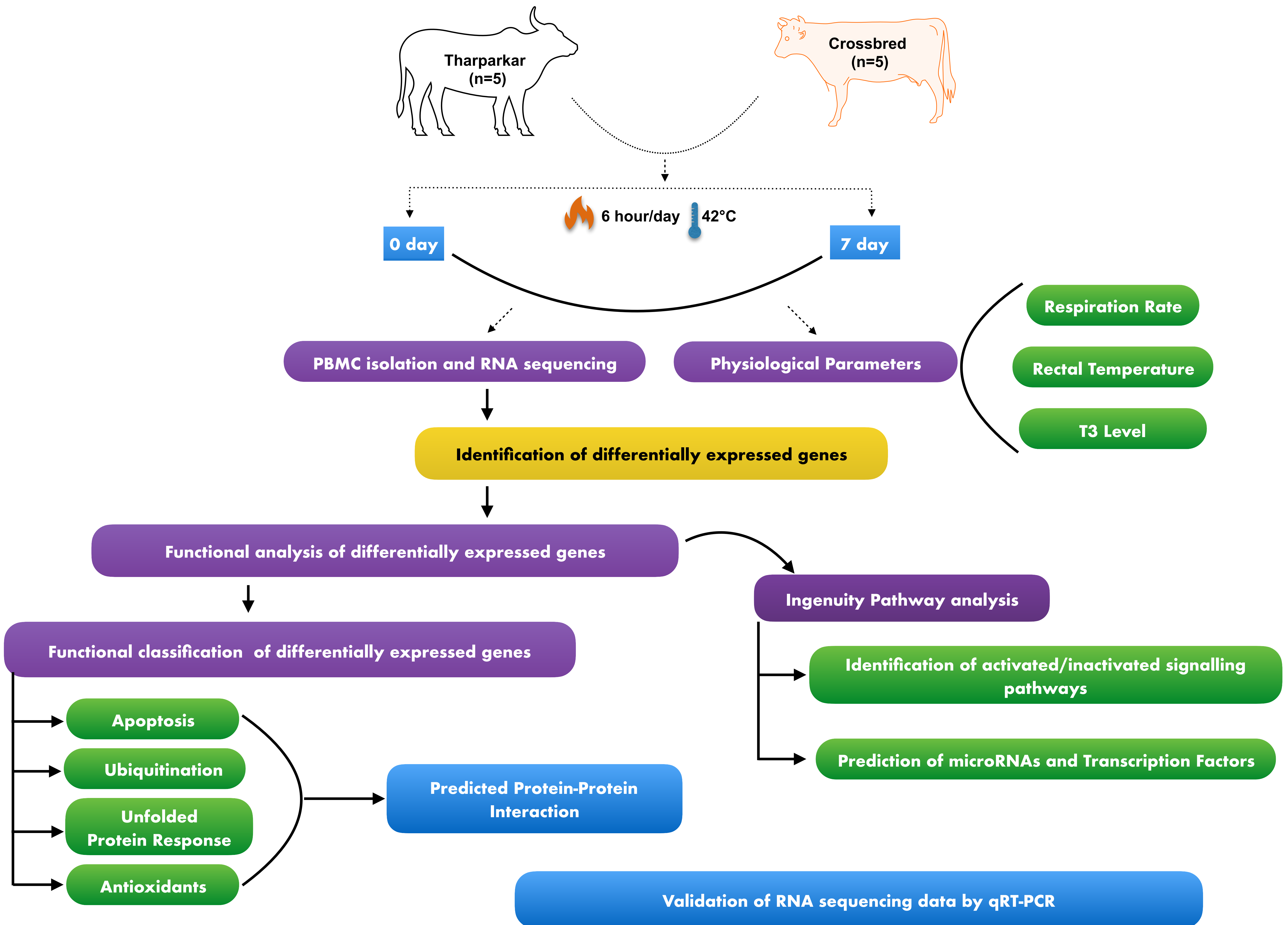
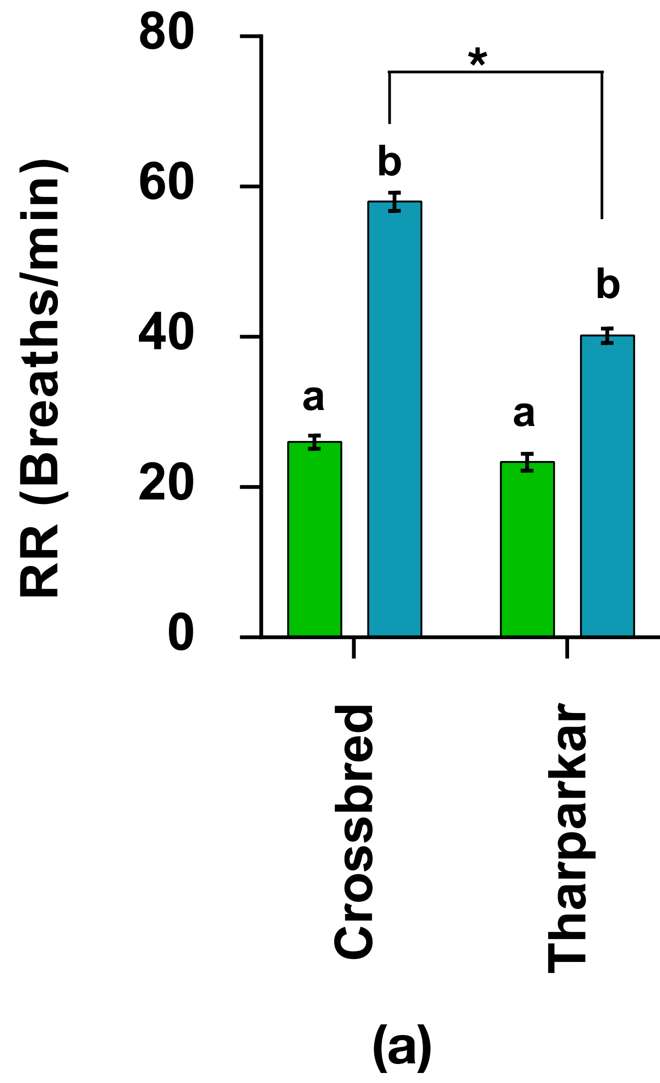
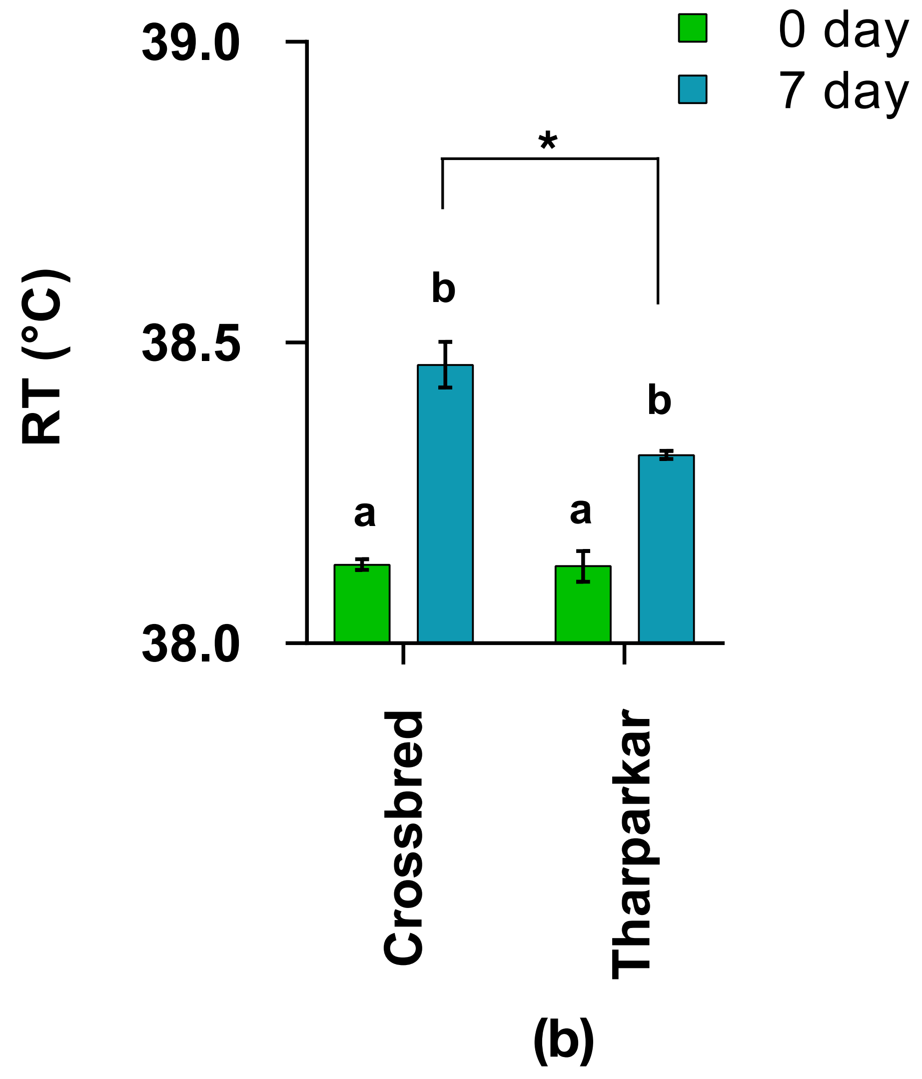
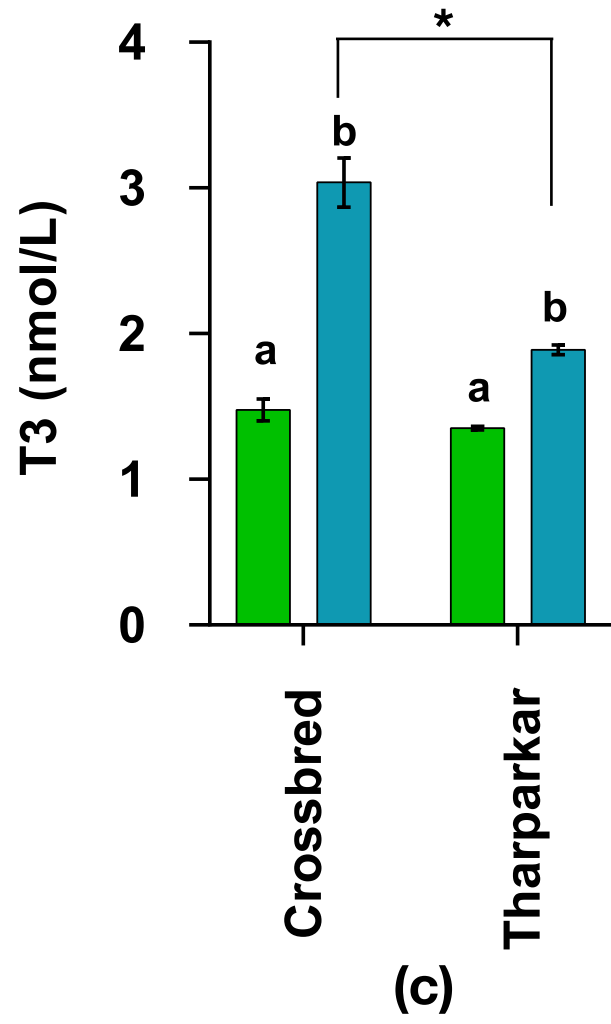
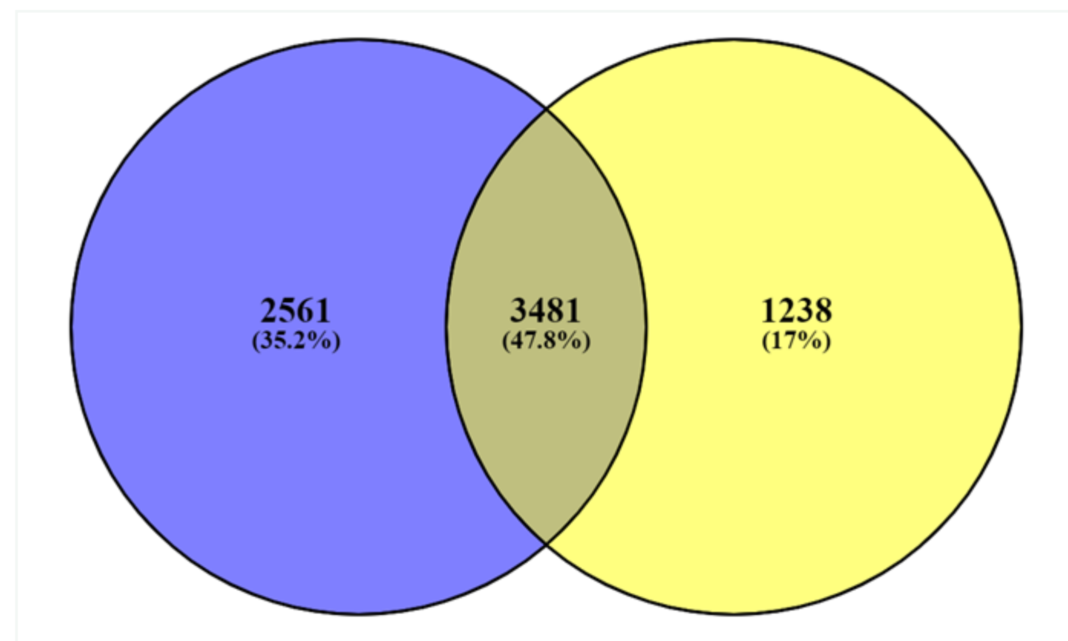


Tharparkar
(n=5)
Crossbred
(n=5)
6 hour/day
42°C
0 day
7 day
PBMC isolation and RNA sequencing
Physiological Parameters
Respiration Rate
Rectal Temperature
T3 Level
Identification of differentially expressed genes
Functional analysis of differentially expressed genes
Ingenuity Pathway analysis
Functional classification of differentially expressed genes
Identification of activated/inactivated signalling pathways
Apoptosis
Ubiquitination
Unfolded Protein Response
Antioxidants
Predicted Protein-Protein Interaction
Prediction of microRNAs and Transcription Factors
Validation of RNA sequencing data by qRT-PCR

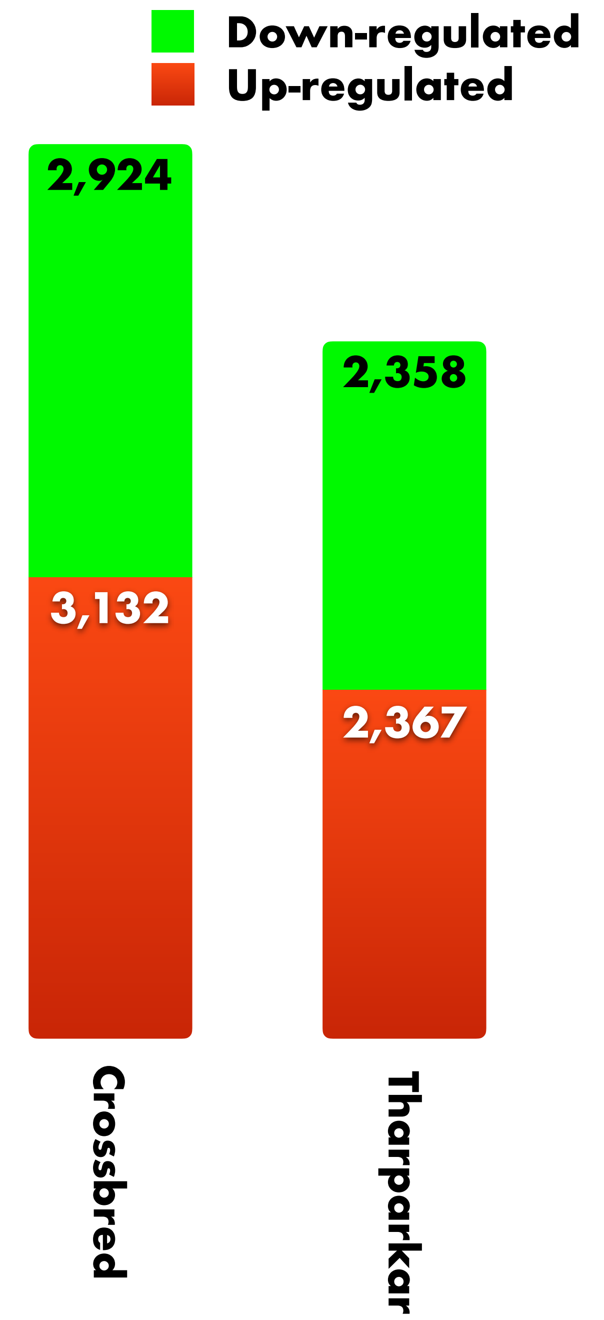

RR (Breaths/min)
80
60
40
20
0
*
a
b
a
b
Crossbred
Tharparkar
(a)


0 day
7 day
RT (°C)
39.0
38.5
38.0
*
a
b
a
b
Crossbred
Tharparkar
(b)


T3 (nmol/L)
4
3
2
1
0
*
a
b
a
b
Crossbred
Tharparkar
(c)



(a)



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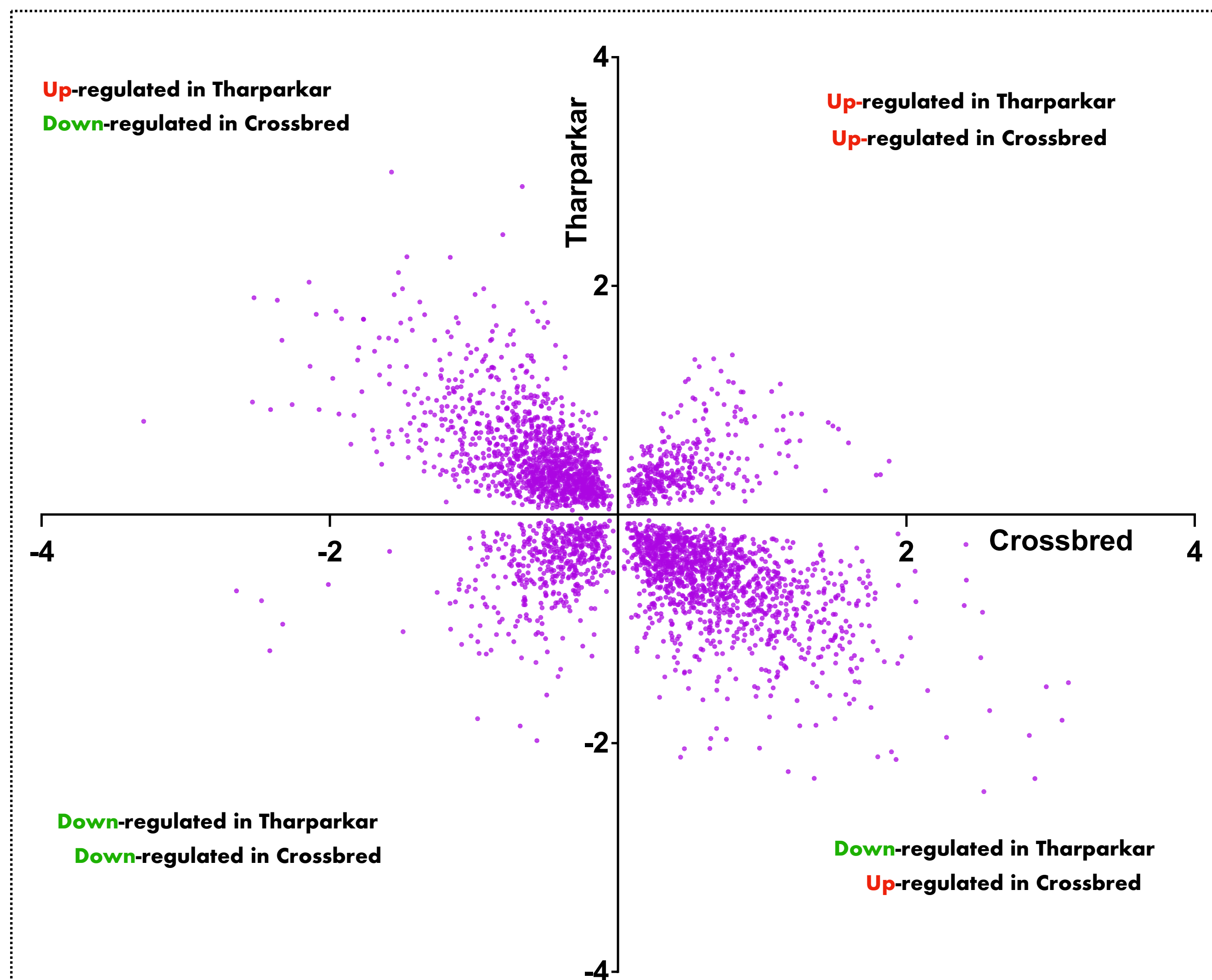

(c)

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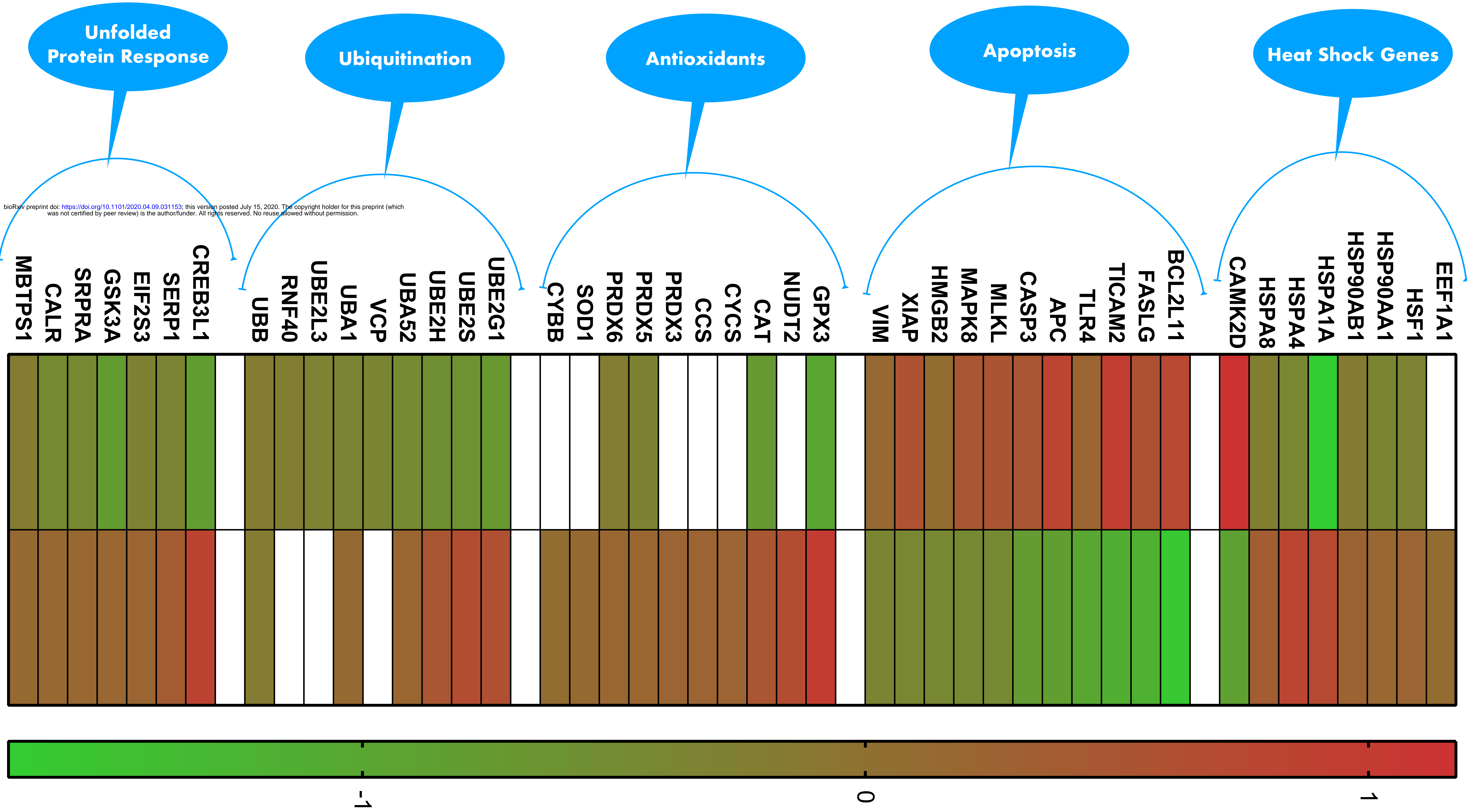
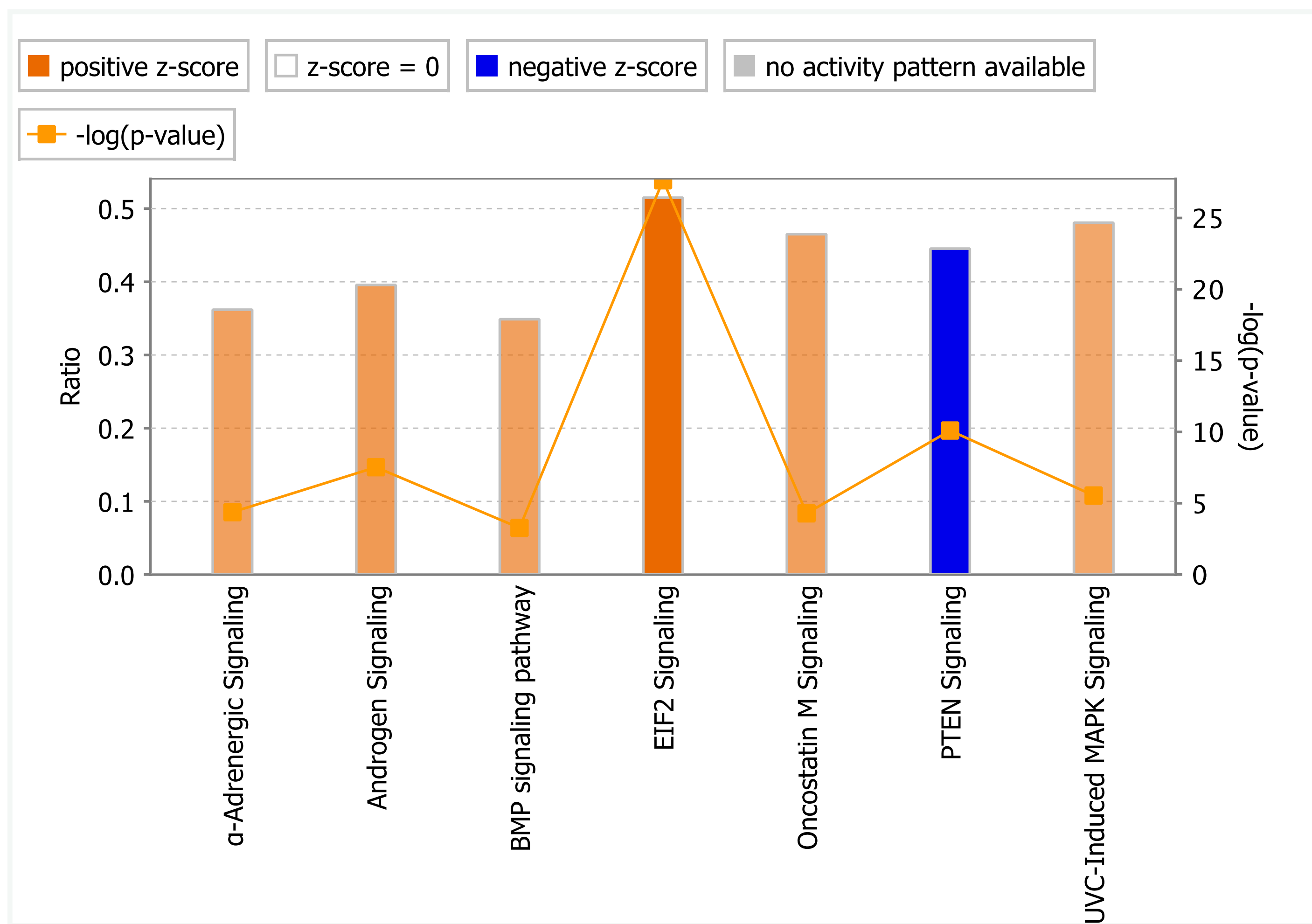


(a)



(b)

OSM
Extracellular space
OSMR β
GP 130
Cytoplasm
Ras
JAK
JAK
STAT 1/3/5
GRB2
SHC
SOS
c-Raf
STAT 1/3/5
MEK 1/2
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Nucleus
ERK 1/2
STAT 1/3/5
STAT 1/3/5
Elk1
MMP1 MMP3 MMP13 TIMP-3 CHI3L1 PLAU MT2A EPAS1

(a)

OSM
Extracellular space
OSMR β
GP 130
Cytoplasm
Ras
JAK
JAK
STAT 1/3/5
GRB2
SHC
SOS
c-Raf
STAT 1/3/5
MEK 1/2
STAT 1/3/5
STAT 1/3/5
ERK 1/2
Nucleus
ERK 1/2
STAT 1/3/5
STAT 1/3/5
Elk1
MMP1 MMP3 MMP13 TIMP-3 CHI3L1 PLAU MT2A EPAS1

(b)

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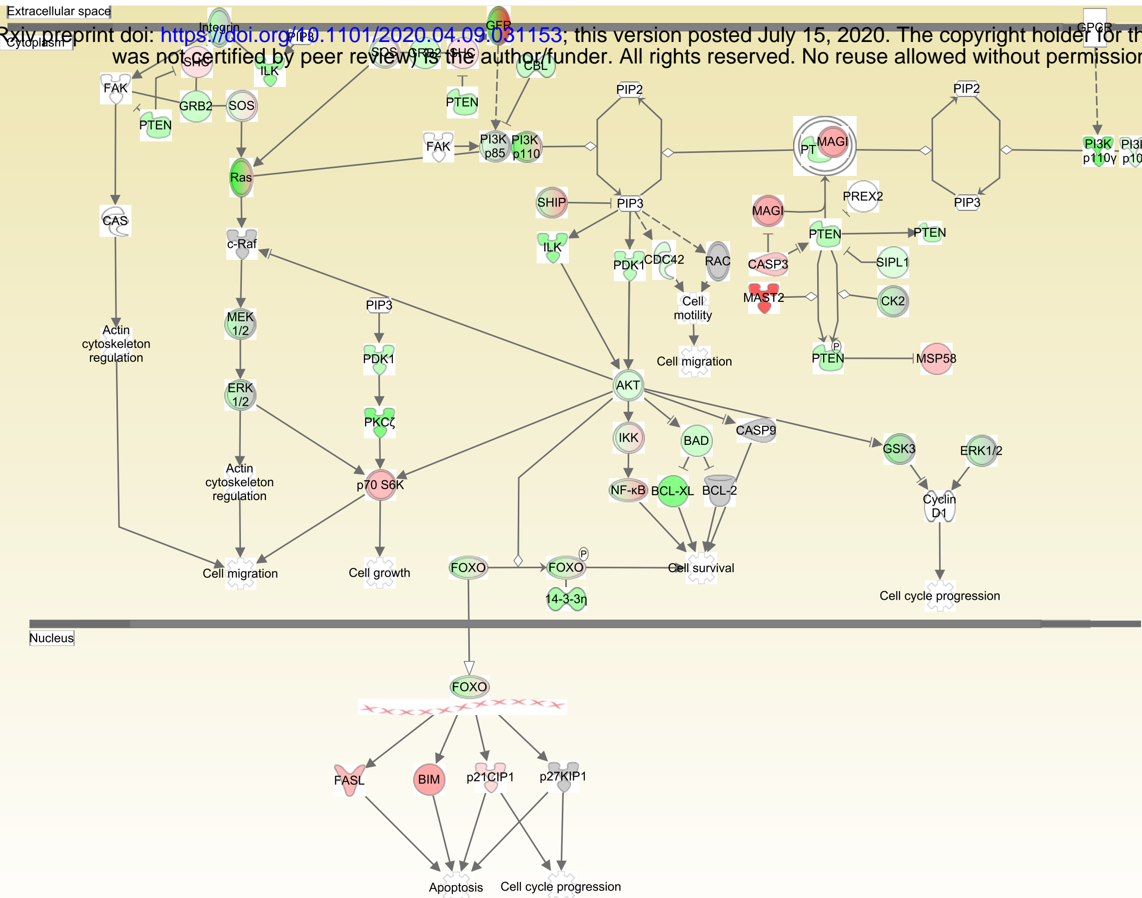
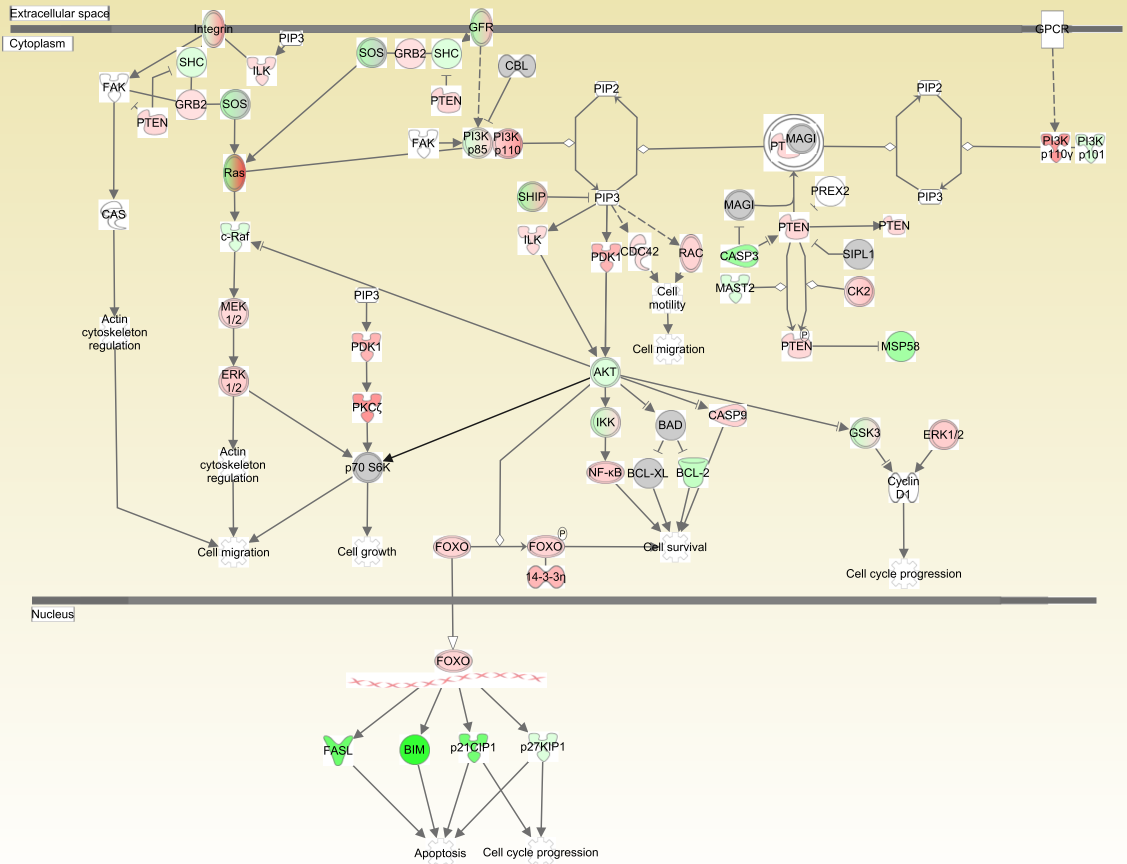


(a)



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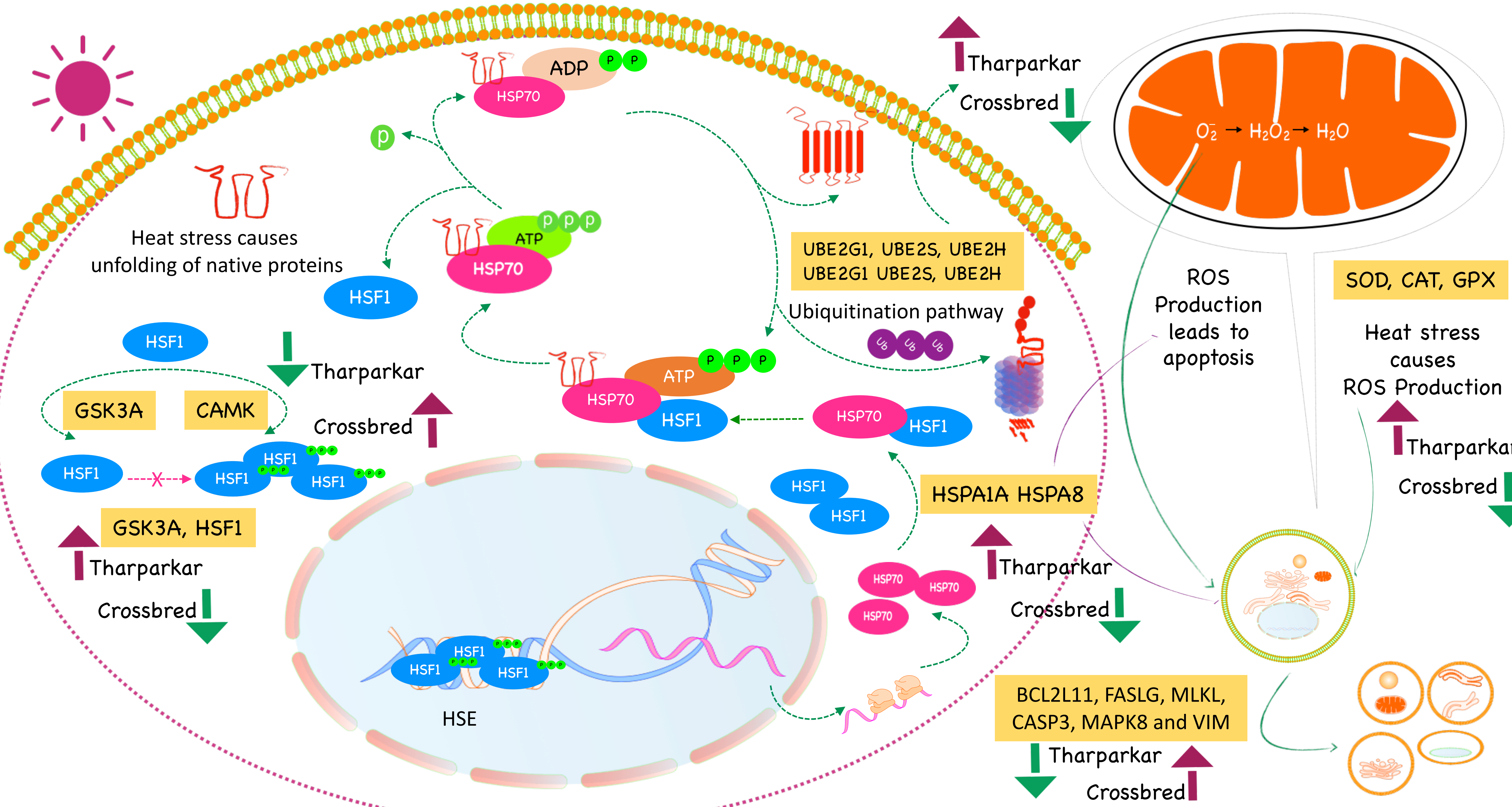

ADP
P P
HSP70
p
Heat stress causes
unfolding of native proteins
ATP
p p p
HSP70
HSF1
HSF1
Tharparkar
Crossbred
GSK3A
CAMK
HSF1
HSF1
HSF1
HSF1
GSK3A, HSF1
Tharparkar
Crossbred
ATP
P P P
HSP70
HSF1
HSP70
HSF1
Tharparkar
Crossbred
UBE2G1, UBE2S, UBE2H
UBE2G1 UBE2S, UBE2H
Ubiquitination pathway
Ub Ub Ub
HSF1
HSF1
HSPA1A HSPA8
Tharparkar
Crossbred
HSP70
HSP70
HSP70
HSF1
HSF1
HSF1
HSE
$O_2^- \rightarrow H_2O_2 \rightarrow H_2O$
ROS
Production
leads to
apoptosis
SOD, CAT, GPX
Heat stress
causes
ROS Production
Tharparkar
Crossbred
BCL2L11, FASLG, MLKL,
CASP3, MAPK8 and VIM
Tharparkar
Crossbred