

Article

Exploratory Genome-Wide Computational Analysis of Candidate MiRNA Regulatory Variants in Thermotolerant Dairy Cows

Maria-Anna Kyrgiafini ¹, Themistoklis Giannoulis ^{2,*}, Ioannis Nanas ³, Eleni Dovolou ⁴, Georgios S. Amiridis ^{3,*} and Zissis Mamuris ¹

¹ Laboratory of Genetics, Comparative and Evolutionary Biology, Department of Biochemistry & Biotechnology, University of Thessaly, 41500 Larissa, Greece; mariankyrgiafini@gmail.com (M.-A.K.); zmamur@uth.gr (Z.M.)

² Laboratory of Biology, Genetics and Bioinformatics, Department of Animal Science, University of Thessaly, 41500 Larissa, Greece

³ Department of Obstetrics and Reproduction, Veterinary Faculty, University of Thessaly, 43100 Karditsa, Greece; gnsnanas@gmail.com

⁴ Laboratory of Animal Reproduction, Department of Animal Science, University of Thessaly, 41500 Larissa, Greece; entovolou@uth.gr

* Correspondence: thgianno@uth.gr (T.G.); gsamir@uth.gr (G.S.A.)

Abstract

Heat stress poses a critical threat to dairy cattle productivity, fertility and welfare, yet the genetic mechanisms underlying individual variation in thermotolerance remain poorly understood. While previous studies have focused predominantly on miRNA expression profiles or coding gene variants, genome-wide identification of variants within miRNA genes has remained largely unexplored. In this exploratory study, pooled whole-genome sequencing was applied to previously characterized thermotolerant and thermosensitive Holstein cows, classified based on reproductive performance under summer heat stress and supported by physiological and endocrine characterization, to identify candidate genetic variants within miRNA genes. Fifteen variants were detected in the thermotolerant sequencing pools but not in the thermosensitive pools under the applied variant-calling and filtering criteria, across 15 distinct miRNA loci, including one potentially novel SNP. Five variants were located within mature miRNA sequences, and several were associated with predicted changes in minimum free energy (ΔG), suggesting potential differences in pre-miRNA secondary structure and thermodynamic stability. Two seed-region variants in bta-miR-2883 and bta-miR-2388-3p were associated with large computationally predicted differences in miRNA target repertoires, with gains of approximately 3000 predicted target genes and losses of approximately 600 per miRNA. Functional enrichment analysis of the computationally predicted target sets identified over-represented biological processes and pathways related to stress-responsive signaling pathways and metabolism among gained targets, and intracellular trafficking, vesicle-mediated transport, and membrane dynamics among lost targets. Collectively, these computational findings identify candidate variants within miRNA genes and generate hypotheses regarding their potential regulatory consequences, without establishing their functional involvement in thermotolerance. Individual-level genotyping, genotype–phenotype association analyses, and functional validation are required to determine their biological relevance.

Keywords: heat stress; miRNA; thermotolerance; whole-genome sequencing; post-transcriptional regulation; *Bos taurus*

Academic Editor: Fangxiong Shi

Received: 7 September 2026

Revised: 22 October 2026

Accepted: 23 October 2026

Published: 26 October 2026

Copyright: © 2026 by the authors. Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Climate change, especially the increase in temperature that is responsible for heat stress (HS), is at the forefront of the dangers that threaten the viability and sustainability of livestock farming. Especially for the dairy sector, HS exerts negative effects on health, reproductive and productive performance of dairy cows [1]. Exposure to elevated temperatures triggers a series of physiological and behavioral adaptations to mitigate the negative effects of HS, including increased respiration and heart rate, reduced feed intake and increased water consumption [2]. High metabolic heat production associated with high milk yield further increases the internal heat load, exacerbating the vulnerability to heat stress [3]. Collectively, these adaptations lead to a negative energy balance, responsible for reduced animal fitness and increased incidences of infectious and metabolic diseases, while significantly compromising milk yield and fertility [4,5]. This physiological discomfort is induced when environmental conditions exceed the upper tolerated threshold of the thermoneutral zone, commonly measured using the Temperature–Humidity Index (THI). Specifically, THI values below 68 indicate thermal comfort, values between 69 and 78 correspond to mild heat stress, values between 79 and 89 to moderate heat stress, and values exceeding 90 to severe heat stress, even leading to death [6,7]. Consequently, heat stress constitutes a complex challenge that adversely affects animal welfare and undermines the sustainability of livestock production systems, resulting in significant economic losses due to impairments in productive efficiency and reproductive performance. Thus, there is a need to elucidate the molecular, cellular and genetic components of thermotolerance, including regulatory pathways involved in stress response, and to integrate genomic information in breeding programs aiming at enhanced heat stress resilience.

Towards this goal, there are several studies that have attempted to shed light on the mechanisms and pathways involved in thermotolerance and highlight specific candidate markers for genetic/genomic selection. Early research primarily focused on the pivotal role of Heat Shock Proteins (HSPs) to elucidate the mechanisms underlying thermotolerance, as these proteins serve as universal cellular indicators responding to a diverse array of stressors [8]. HSPs are broadly classified by molecular weight into small (<40 kDa) and large (>40 kDa) families, both of which exhibit significant mRNA upregulation under heat stress (HS) across multiple cell types [9–11]. Their expression and transcriptional regulation via Heat Shock Factors (HSFs) have therefore been proposed as key molecular markers for thermotolerance and genetic selection [12–14]. However, while HSPs represent a core component of the cellular heat stress response, accumulating evidence indicates that thermotolerance arises from a far more complex and integrated regulatory network. Whole-genome sequencing and genome-wide association studies (GWASs) have identified heat stress-associated variants in genes such as *DNAJC27*, *DNAJA4*, *DNAJC21*, *DNAJB1*, and *HSPA1A*, implicating pathways linked not only to protein homeostasis but also to neuronal and immune system function [15–17]. Furthermore, transcriptomic studies have revealed tissue-specific adaptations, including alterations in hepatic oxidative phosphorylation and mitochondrial function that contribute to negative energy balance under heat stress [18], as well as long-term developmental and signaling alterations induced by preweaning thermal exposure [19]. Given the breadth and complexity of these heat stress-responsive pathways, precise post-transcriptional regulation is essential for maintaining cellular homeostasis and survival. In this context, miRNAs have emerged as primary regulatory candidates governing these complex biological processes, positioning them as promising candidates for understanding and modulating thermotolerance.

MicroRNAs (miRNAs) are endogenous small non-coding RNAs that regulate gene expression post-transcriptionally by binding to the 3' UTR of target mRNAs, inhibiting translation or promoting their degradation [20]. In recent years, miRNAs have emerged as central regulators of stress responses and reproductive function in livestock [21], with

differential expression of specific miRNAs documented in cattle tissues exposed to heat stress, implicating these molecules in the modulation of metabolic, immune, and developmental processes as well as HSP synthesis [22,23]. Beyond their regulatory roles, miRNAs are increasingly recognized as promising biomarkers of stress resilience and reproductive performance due to their stability in biological fluids and their ability to reflect physiological states [21,24].

Despite growing evidence that miRNAs play important roles in the physiological response to heat stress and reproductive processes, most existing studies have primarily focused on miRNA expression profiles rather than on genetic variation within miRNA genes. Similarly, genetic studies of thermotolerance in cattle have largely concentrated on polymorphisms in protein-coding genes, such as heat shock proteins and other stress-related loci, while sequence variation within miRNA genes remains comparatively understudied. However, single-nucleotide polymorphisms (SNPs) located within miRNA genes, particularly in precursor, mature, or seed regions, can influence miRNA biogenesis, stability, and target recognition, ultimately reshaping post-transcriptional regulatory networks [25]. Nevertheless, the contribution of miRNA sequence variation to heat stress adaptation in dairy cattle, as well as its potential utility as a biomarker for identifying thermotolerant animals, remains largely unexplored. Addressing this gap could provide novel insights into the molecular mechanisms underlying thermotolerance and support the development of genomics-assisted breeding strategies for climate-resilient livestock.

Thus, given the increasing impact of heat stress on dairy production and the limited understanding of how genetic variation within regulatory non-coding regions may relate to heat-stress responsiveness, further investigation into miRNA-mediated mechanisms is warranted. Although previous studies in cattle have primarily focused on differential miRNA expression profiles under heat stress conditions or on coding variants within protein-coding genes, the genome-wide identification and characterization of miRNA regulatory variants potentially relevant to heat-stress responses remain largely unexplored. Little is known about how sequence variation within miRNA genes may influence miRNA structure, maturation, target recognition, and downstream regulatory networks potentially relevant to heat-stress responses. Therefore, the present exploratory study aimed to identify candidate variants within miRNA loci from pooled WGS data of previously characterized thermotolerant and thermosensitive Holstein cows, defined primarily by reproductive performance under heat-stress conditions and supported by physiological and endocrine characterization, and to investigate their potential regulatory consequences through *in silico* analyses. By identifying variants detected in the thermotolerant sequencing pools but not in the thermosensitive pools under the applied filtering criteria and evaluating their predicted effects on miRNA structure, target recognition, and downstream biological pathways, this research provides an exploratory assessment of the potential regulatory consequences of candidate miRNA variants and prioritizes them for future individual-level genotyping, genotype–phenotype association analyses, and functional validation in cattle.

2. Materials and Methods

2.1. Animals and Phenotypic Characterization

The procedures of this study followed the standards set by the European regulations and the University of Thessaly regarding animal welfare. No license was issued as the methods were not invasive and caused no pain or discomfort to the animals. A detailed description of animal management, experimental design, and phenotypic characterization has been published previously by our group [15]. In brief, the study included 80 lactating Holstein cows reared on three commercial dairy farms in Thessaly, Central Greece. All farms employed similar housing, feeding, and cooling practices, utilizing automated fan-

and-sprinkler cooling systems that activated at a temperature–humidity index (THI) of 68. The study was conducted during the summer of 2020 (June–August), and only cows receiving their first artificial insemination (AI) during June and July of the same year under heat stress conditions ($\text{THI} > 72$) were included. During the study period, THI data were obtained from the electronic controller of the cooling system. The analysis revealed that the mean daily THI steadily exceeded 72, with an average of 74.3 ± 6.9 , based on 24 hourly measurements each day. The lowest THI values were observed between 4:00 AM and 5:00 AM (67.4 ± 1.9), while the highest values occurred between 5:00 PM and 6:00 PM (83.4 ± 4.3). All animals were inseminated following estrus and ovulation synchronization using an OVSYNCH protocol, with pregnancy diagnosed through pregnancy-associated glycoprotein measurement and confirmed by rectal palpation. To minimize sire effects, the same bull was used for inseminations within each farm. The thermotolerant group comprised 20 cows from Farm A and 10 cows from each of Farms B and C, and the thermosensitive group had the same composition (20, 10 and 10 cows, respectively). Because the same bull was used for all inseminations within each farm, sire is nested within farm, and the two groups were therefore identical in their farm and sire composition. Based on reproductive performance under heat stress, cows that conceived at the first AI were classified as thermotolerant, while those that failed to conceive after at least two consecutive AIs were classified as thermosensitive. Although conception is a multifactorial reproductive outcome that may also be influenced by reproductive factors unrelated to heat stress, the biological relevance of this classification is further supported by previous physiological and endocrine characterization of the same experimental population. All enrolled animals underwent weekly detailed general examination and ultrasonographic examinations to assess postpartum uterine involution and ovarian resumption. Uterine health was evaluated using Metrichick (Simcro Tech Limited, New Zealand), ultrasonography, and endometrial cytology, as outlined by Nanas et al. [26]. Any animal diagnosed with general health issues, or with any type of uterine infection by day 60 postpartum, was excluded from the analysis. At first AI, there were no significant differences ($p > 0.05$) in parity, body condition score (BCS), days in milk, or milk yield between the groups [26]. These clinical monitoring procedures, exclusion criteria, and comparable baseline characteristics were intended to minimize the influence of identifiable health-related and reproductive confounding factors on conception outcomes, although they could not eliminate all factors unrelated to heat stress. Under heat-stress conditions, thermotolerant cows exhibited lower circulating cortisol and HSP70 concentrations, and a greater preovulatory LH response compared with thermosensitive cows, consistent with differences in physiological responses under heat stress conditions [27]. Thus, the terms “thermotolerant” and “thermosensitive” are used herein to denote these previously characterized phenotypic groups rather than thermotolerance defined solely by conception outcome.

It should be noted that the present study utilized the same experimental population and pooled whole-genome sequencing (WGS) dataset previously investigated by our group [15]. However, whereas the previous study focused on genetic variation in heat-shock protein-related genes, the current analysis specifically examines variants located within miRNA genomic regions and their potential regulatory consequences.

2.2. Sample Preparation

For the genomic analysis, blood samples were collected in EDTA tubes, transported on ice, and stored at $-20\text{ }^{\circ}\text{C}$ until DNA extraction. Total genomic DNA was isolated using the PureLink Genomic DNA Mini Kit (Invitrogen, USA), followed by quality assessment via agarose gel electrophoresis and quantification with the Qubit dsDNA BR Assay Kit (Invitrogen, USA). To perform pool-sequencing, forty animals from each experimental group (thermotolerant and thermosensitive) were distributed into four distinct pools,

each consisting of equimolar DNA from ten individuals, resulting in a final concentration of 100 ng/ μ L and a total quantity of 2 μ g per pool.

Animals were grouped according to their phenotypic classification and selected to have comparable phenotypic characteristics within each pool. This strategy was intended to facilitate exploratory variant discovery at the pooled level rather than individual-level genotyping. Consequently, individual genetic differences could not be resolved from the pooled sequencing data, and the presence of a variant within a pool could not be attributed to a specific animal. Pools were assembled within each farm, i.e., two pools from Farm A and one pool from each of Farms B and C per group.

2.3. Whole-Genome Sequencing and Bioinformatics Analysis

Genomic DNA samples were shipped to Novogene, where whole-genome sequencing was performed on the Illumina HiSeq 3000 platform (Illumina, San Diego, USA), with a target mean sequencing coverage of 100 $\times$ per pool, corresponding to a theoretical average sequencing contribution of approximately 10 $\times$ per animal under the assumption of equal DNA representation within each pool, rather than directly measured individual-level coverage. Raw FASTQ files were initially assessed for quality using FASTQC (v.0.12.0), and low-quality reads and adapter sequences were removed using Trimmomatic (v0.39) [28] with a PHRED quality threshold of 30. High-quality paired-end reads were mapped to the ARS-UCD1.2 reference genome [29] using BWA-MEM (v0.7.17) [30]. PCR duplicates were identified and removed using Picard, and BAM file processing, including conversion, sorting, and indexing, was performed with SAMtools (v1.22.3) [31]. Sequencing quality control metrics were assessed separately for each of the eight DNA pools, including the total number of read pairs, mapping rate, mean sequencing depth, and the proportion of the reference genome covered at sequencing depths of at least 10 $\times$ and 30 $\times$. Detailed pool-specific sequencing quality metrics are provided in Supplementary Table S1. For variant calling, the four BAM files corresponding to the thermotolerant pools (TT1–TT4) were merged into one group-level BAM file, while the four BAM files corresponding to the thermosensitive pools (TS1–TS4) were merged into a second group-level BAM file. Variant calling was subsequently performed separately on the two merged BAM files using FreeBayes (v1.3.6) [32]. Resulting variant call format (VCF) files were filtered using VCFtools (v0.1.16) to retain high-confidence single-nucleotide polymorphisms (SNPs) based on a minimum quality score of 30 ($\text{minQ} \geq 30$), a minimum allele count of 1 ($\text{mac} \geq 1$), and a minimum read depth of 4 ($\text{minDP} \geq 4$) [33]. Finally, variants detected exclusively in the thermotolerant or thermosensitive pooled sequencing datasets were identified using BCFtools isec (v1.15) [34]. The 15 candidate miRNA variants identified in the thermotolerant dataset were additionally confirmed as detected in all four original TT pools (TT1–TT4) and not detected in any of the four TS pools (TS1–TS4) under the applied detection criteria. The minimum allele count threshold of one was retained to allow exploratory detection of potentially low-frequency variants. Given the pooled sequencing design, individual genotypes and genotype or allele frequencies could not be determined; therefore, the identified variants were considered exploratory candidates rather than variants formally associated with the thermotolerance phenotype. Functional annotation of variants was performed using the Variant Effect Predictor (VEP, v108) [35] to assess their genomic location and predicted impact.

2.4. In Silico Analysis and Investigation of Mirnas

To explore the potential role of miRNA-related genetic variation in thermotolerance, an in silico analysis was conducted focusing on candidate variants detected exclusively in the thermotolerant pooled sequencing dataset. A custom script was used to identify these variants mapping within miRNA genes for subsequent functional characterization. To

assess the potential structural and regulatory consequences of the identified miRNA-related variants, the miRNASNP v4 database [36] was employed to determine whether the variants were located within primary miRNA (pri-miRNA), precursor miRNA (pre-miRNA), or mature miRNA regions. Additionally, the potential effects of SNPs on pre-miRNA secondary structure were evaluated using minimum free energy (MFE) predictions available through miRNASNP v4 [36], based on RNAfold [37]. The change in folding free energy (ΔG) was defined as $MFE(\text{mutant}) - MFE(\text{wild-type})$, with all values expressed in kcal/mol. Accordingly, positive ΔG values indicate a less stable predicted pre-miRNA hairpin in the variant sequence relative to the wild-type sequence, whereas negative values indicate a more stable one. Wild-type MFE values were retrieved from miRNASNP v4 [36], and the corresponding mutant MFE values were derived from the reported ΔG values. For variants located within the seed regions of miRNAs, miRNASNP v4 [36] was further utilized to assess their potential effects on miRNA–mRNA target recognition. The database employs two complementary target prediction algorithms, TargetScan 8.0 and miRmap (v1.1), to independently predict miRNA binding sites within the 3′ untranslated regions (3′UTRs) of target genes. For each seed-region variant, target predictions are compared between the wild-type miRNA sequence, containing the reference allele, and the corresponding variant sequence, containing the alternative allele. According to the criteria implemented in miRNASNP v4 [36], a target interaction is classified as lost when it is predicted by both algorithms for the wild-type sequence but by neither algorithm for the variant sequence. Conversely, a gained interaction is defined as one predicted by both algorithms for the variant sequence but by neither algorithm for the wild-type sequence. For miRNAs affected by variants predicted to influence target recognition, downstream target genes were retrieved based on miRNASNP v4 [36] predictions. These gained and lost targets represent computationally predicted changes in miRNA–mRNA interactions rather than experimentally validated regulatory relationships. Functional enrichment analyses were subsequently performed on the resulting target gene sets using ShinyGO v0.85 [38] to explore the biological processes and pathways potentially impacted by the identified miRNA variants. Gene Ontology (GO) enrichment analysis [39] was conducted to identify overrepresented biological processes (BPs), molecular functions (MFs), and cellular components (CCs), while Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis [40] was used to identify significantly enriched pathways. Since the analysis was based on whole-genome sequencing data rather than on gene expression data (e.g., RNA-Seq), no expression-based filtering was applied, and all annotated *Bos taurus* genes were used as the background gene set for the GO and KEGG enrichment analyses. The gained and lost target gene sets were analyzed separately, and *p*-values were corrected for multiple testing using the false discovery rate (FDR).

3. Results

3.1. Whole-Genome Sequencing and Variant Overview

Whole-genome sequencing generated between 910 and 1067 million read pairs per DNA pool, with mapping rates ranging from 96.7% to 98.4%. Mean sequencing depth ranged from 96.2× to 103.5×, consistent with the targeted coverage of approximately 100× per pool. The proportion of the reference genome covered at depths of at least 10× ranged from 98.7% to 99.1%, while coverage at $\geq 30\times$ ranged from 97.1% to 98.0%. Overall, sequencing quality metrics were relatively consistent across the thermotolerant and thermosensitive pools. Detailed sequencing statistics for each pool are presented in Supplementary Table S1.

Whole-genome sequencing analysis identified a total of 11,666,181 single-nucleotide polymorphisms (SNPs) in the thermotolerant pooled sequencing dataset and 11,538,714 SNPs in the thermosensitive pooled sequencing dataset. Among these variants, 1,083,403

SNPs were detected exclusively in the thermotolerant pools, while 955,936 SNPs were detected exclusively in the thermosensitive pools; the remaining variants were shared between the two groups (Figure 1). Variants detected exclusively in the thermotolerant pools provided the basis for subsequent analyses aimed at identifying candidate regulatory variants within miRNA genes and exploring their potential functional relevance.

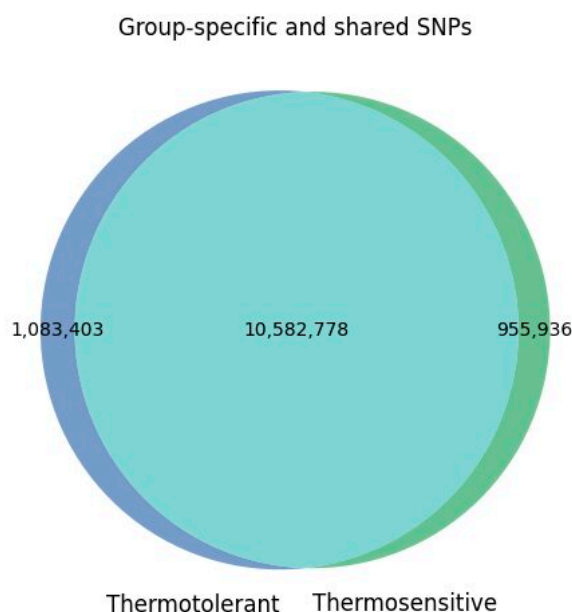


Figure 1. Venn diagram illustrating the distribution of single-nucleotide polymorphisms (SNPs) detected in the thermotolerant (blue) and thermosensitive (green) pooled sequencing datasets. Variants detected exclusively in either group and variants shared between the two groups are shown.

Functional annotation of SNPs detected exclusively in the thermotolerant pools revealed that most variants were in non-coding regions, with 46.70% mapping to intergenic regions and 51.65% to intronic regions. A smaller proportion of variants were found in coding and untranslated regions, including synonymous (0.63%) and non-synonymous (0.49%) variants, as well as variants in the 3' untranslated region (UTR; 0.38%) and 5' UTR (0.15%).

3.2. Identification of miRNA-Associated Variants

Using a custom script, candidate variants detected exclusively in the thermotolerant pools and mapping to miRNA genes were identified. In total, 15 variants were mapped to 15 distinct miRNAs. All 15 candidate variants were detected in all four thermotolerant sequencing pools and were not detected in any of the four thermosensitive pools under the applied variant-calling and filtering criteria. Detailed information on genomic location, variant identity, reference and alternative alleles, and affected miRNAs is provided in Table 1. It should be noted that pool-level detection does not establish individual genotypes or allele frequencies.

Table 1. Candidate variants detected exclusively in the thermotolerant pooled sequencing dataset and mapping within miRNA loci, including genomic location, variant identity, affected miRNA and region, predicted minimum free energy (ΔG) change, and number of gained or lost miRNA target genes (where applicable). MiRNA gene/precursor loci are designated using the “bta-mir-” nomenclature, whereas mature miRNA products are designated using “bta-miR-”, followed by the corresponding -5p or -3p suffix where applicable. “–” indicates that the corresponding in silico prediction was not available or applicable for the indicated variant. REF and ALT indicate the reference and alternative alleles, respectively, according to the *Bos taurus* ARS-UCD1.2 reference genome. ΔG represents the difference in predicted minimum free energy (MFE) between the mutant and wild-type pre-miRNA sequences, calculated as $\Delta G = \text{MFE}(\text{mutant}) - \text{MFE}(\text{wild-type})$. Detailed MFE values for both sequences are provided in Supplementary Table S2.

Genomic Region	SNP ID	REF Allele	ALT Allele	miRNA Locus	Region	ΔG	miRNA Target Genes (Gain/Loss)
1:1,666,094–1,666,094	rs383925958	G	A	bta-mir-12,045	pri-miRNA/pre-miRNA	–	–
3:105,457,752–105,457,752	Novel	T	C	bta-mir-30c	pri-miRNA/pre-miRNA	–	–
3:7,932,297–7,932,297	rs380243757	C	T	bta-mir-2285t	mature miRNA	–	–
4:76,017,522–76,017,522	rs379686115	T	C	bta-mir-2420	pri-miRNA/pre-miRNA	10.7	–
5:109,644,802–109,644,802	rs109562598	G	A	bta-mir-12,001	pri-miRNA/pre-miRNA	–0.4	–
6:115,667,948–115,667,948	rs133612666	G	A	bta-mir-2450d	mature miRNA (3p)	19.1	–
7:108,839,700–108,839,700	rs385411830	A	T	bta-mir-2285bv	mature miRNA (3p)	–	–
8:101,253,139–101,253,139	rs379892150	G	T	bta-mir-7863	pri-miRNA/pre-miRNA	–	–
9:25,151,106–25,151,106	rs207677665	C	T	bta-mir-2284o	pri-miRNA/pre-miRNA	–	–
18:8,132,166–8,132,166	rs207829955	C	T	bta-mir-2883	mature miRNA (3p)/seed	–1.7	2940 gain/664 loss
20:15,245,801–15,245,801	rs383989638	C	T	bta-mir-320a-2	pri-miRNA/pre-miRNA	–	–
21:55,229,288–55,229,288	rs383423817	T	A	bta-mir-3432a-1	pri-miRNA/pre-miRNA	–	–
22:56,110,860–56,110,860	rs797185658	C	T	bta-mir-11,994	pri-miRNA/pre-miRNA	–	–
23:31,828,956–31,828,956	rs469674557	G	A	bta-mir-6531	pri-miRNA/pre-miRNA	–	–
25:34,785,340–34,785,340	rs208131360	G	C	bta-mir-2388	mature miRNA (3p)/seed	6.4	3,169 gain/559 loss

Functional annotation revealed that a subset of variants ($n = 5$) was located within mature miRNA sequences, suggesting a potential direct impact on miRNA function and interactions. The remaining variants were mapped to pri-miRNA or pre-miRNA regions. Among the identified miRNA-associated variants, 14 were previously reported, while one represented a novel variant. All miRNA-associated variants were selected for subsequent functional evaluation using miRNASNP v4 [36].

3.3. Variants and Predicted Effects on miRNA Structure

Thermotolerant-specific variants within mature miRNA sequences or pre-miRNA regions were further evaluated using miRNASNP v4 [36] and RNAfold [37] to assess their potential effects on the structural properties of miRNAs. Several variants were associated with predicted changes in minimum free energy (ΔG), suggesting potential alterations in miRNA secondary structure and stability (Table 1).

Pronounced predicted differences in minimum free energy (ΔG) were observed for variants in bta-mir-2420 ($\Delta G = 10.7$), while a more modest predicted difference was detected for bta-mir-12001 ($\Delta G = -0.4$). These findings suggest that the corresponding variants may alter the predicted pre-miRNA secondary structure and thermodynamic stability, although any effects on miRNA maturation remain to be experimentally established. Additionally, seed-region variants in bta-mir-2883 ($\Delta G = -1.7$) and bta-mir-2388 ($\Delta G = 6.4$) were also associated with predicted changes in ΔG , indicating potential differences in the predicted secondary structures of the corresponding pre-miRNA hairpins. A variant within the mature sequence of bta-miR-2450d was associated with a substantial predicted change in pre-miRNA folding free energy ($\Delta G = 19.1$), suggesting a potential impact on

the predicted secondary structure and thermodynamic stability of the corresponding precursor hairpin. The corresponding wild-type and mutant MFE values are provided in Supplementary Table S2. These computational predictions suggest potential effects on pre-miRNA folding but do not establish changes in miRNA stability, maturation, or biological function. The predicted secondary structures of the wild-type and SNP-containing pre-miRNA hairpins of bta-mir-2450d are illustrated in Figure 2, highlighting potential differences in precursor folding associated with the identified variant. Overall, structural alterations in miRNAs may influence their stability and/or interactions with components of the RNA-induced silencing complex.

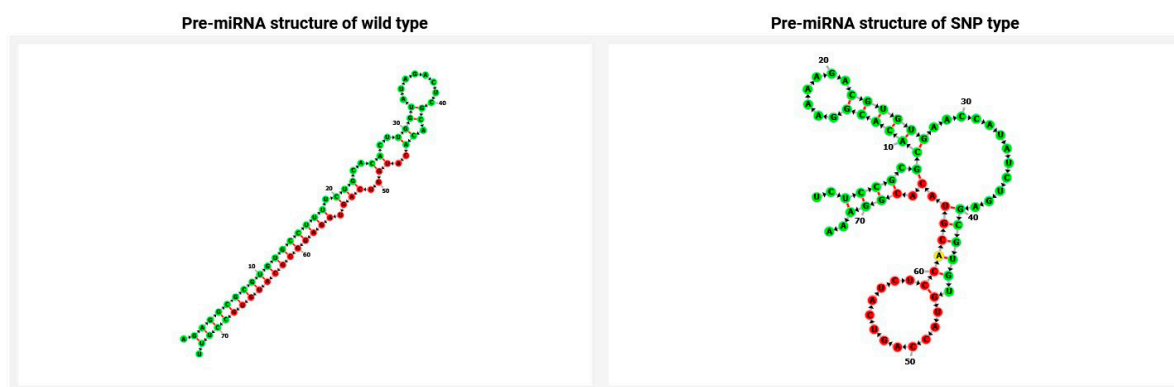


Figure 2. Predicted secondary structures of the wild-type (**left**) and SNP-containing (**right**) pre-miRNA of bta-mir-2450d were generated using RNAfold [37]. The structures represent the pre-miRNA hairpin rather than the primary miRNA transcript (pri-miRNA) or the mature miRNA. Nucleotide positions are indicated, and differences in folding patterns between the wild-type and variant sequences are shown.

3.4. miRNA–mRNA Interactions and Functional Enrichment of Target Genes

Variants within miRNA seed regions have the potential to alter miRNA–mRNA interactions, as the seed sequence is critical for target recognition and binding specificity [41]. To investigate the potential effects on miRNA target recognition of seed-region variants detected in the thermotolerant sequencing pools but not in the thermosensitive pools, predicted miRNA target genes were retrieved using miRNASNP v4 [36].

Two variants detected in the thermotolerant pools but not in the thermosensitive pools, located within miRNA seed regions, were identified (Table 1). The variant rs207829955 in bta-miR-2883-3p was associated with a predicted gain of 2940 target genes and a predicted loss of 664 target genes, while rs208131360 affecting the seed region of bta-miR-2388-3p was associated with a predicted gain of 3169 target genes and a predicted loss of 559 target genes. These findings indicate large predicted differences in miRNA target repertoires associated with seed-region sequence variation.

To explore the potential biological implications of these predicted differences in miRNA target recognition, the predicted gained and lost target gene sets were subsequently analyzed separately using ShinyGO v0.85 [38]. The analysis focused on the functional enrichment of Gene Ontology [39,42] terms and KEGG pathways [40,43,44].

KEGG pathway analysis of lost target genes revealed significant enrichment in pathways related to endocytosis and bacterial invasion of epithelial cells (FDR < 0.01), both of which are associated with membrane dynamics and vesicle trafficking (Figure S3). Consistent with these findings, Gene Ontology cellular component analysis demonstrated strong enrichment for endocytic vesicles, recycling endosome membranes, cytoplasmic vesicles, endosomes, and vesicle membranes, as well as components of the actin cytoskeleton and cell junctions. Additional enriched cellular components included nuclear-

associated structures (Figure S4). Gene Ontology molecular function enrichment primarily reflected broad binding-related activities, as presented in Figure S5. At the biological process level, lost target genes were significantly enriched for processes involved in protein localization, intracellular transport, vesicle-mediated transport, and membrane organization, as well as the regulation of cellular responses to stimuli and intracellular signaling pathways (Figure 3). Collectively, these enrichment results identify intracellular trafficking and membrane-associated processes among the biological functions represented in genes predicted to lose miRNA targeting. These predictions do not establish changes in gene expression or pathway activity.

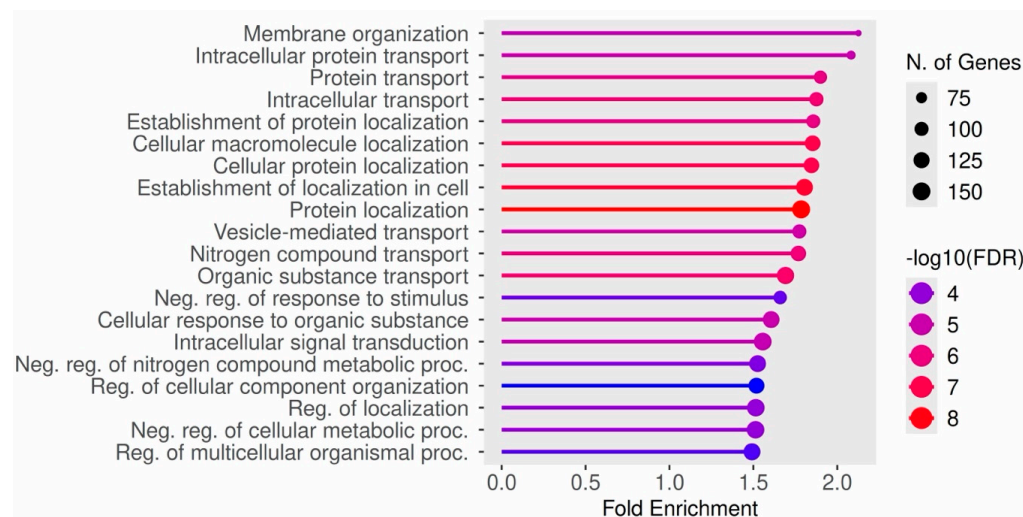


Figure 3. Dot plot showing significantly enriched Gene Ontology (GO) biological process terms for target genes predicted to be lost due to seed-region variants in miRNAs identified exclusively in thermotolerant cows. The size of each dot represents the number of genes associated with the corresponding GO term, while color indicates the level of statistical significance. The x-axis shows the fold enrichment, and the y-axis lists the enriched GO biological process terms. *p*-values were corrected for multiple testing using the false discovery rate (FDR).

In contrast, the gained target genes were enriched in a distinct yet complementary set of pathways. KEGG analysis revealed significant enrichment in stress- and metabolism-related signaling pathways, including the MAPK, AMPK, PI3K–Akt, Ras, and Rap1 signaling pathways, as well as pathways associated with amino acid metabolism, such as valine, leucine, and isoleucine degradation. Additional enriched pathways included those related to phosphatidylinositol signaling, sphingolipid signaling, and insulin resistance (Figure 4). These enrichment results describe functional annotations of computationally predicted miRNA targets and do not demonstrate altered pathway activity or metabolic adaptation under heat stress conditions.

Gene Ontology enrichment analysis further supported these findings, revealing that gained target genes were strongly enriched for cellular components associated with the endoplasmic reticulum, Golgi apparatus, vesicles, and organelle membranes (Figure S6). The enrichment analysis at the molecular function level highlighted binding- and kinase-related terms (Figure S7). At the biological process level, enriched terms included protein transport and localization, intracellular transport, vesicle-mediated transport, cellular response to stress, and intracellular signal transduction (Figure 5). Collectively, these enrichment results identify signaling, metabolic, and intracellular transport-related processes among the biological functions represented in genes predicted to gain miRNA targeting. These findings provide a basis for further investigation of potential regulatory interactions without establishing their functional consequences.

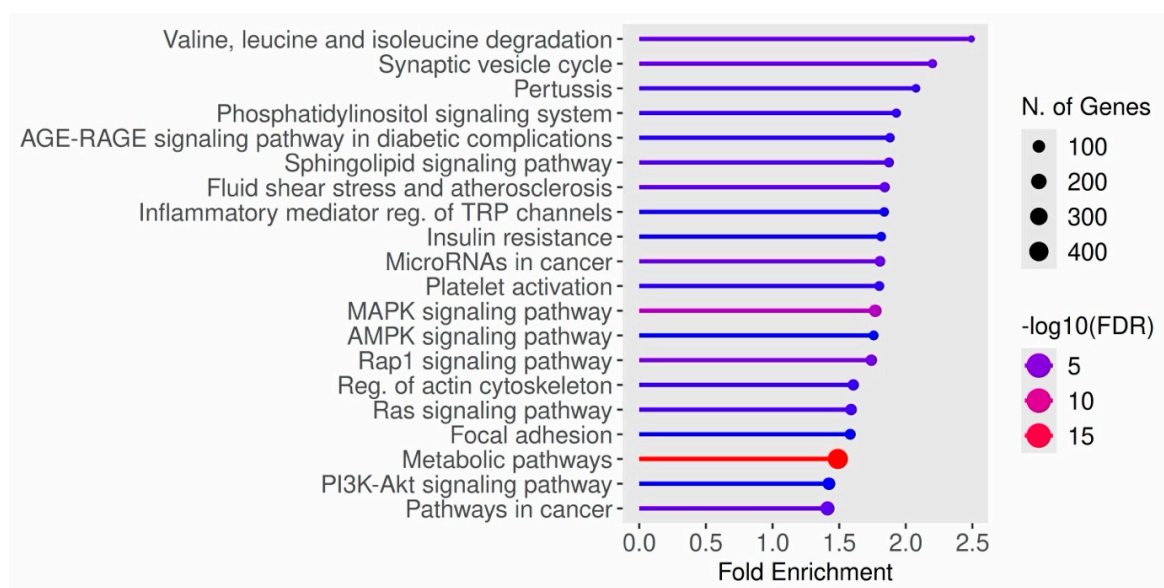


Figure 4. Dot plot showing significantly enriched KEGG terms for target genes predicted to be gained due to seed-region variants in miRNAs identified exclusively in thermotolerant cows. The size of each dot represents the number of genes associated with the corresponding KEGG term, while color indicates the level of statistical significance. The *x*-axis shows the fold enrichment, and the *y*-axis lists the enriched KEGG terms. *p*-values were corrected for multiple testing using the false discovery rate (FDR).

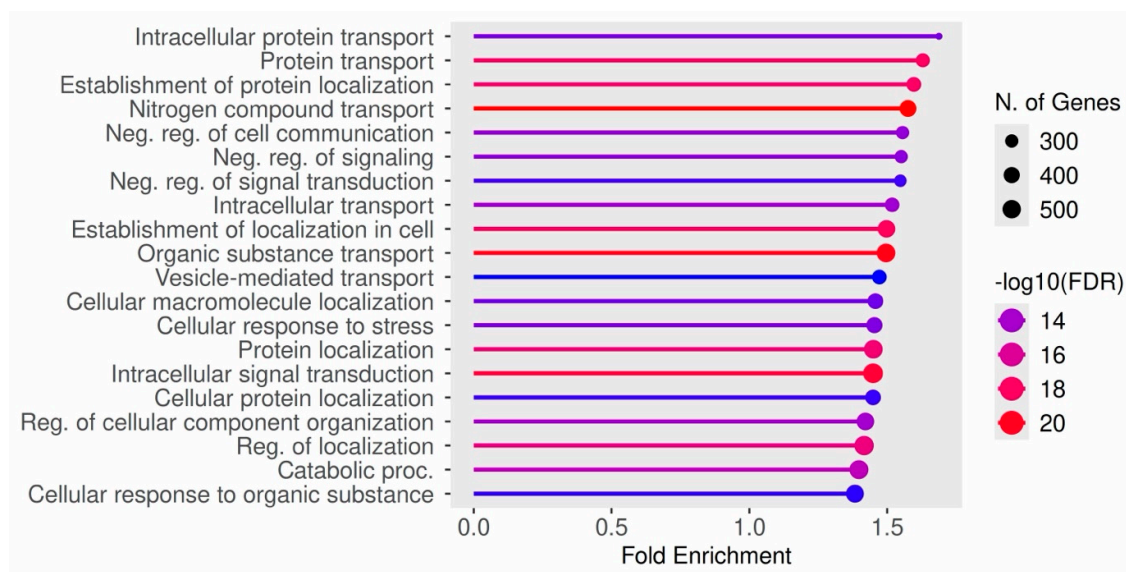


Figure 5. Dot plot showing significantly enriched Gene Ontology (GO) biological process terms for target genes predicted to be gained due to seed-region variants in miRNAs identified exclusively in thermotolerant cows. The size of each dot represents the number of genes associated with the corresponding GO term, while color indicates the level of statistical significance. The *x*-axis shows the fold enrichment, and the *y*-axis lists the enriched GO biological process terms. *p*-values were corrected for multiple testing using the false discovery rate (FDR).

4. Discussion

Heat stress poses a significant challenge for dairy cattle, impairing productivity, fertility, and animal welfare through widespread physiological and cellular disruption [1,45]. While numerous studies have characterized transcriptional and physiological responses to heat stress [18,19,23,46,47], the potential contribution of genetic variation in post-

transcriptional regulatory elements, such as microRNAs (miRNAs), remains less well defined. In this exploratory study, we identified 15 candidate variants—including one potentially novel SNP—within miRNA genes that were detected in the thermotolerant sequencing pools but not in the thermosensitive pools under the applied variant-calling and filtering criteria. We subsequently investigated their predicted structural and functional consequences. Several variants were associated with predicted differences in pre-miRNA secondary structure, while seed-region variants were associated with computationally predicted differences in miRNA target repertoires. Functional enrichment analysis identified distinct sets of biological pathways among genes predicted to gain or lose miRNA targeting, including stress-responsive signaling, metabolic regulation, intracellular trafficking, and membrane-associated processes. These findings suggest potential differences in miRNA target recognition associated with the identified variants but do not establish changes in pathway activity or cellular function.

4.1. Candidate miRNA Variants Potentially Relevant to Thermotolerance

At the first level of our analysis, pooled whole-genome sequencing enabled the identification of candidate genetic variants detected exclusively in the thermotolerant pools, providing a set of loci for further investigation in relation to heat stress resilience. Whole-genome sequencing (WGS) offers an important approach for investigating the genetic architecture of complex traits, such as thermotolerance, as it enables the detection of both known and novel variants across coding and non-coding genomic regions [48,49]. Unlike traditional array-based Genome-Wide Association Studies (GWASs), which rely on common variants tagged on a chip, WGS can identify both known and novel variations across an entire population [50]. Recent advancements have demonstrated that very low-depth WGS (even at very low coverage) can be a cost-effective alternative to GWAS chips, maintaining high genotype accuracy and often providing superior power to detect association signals [50,51]. WGS is particularly effective at assessing the contribution of rare variants, which are often the causal drivers of phenotypic diversity in complex traits like environmental adaptation [52,53]. While common GWAS approaches primarily identify loci based on linkage disequilibrium, WGS allows for a more direct assessment of potential causal mutations, facilitating a deeper understanding of the molecular mechanisms behind traits like heat resilience [48,54]. These advantages refer to individual-level sequencing designs; the pooled design applied here supports variant discovery but not individual-level genotyping or association testing. Unlike environmentally responsive expression changes, DNA sequence variants represent genomic features that can be investigated as potential genetic markers. However, the inheritance patterns, association with thermotolerance, and predictive value of the specific variants identified in this study remain to be established through individual-level genotyping and validation in independent populations [55]. In the present study, the identification of candidate variants within miRNA loci provides an additional layer of genetic information that complements previous studies focusing predominantly on protein-coding genes or heat-responsive miRNA expression. To our knowledge, the variants identified here have not previously been investigated in relation to thermotolerance in dairy cattle. Moreover, several were located within functionally relevant regions of miRNA genes, including mature and seed sequences, supporting their prioritization for subsequent individual-level genotyping and functional investigation.

Among the identified candidates, the detection of a novel SNP within a miRNA gene is particularly noteworthy and warrants further investigation. As this variant has not yet been individually genotyped, its frequency and distribution within the study population remain unknown. Individual-level validation will therefore be required before its potential relationship with thermotolerance can be evaluated.

Taken together, these findings identify candidate miRNA gene variants for individual-level genotyping, and any utility of these variants as biomarkers of thermotolerance remains to be established. Importantly, their association with physiological or reproductive indicators of heat-stress responsiveness has not been demonstrated and will require individual-level genotype–phenotype analyses and independent validation. Further validation in independent populations, along with the integration of phenotypic and transcriptomic data, will be required to evaluate their predictive value and applicability in breeding programs aimed at improving heat stress resilience under increasingly challenging climatic conditions. Importantly, the pooled WGS dataset analyzed here originates from the same experimental population previously investigated by our group [15]. However, the present study addresses a distinct research question by specifically investigating genetic variants within miRNA loci, which were not examined in our previous analysis. Unlike the earlier study, which focused on variants in heat-shock protein-related genes, the current investigation explores the potential regulatory consequences of miRNA-associated variants through computational analyses of pre-miRNA secondary structure, miRNA–target interactions, and downstream biological pathways. In our previous study, pooled sequencing was similarly used as an initial variant-discovery approach, followed by individual-level genotyping of selected candidate SNPs in the broader phenotypically characterized population. This second-stage analysis confirmed the presence of selected pool-derived variants in individual animals and identified significant associations between variants in heat-shock protein genes and the thermotolerance phenotype [15]. These previous findings support the utility of the present pooled WGS dataset as a resource for candidate variant discovery. Nevertheless, the specific miRNA variants identified in the current study have not been individually genotyped, and their presence and allele frequencies at the individual level remain to be confirmed.

4.2. Heat Stress-Associated miRNAs: Evidence from Previous Studies

Several miRNAs harboring candidate variants detected in the thermotolerant pools in our dataset have been implicated in bovine heat stress responses, primarily through differential expression studies in heat-stressed animals. Notably, bta-mir-30c and bta-mir-320a-2 were reported among miRNAs showing differential expression under thermal stress in the peripheral blood mononuclear cells (PBMCs) of crossbred cattle (*Bos taurus* × *Bos indicus*), supporting a potential role for these miRNAs in systemic stress adaptation [56]. Consistent with these findings, bta-mir-30c, bta-miR-320a-1, and bta-miR-320a-2 were also found to be differentially expressed in the Sahiwal (*Bos indicus*) breed during thermal stress [57]. Further evidence linking bta-miR-320a to heat stress comes from serum profiling studies in Holstein cows, which revealed that this miRNA was significantly differentially expressed under heat stress conditions [58]. Additionally, bta-miR-320a was reported to be downregulated in preovulatory follicular fluid extracellular vesicles collected during summer compared to winter in Holstein dairy cows, suggesting a potential role in heat stress-related reproductive adaptation [59]. Moreover, bta-mir-3432a was identified as differentially expressed in both Holstein and Jersey cattle under heat stress conditions [60], providing biological context for the candidate variant detected in bta-mir-3432a-1 in the thermotolerant pools.

Beyond individual miRNAs, previous serum profiling under heat stress has shown that the miR-2284 and miR-2285 families are among the most abundant miRNA families detected in Holstein cows [58], providing supportive context for the presence of variants in bta-mir-2284o and bta-mir-2285t/bv in thermotolerant animals. Notably, bta-miR-2285t was identified as one of the most highly differentially expressed miRNAs in the mammary tissue of Holstein cattle exposed to heat stress compared to normal conditions [22]. The miR-2285 family, which comprises over 40 members distributed across the bovine genome,

has also been implicated in stress-related adaptation mechanisms in other species. For example, miR-2285o-3p contributes to high-altitude adaptation in yaks by attenuating hypoxia-induced apoptosis through targeting *caspase-3* [61]. Members of the miR-2284 family have been found to be differentially expressed between heat-stressed and non-heat-stressed lactating Holstein cows, too [62].

Importantly, several other miRNAs identified in this study (e.g., bta-mir-2883, bta-mir-12045, bta-mir-11994, bta-mir-2388, bta-mir-2450d) have limited or currently unavailable evidence linking them to heat stress in dairy cattle. This highlights the novelty of our findings and the importance of exploring regulatory genetic variation beyond expression-based signatures. Nevertheless, some of these miRNAs have been associated with productive or reproductive traits known to be adversely affected by heat stress. For instance, bta-mir-12001 is characterized as a sperm-borne miRNA associated with fertility in dairy cattle [63], while bta-mir-2420 has also been linked to sperm quality and fertility-related traits [64]. Additionally, bta-mir-7863 has been associated with milk production and shows differential expression under various farm management systems [65]. It has also been proposed as a mammary biomarker for mastitis caused by *Staphylococcus aureus* and *Escherichia coli* [66]. Similarly, bta-miR-6531 is highly enriched in low-motility sperm and has been shown to regulate calcium influx in bovine Leydig cells, linking it to male reproductive function [67].

Overall, the partial overlap between our candidate miRNAs and those previously reported as heat-responsive provides biological context for our findings. Additionally, the identification of candidate variants in miRNAs that have not previously been linked to heat stress, together with the detection of a novel SNP, expands the catalogue of miRNA-related candidates for further investigation in the context of thermotolerance. Individual-level genotyping and functional validation will be required to determine whether these variants are reproducibly associated with the phenotype.

4.3. Potential miRNA-Mediated Regulatory Mechanisms Associated with Heat Stress Responses

Analysis of predicted miRNA–mRNA interaction changes associated with candidate variants detected in the thermotolerant pools suggested potential differences in post-transcriptional regulatory networks. However, it is important to distinguish between computationally predicted changes in miRNA target recognition and experimentally demonstrated regulatory effects. A predicted gain of miRNA targeting indicates a potential new interaction between an miRNA and a target transcript, whereas a predicted loss indicates the possible disruption of an existing interaction. The relatively large number of predicted gained and lost target genes may be attributed, at least in part, to the central role of the miRNA seed region in target recognition. A single-nucleotide substitution within this region may alter sequence complementarity with numerous mRNA 3'UTRs, resulting in extensive computationally predicted changes in the target repertoire. Importantly, the magnitude of these predicted changes also depends on the characteristics and criteria of the computational algorithms employed and should not be interpreted as evidence of widespread functional alterations. In addition, because the gained and lost target sets each comprise several hundred to several thousand genes, the enrichment analyses may identify broad biological terms (e.g., protein transport and intracellular signaling) that are commonly represented across different gene sets. Such enrichment patterns should therefore be interpreted cautiously and should not be considered evidence of altered biological pathway activity. Neither outcome directly establishes changes in target gene expression, pathway activation or inhibition, or cellular phenotype. Therefore, the functional enrichment results presented here should be interpreted as hypothesis-generating observations rather than evidence of altered pathway activity in thermotolerant cows.

Heat stress is known to disrupt cellular homeostasis through effects on metabolism, mitochondrial function, intracellular trafficking, and cell survival, necessitating adaptive regulatory responses at multiple molecular levels [1,47].

A notable feature of the analysis was the functional divergence between lost and gained miRNA targets. Genes predicted to gain miRNA targeting due to the identified seed-region variants were enriched in pathways associated with stress-responsive signaling and metabolic control, including MAPK, AMPK, PI3K–Akt, Ras, and Rap1 signaling, as well as amino acid degradation and insulin resistance pathways. These signaling networks are well established as central regulators of cellular stress responses, energy sensing, and survival under thermal and metabolic stress. In particular, the PI3K–Akt signaling pathway has been shown to play a protective role under heat stress by modulating cell survival, metabolism, and apoptosis, as well as by supporting antioxidant capacity and proteostasis [68–70]. Similarly, Ras and Rap1 small GTPases function as key regulators of stress-responsive signaling, acting as molecular switches that regulate protein expression, metabolic shifts, and survival mechanisms in response to elevated temperatures [71,72]. A study in cattle also shows that Ras and Rap1 pathways are among the over-represented pathways in crossbred cattle under chronic heat stress [46]. The enrichment of kinase- and binding-related molecular functions further identifies signaling-related processes among the predicted miRNA targets, although the direction and functional consequences of any potential regulatory changes remain unknown. Enrichment of insulin resistance and amino-acid degradation pathways among gained targets is consistent with previously described metabolic shifts in heat-stressed dairy cows, where nutrient partitioning and energy efficiency are prioritized to sustain cellular function [73,74]. Notably, genes predicted to gain miRNA targeting were also enriched in phosphatidylinositol and sphingolipid signaling pathways, identifying additional candidate processes of potential relevance to cellular responses under heat stress. Beyond their signaling roles, sphingolipids are critical structural components of cellular membranes and key determinants of membrane fluidity and microdomain organization [75]. Modulation of sphingolipid metabolism has been shown to influence membrane stability, stress signaling, and apoptotic responses under thermal and oxidative stress conditions [76]. Collectively, the enrichment of stress-responsive and metabolic pathways among predicted gained miRNA targets highlights biological processes potentially relevant to heat-stress responses, without establishing that the identified variants alter their post-transcriptional regulation. Although these pathways have established roles in cellular stress responses, their enrichment among predicted targets does not demonstrate increased pathway activity, enhanced stress adaptation, or functional changes in the corresponding regulatory networks.

In addition, gained miRNA targets were enriched for biological processes related to protein transport, protein localization, and vesicle-mediated transport, as well as for cellular components associated with the endoplasmic reticulum, Golgi apparatus, and organelle membranes. These enrichment patterns highlight intracellular protein-handling processes as potentially relevant to the predicted miRNA target repertoires. However, they do not establish whether miRNA-mediated regulation of these processes is altered under heat stress conditions.

In contrast, genes predicted to lose miRNA targeting due to the identified seed-region variants were predominantly enriched for intracellular trafficking and membrane-associated processes, including endocytosis, recycling endosomes, vesicle-mediated transport, protein localization, and cytoskeletal organization. These processes are essential for dynamic cellular remodeling, receptor recycling, and intracellular transport, particularly under stress conditions requiring rapid structural and functional adjustments [77]. The enrichment of intracellular trafficking and membrane-associated processes among genes predicted to lose miRNA targeting suggests that these cellular functions

may be relevant to the potential regulatory consequences of the identified variants. However, predicted loss of miRNA targeting does not necessarily result in increased target gene expression or altered pathway function. Accordingly, these observations should be considered as potential regulatory hypotheses requiring experimental confirmation rather than evidence of functional deregulation or adaptive cellular remodeling. Importantly, similar vesicle-mediated processes have been implicated in cellular responses to environmental and metabolic stress, supporting the biological plausibility of this pattern [78,79]. Recent evidence supports the functional importance of extracellular vesicles (EVs) in heat stress responses in dairy cattle. A study in Holstein Friesian and Brown Swiss cows demonstrated that small EVs isolated from milk under heat stress conditions differentially modulate mammary epithelial cell viability, reactive oxygen species accumulation, and expression of stress-associated genes *in vitro*, indicating that EV cargo can influence cellular phenotypes under thermal challenge. In this study, EVs from heat-stressed Brown Swiss cows, a breed with comparatively greater thermotolerance, triggered cellular defense responses, suggesting that vesicle-mediated intercellular communication may contribute to breed-specific differences in heat stress resilience [80].

Taken together, these findings provide a basis for a hypothetical regulatory framework (Figure 6) illustrating potential changes in miRNA–mRNA interactions associated with the identified variants. In this model, genes predicted to gain miRNA targeting are enriched in stress-responsive signaling and metabolic pathways, whereas genes predicted to lose miRNA targeting are predominantly associated with intracellular trafficking and membrane-related processes. Importantly, this distinction reflects differences in computationally predicted target repertoires rather than experimentally demonstrated changes in miRNA-mediated repression or pathway activity. The proposed framework should therefore be regarded as a hypothesis-generating representation of potential regulatory interactions, requiring further experimental investigation to determine whether these predictions have functional consequences under heat stress conditions.

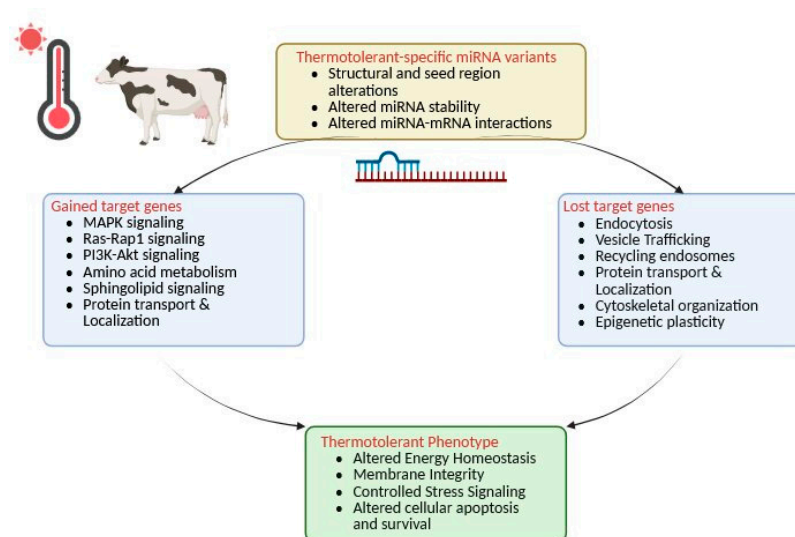


Figure 6. Hypothetical model illustrating computationally predicted changes in miRNA target repertoires associated with candidate variants identified in thermotolerant DNA pools. The model summarizes the biological pathways enriched among genes predicted to gain or lose miRNA targeting and does not imply experimentally confirmed changes in gene expression, pathway activity, or cellular function.

It should also be acknowledged that miRNA-mediated regulation represents a highly complex and context-dependent process influenced by multiple biological factors,

including tissue specificity, environmental conditions, transcript abundance, RNA secondary structure, and the coordinated action of multiple regulatory molecules. Consequently, predicted miRNA–mRNA interactions and enrichment patterns should be interpreted with caution, as computational target prediction approaches may include false-positive or context-specific interactions that are not necessarily functionally active *in vivo*. Furthermore, the presence of sequence variants within miRNA regions does not directly confirm altered regulatory activity, but rather indicates the potential for modified miRNA maturation, stability, or target recognition. Therefore, the proposed regulatory mechanisms described in the present study should be considered hypothesis-generating and will require future experimental validation through expression profiling, functional assays, and independent population studies.

4.4. Strengths and Limitations

Several strengths and limitations of this study should be acknowledged. Key strengths include the use of a well-characterized dairy cattle population with previously characterized thermotolerant and thermosensitive phenotypic groups under real farm conditions, the application of WGS to broadly characterize genetic variation—including variants within miRNA genes not typically assessed by array-based GWAS—and the integrative bioinformatic framework combining variant discovery with computational predictions of miRNA secondary structure, target recognition, and functional enrichment. Together, these features provide a framework for prioritizing candidate miRNA variants for further investigation. Regarding limitations, the functional consequences of the identified variants were inferred solely through *in silico* tools, and no experimental validation was performed. In particular, the predicted miRNA–mRNA interactions and potential changes in target recognition have not been experimentally validated and should therefore be regarded as computational predictions rather than confirmed regulatory interactions. Furthermore, these predictions were not supported by experimental measurements of miRNA expression, target transcript abundance, or pathway activity. Consequently, the enrichment results cannot establish whether the identified variants lead to increased or decreased regulation of individual genes or to functional changes in the corresponding biological pathways. Future experimental studies are required to validate the predicted miRNA–target interactions and determine their potential biological relevance under heat stress conditions. An important limitation of the present study is the pooled WGS design, which does not provide individual-level genotypes and therefore precludes formal estimation and comparison of genotype and allele frequencies between the phenotypic groups. Although reproductive performance and selected physiological and endocrine indicators, including circulating HSP70 and cortisol levels, were previously assessed in the same experimental population [15], the absence of individual-level genotypes for the identified miRNA variants also precluded direct genotype–phenotype association analyses. Consequently, the potential relationships between these candidate variants and physiological or reproductive indicators of heat-stress response remain to be established. Moreover, because the variant-filtering strategy allowed a minimum allele count of one, the possibility that some low-frequency variants were supported by a limited number of sequencing reads cannot be excluded. Although the sequencing data underwent quality-control and variant-filtering procedures, variant-specific read-level support was not independently assessed in the present analysis. Therefore, the consistency of variant detection across the four thermotolerant pools should not be interpreted as a substitute for detailed read-level verification, particularly for the potentially novel variant and the two variants located within miRNA seed regions. Accordingly, variants detected exclusively in the thermotolerant pools should be regarded as exploratory candidates rather than individually validated or formally associated thermotolerance markers. Individual-level

genotyping in the present population, followed by validation in independent populations, will be necessary to confirm these findings. In particular, the novel variant and the two seed-region variants identified in bta-miR-2883-3p and bta-miR-2388-3p represent priority candidates for such validation. The study was conducted across three Holstein dairy farms under specific environmental and management conditions, limiting generalizability to other breeds or production systems, and the variants were not validated in an independent cohort. Moreover, because DNA was sequenced in pools, farm and sire could not be incorporated as individual-level covariates in genotype–phenotype association analyses. In addition, no miRNA or mRNA expression data were available for these animals to determine whether the predicted regulatory changes occur in the thermotolerant group. Furthermore, the analysis focused exclusively on miRNA–mRNA interactions without accounting for competing endogenous RNA (ceRNA) networks, which may modulate miRNA availability and activity. Additionally, thermotolerance represents a complex, multidimensional trait, whereas the primary criterion used for phenotypic classification in the present population was reproductive performance under heat-stress conditions. Although previous physiological and endocrine characterization supports differential heat-stress responsiveness between these groups, reproductive outcome may also be influenced by factors unrelated to thermotolerance. Future studies integrating ceRNA network analysis and core heat-response gene interactions could provide a more comprehensive picture of the miRNA-mediated regulatory landscape underlying thermotolerance. Importantly, individual-level genotyping should be integrated with the available physiological, endocrine, and reproductive data from the same experimental population, including circulating HSP70 and cortisol levels, to investigate potential genotype–phenotype associations and assess the relevance of these candidate variants to heat-stress resilience. Finally, incomplete annotation of bovine miRNA genes and their target repertoires may have constrained the interpretation of certain variants.

5. Conclusions

This exploratory study provides a genome-wide computational investigation of miRNA genetic variation potentially relevant to thermotolerance in dairy cattle, identifying 15 candidate variants detected in the thermotolerant sequencing pools but not in the thermosensitive pools under the applied variant-calling and filtering criteria. In silico analyses indicated that some of these variants may be associated with predicted differences in pre-miRNA secondary structure and miRNA target recognition, with functional enrichment of computationally predicted target genes identifying biological processes and pathways related to stress-responsive signaling, metabolism, intracellular trafficking, and membrane-associated processes. These results represent computational hypotheses regarding potential regulatory consequences and do not demonstrate altered miRNA-mediated regulation, biological pathway activity, or mechanisms of heat tolerance. These findings provide a framework for prioritizing candidate miRNA variants for subsequent genetic and functional investigation, without establishing their contribution to heat-stress resilience. However, given the pooled sequencing design and the absence of individual-level genotyping and functional validation, the identified variants should be regarded as exploratory candidates rather than validated markers of thermotolerance. Importantly, although differences in HSP70, cortisol, and preovulatory LH responses were previously reported between the phenotypic groups, the present study cannot determine whether individual animals exhibiting these physiological characteristics carry the identified variants. Consequently, direct genotype–phenotype associations have not been established, and the physiological and genomic findings remain unlinked at the individual level. Future individual-level genotyping, particularly of the novel SNP and the seed-region variants in bta-miR-2883 and bta-miR-2388-3p, combined with analyses of their associations

with physiological and reproductive indicators, functional validation and evaluation in independent cattle populations, will be required to establish their potential relevance to thermotolerance and their possible utility in future genomic selection strategies.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/agriculture16212341/s1>. Table S1: Whole-genome sequencing quality control metrics for thermotolerant and thermosensitive DNA pools. TT, thermotolerant; TS, thermosensitive; M, million read pairs. Each pool comprised DNA from ten individual animals. Mean depth indicates average sequencing coverage, expressed as fold coverage ($\times$). Coverage $\geq 10\times$ and $\geq 30\times$ represent the percentages of the reference genome covered by at least 10 and 30 sequencing reads, respectively; Table S2: Predicted minimum free energy (MFE) values for wild-type and SNP-containing pre-miRNA sequences, together with the corresponding changes in folding free energy (ΔG). All values are expressed in kcal/mol. ΔG was calculated as MFE(mutant) – MFE(wild-type); Figure S1: Complete KEGG pathway enrichment results for genes predicted to lose miRNA targeting due to seed-region variants identified in thermotolerant DNA pools; Figure S2: Complete Gene Ontology (GO) Cellular Component (CC) enrichment results for genes predicted to lose miRNA targeting due to seed-region variants identified in thermotolerant DNA pools; Figure S3: Complete Gene Ontology (GO) Molecular Function (MF) enrichment results for genes predicted to lose miRNA targeting due to seed-region variants identified in thermotolerant DNA pools; Figure S4: Complete Gene Ontology (GO) Cellular Component (CC) enrichment results for genes predicted to gain miRNA targeting due to seed-region variants identified in thermotolerant DNA pools; Figure S5: Complete Gene Ontology (GO) Molecular Function (MF) enrichment results for genes predicted to gain miRNA targeting due to seed-region variants identified in thermotolerant DNA pools.

Author Contributions: M.-A.K.: methodology, investigation, writing—original draft, data curation; T.G.: methodology, writing—original draft, data curation; I.N.: investigation, writing—review and editing; E.D.: methodology, writing—review and editing; G.S.A.: investigation, writing—review and editing, supervision; Z.M.: conceptualization, writing—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: The raw whole-genome sequencing data presented in this study are openly available in the NCBI BioProject database at <https://www.ncbi.nlm.nih.gov/bioproject/>, accession number PRJNA747189 (accessed on 26 October 2026). The corresponding BioSample accession numbers for the four thermotolerant pools are SAMN20283126 (TT1), SAMN20283127 (TT2), SAMN20283128 (TT3), and SAMN20283129 (TT4), while those for the four thermosensitive pools are SAMN20283130 (TS1), SAMN20283131 (TS2), SAMN20283132 (TS3), and SAMN20283133 (TS4).

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Dovolou, E.; Giannoulis, T.; Nanas, I.; Amiridis, G.S. Heat Stress: A Serious Disruptor of the Reproductive Physiology of Dairy Cows. *Animals* **2023**, *13*, 1846. <https://doi.org/10.3390/ani13111846>.
2. Oliveira, C.P.; Sousa, F.C.d.; da Silva, A.L.; Schultz, É.B.; Valderrama Londoño, R.I.; de Souza, P.A.R. Heat Stress in Dairy Cows: Impacts, Identification, and Mitigation Strategies—A Review. *Animals* **2025**, *15*, 249. <https://doi.org/10.3390/ani15020249>.
3. Rhoads, M.L.; Rhoads, R.P.; VanBaale, M.J.; Collier, R.J.; Sanders, S.R.; Weber, W.J.; Crooker, B.A.; Baumgard, L.H. Effects of Heat Stress and Plane of Nutrition on Lactating Holstein Cows: I. Production, Metabolism, and Aspects of Circulating Somatotropin. *J. Dairy Sci.* **2009**, *92*, 1986–1997. <https://doi.org/10.3168/jds.2008-1641>.
4. Wheelock, J.B.; Rhoads, R.P.; VanBaale, M.J.; Sanders, S.R.; Baumgard, L.H. Effects of Heat Stress on Energetic Metabolism in Lactating Holstein Cows. *J. Dairy Sci.* **2010**, *93*, 644–655.
5. Wolfenson, D.; Roth, Z. Impact of Heat Stress on Cow Reproduction and Fertility. *Anim. Front.* **2018**, *9*, 32–38. <https://doi.org/10.1093/af/vfy027>.

6. Bernabucci, U.; Lacetera, N.; Baumgard, L.H.; Rhoads, R.P.; Ronchi, B.; Nardone, A. Metabolic and Hormonal Acclimation to Heat Stress in Domesticated Ruminants. *Animal* **2010**, *4*, 1167–1183. <https://doi.org/10.1017/S175173111000090X>.
7. Pinto, S.; Hoffmann, G.; Ammon, C.; Amon, T. Critical THI Thresholds Based on the Physiological Parameters of Lactating Dairy Cows. *J. Therm. Biol.* **2020**, *88*, 102523. <https://doi.org/10.1016/j.jtherbio.2020.102523>.
8. Liu, S.; Liu, Y.; Bao, E.; Tang, S. The Protective Role of Heat Shock Proteins against Stresses in Animal Breeding. *Int. J. Mol. Sci.* **2024**, *25*, 8208. <https://doi.org/10.3390/ijms25158208>.
9. Khan, A.; Dou, J.; Wang, Y.; Jiang, X.; Khan, M.Z.; Luo, H.; Usman, T.; Zhu, H. Evaluation of Heat Stress Effects on Cellular and Transcriptional Adaptation of Bovine Granulosa Cells. *J. Anim. Sci. Biotechnol.* **2020**, *11*, 25. <https://doi.org/10.1186/s40104-019-0408-8>.
10. Kim, W.-S.; Nejad, J.G.; Roh, S.-G.; Lee, H.-G. Heat-Shock Proteins Gene Expression in Peripheral Blood Mononuclear Cells as an Indicator of Heat Stress in Beef Calves. *Animals* **2020**, *10*, 895.
11. Stamperna, K.; Giannoulis, T.; Nanas, I.; Kalemkeridou, M.; Dadouli, K.; Moutou, K.; Amiridis, G.S.; Dovolou, E. Short Term Temperature Elevation during IVM Affects Embryo Yield and Alters Gene Expression Pattern in Oocytes, Cumulus Cells and Blastocysts in Cattle. *Theriogenology* **2020**, *156*, 36–45.
12. Archana, P.R.; Aleena, J.; Pragna, P.; Vidya, M.K.; Abdul Niyas, P.A.; Bagath, M.; Krishnan, G.; Manimaran, A.; Beena, V.; Kurien, E.K.; et al. Role of Heat Shock Proteins in Livestock Adaptation to Heat Stress. *J. Dairy Vet. Anim. Res.* **2017**, *5*, 00127. <https://doi.org/10.15406/jdvar.2017.05.00127>.
13. Cheruiyot, E.K.; Haile-Mariam, M.; Cocks, B.G.; MacLeod, I.M.; Xiang, R.; Pryce, J.E. New Loci and Neuronal Pathways for Resilience to Heat Stress in Cattle. *Sci. Rep.* **2021**, *11*, 16619.
14. Sailo, L.; Gupta, I.; Archana, V.; Ramendra, D.; Chaudhari, M.V. Association of Single Nucleotide Polymorphism of Hsp90ab1 Gene with Thermotolerance and Milk Yield in Sahiwal Cows. *Afr. J. Biochem. Res.* **2015**, *9*, 99–103.
15. Kalemkeridou, M.; Nanas, I.; Moutou, K.; Amiridis, G.S.; Tsiourlianos, A.; Dovolou, E.; Mamuris, Z.; Giannoulis, T. Genetic Diversity and Thermotolerance in Holstein Cows: Pathway Analysis and Marker Development Using Whole-Genome Sequencing. *Reprod. Domest. Anim.* **2022**, *58*, 146–157.
16. Luo, H.; Hu, L.; Brito, L.F.; Dou, J.; Sammad, A.; Chang, Y.; Ma, L.; Guo, G.; Liu, L.; Zhai, L.; et al. Weighted Single-Step GWAS and RNA Sequencing Reveals Key Candidate Genes Associated with Physiological Indicators of Heat Stress in Holstein Cattle. *J. Anim. Sci. Biotechnol.* **2022**, *13*, 108. <https://doi.org/10.1186/s40104-022-00748-6>.
17. Otto, P.I.; Guimarães, S.E.F.; Verardo, L.L.; Azevedo, A.L.S.; Vandenplas, J.; Sevillano, C.A.; Marques, D.B.D.; Pires, M.D.F.A.; de Freitas, C.; Verneque, R.S.; et al. Genome-Wide Association Studies for Heat Stress Response in Bos taurus × Bos indicus Crossbred Cattle. *J. Dairy Sci.* **2019**, *102*, 8148–8158. <https://doi.org/10.3168/jds.2018-15305>.
18. Li, G.; Yu, X.; Portela Fontoura, A.B.; Javaid, A.; de la Maza-Escola, V.S.; Salandy, N.S.; Fubini, S.L.; Grilli, E.; McFadden, J.W.; Duan, J.E. Transcriptomic Regulations of Heat Stress Response in the Liver of Lactating Dairy Cows. *BMC Genom.* **2023**, *24*, 410.
19. Laporta, J.; Dado-Senn, B.; Guadagnin, A.R.; Liu, L.; Peñagaricano, F. Preweaning Heat Stress Alters Liver Transcriptome and DNA Methylation in Dairy Calves. *J. Dairy Sci.* **2025**, *108*, 4390–4402.
20. O'Brien, J.; Hayder, H.; Zayed, Y.; Peng, C. Overview of MicroRNA Biogenesis, Mechanisms of Actions, and Circulation. *Front. Endocrinol.* **2018**, *9*, 402.
21. Miretti, S.; Lecchi, C.; Ceciliani, F.; Baratta, M. MicroRNAs as Biomarkers for Animal Health and Welfare in Livestock. *Front. Vet. Sci.* **2020**, *7*, 578193.
22. Li, Q.; Yang, C.; Du, J.; Zhang, B.; He, Y.; Hu, Q.; Li, M.; Zhang, Y.; Wang, C.; Zhong, J. Characterization of miRNA Profiles in the Mammary Tissue of Dairy Cattle in Response to Heat Stress. *BMC Genom.* **2018**, *19*, 975.
23. Raza, S.H.A.; Abdelnour, S.A.; Dhshan, A.I.; Hassanin, A.A.; Noreldin, A.E.; Albadrani, G.M.; Abdel-Daim, M.M.; Cheng, G.; Zan, L. Potential Role of Specific MicroRNAs in the Regulation of Thermal Stress Response in Livestock. *J. Therm. Biol.* **2021**, *96*, 102859.
24. Ali, M.E.; Al-Saeed, F.A.; Ahmed, A.E.; Gao, M.; Wang, W.; Lv, H.; Hua, G.; Yang, L.; Abdelrahman, M. MicroRNA as Biomarkers for Physiological and Stress Processing in the Livestock. *Reprod. Domest. Anim.* **2025**, *60*, e70034. <https://doi.org/10.1111/rda.70034>.
25. Cammaerts, S.; Strazisar, M.; De Rijk, P.; Del Favero, J. Genetic Variants in MicroRNA Genes: Impact on MicroRNA Expression, Function, and Disease. *Front. Genet.* **2015**, *6*, 186.
26. Nanas, I.; Dokou, S.; Athanasiou, L.V.; Dovolou, E.; Chouzouris, T.M.; Vasilopoulos, S.; Grigoriadou, K.; Giannenas, I.; Amiridis, G.S. Feeding Flaxseed and Lupins during the Transition Period in Dairy Cows: Effects on Production Performance, Fertility and Biochemical Blood Indices. *Animals* **2023**, *13*, 1972. <https://doi.org/10.3390/ani13121972>.

27. Nanas, I.; Chouzouris, T.M.; Dadouli, K.; Dovolou, E.; Stamperna, K.; Barbagianni, M.; Valasi, I.; Tsiaras, A.; Amiridis, G.S. A Study on Stress Response and Fertility Parameters in Phenotypically Thermotolerant and Thermosensitive Dairy Cows during Summer Heat Stress. *Reprod. Domest. Anim.* **2020**, *55*, 1774–1783.
28. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics* **2014**, *30*, 2114–2120. <https://doi.org/10.1093/bioinformatics/btu170>.
29. Rosen, B.D.; Bickhart, D.M.; Schnabel, R.D.; Koren, S.; Elsik, C.G.; Tseng, E.; Rowan, T.N.; VanRaden, P.M.; Null, D.J.; Low, W.Y.; et al. De Novo Assembly of the Cattle Reference Genome with Single-Molecule Sequencing. *GigaScience* **2020**, *9*, g1aa021. <https://doi.org/10.1093/gigascience/g1aa021>.
30. Li, H.; Durbin, R. Fast and Accurate Short Read Alignment with Burrows-Wheeler Transform. *Bioinformatics* **2009**, *25*, 1754–1760. <https://doi.org/10.1093/bioinformatics/btp324>.
31. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R. The Sequence Alignment/Map Format and SAMtools. *Bioinformatics* **2009**, *25*, 2078–2079. <https://doi.org/10.1093/bioinformatics/btp352>.
32. Garrison, E.; Marth, G. Haplotype-Based Variant Detection from Short-Read Sequencing. *arXiv* **2012**, arXiv:1207.3907. <https://doi.org/10.48550/arXiv.1207.3907>.
33. Danecek, P.; Auton, A.; Abecasis, G.; Albers, C.A.; Banks, E.; DePristo, M.A.; Handsaker, R.E.; Lunter, G.; Marth, G.T.; Sherry, S.T.; et al. The Variant Call Format and VCFtools. *Bioinformatics* **2011**, *27*, 2156–2158. <https://doi.org/10.1093/bioinformatics/btr330>.
34. Danecek, P.; Bonfield, J.K.; Liddle, J.; Marshall, J.; Ohan, V.; Pollard, M.O.; Whitwham, A.; Keane, T.; McCarthy, S.A.; Davies, R.M.; et al. Twelve Years of SAMtools and BCFtools. *GigaScience* **2021**, *10*, giab008. <https://doi.org/10.1093/gigascience/giab008>.
35. McLaren, W.; Gil, L.; Hunt, S.E.; Riat, H.S.; Ritchie, G.R.S.; Thormann, A.; Flicek, P.; Cunningham, F. The Ensembl Variant Effect Predictor. *Genome Biol.* **2016**, *17*, 122. <https://doi.org/10.1186/s13059-016-0974-4>.
36. Cao, W.; He, J.; Feng, J.; Wu, X.; Wu, T.; Wang, D.; Min, C.; Niu, X.; Gao, Z.; Guo, A.-Y.; et al. miRNASNP-v4: A Comprehensive Database for miRNA-Related SNPs across 17 Species. *Nucleic Acids Res.* **2025**, *53*, D1066–D1074. <https://doi.org/10.1093/nar/gkae888>.
37. Lorenz, R.; Bernhart, S.H.; Höner zu Siederdissen, C.; Tafer, H.; Flamm, C.; Stadler, P.F.; Hofacker, I.L. ViennaRNA Package 2.0. *Algorithms Mol. Biol.* **2011**, *6*, 26. <https://doi.org/10.1186/1748-7188-6-26>.
38. Ge, S.X.; Jung, D.; Yao, R. ShinyGO: A Graphical Gene-Set Enrichment Tool for Animals and Plants. *Bioinformatics* **2020**, *36*, 2628–2629. <https://doi.org/10.1093/bioinformatics/btz931>.
39. Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T.; et al. Gene Ontology: Tool for the Unification of Biology. *Nat. Genet.* **2000**, *25*, 25–29. <https://doi.org/10.1038/75556>.
40. Kanehisa, M.; Goto, S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res.* **2000**, *28*, 27–30. <https://doi.org/10.1093/nar/28.1.27>.
41. Kehl, T.; Backes, C.; Kern, F.; Fehlmann, T.; Ludwig, N.; Meese, E.; Lenhof, H.-P.; Keller, A. About miRNAs, miRNA Seeds, Target Genes and Target Pathways. *Oncotarget* **2017**, *8*, 107167–107175. <https://doi.org/10.18632/oncotarget.22363>.
42. The Gene Ontology Consortium. The Gene Ontology Knowledgebase in 2026. *Nucleic Acids Res.* **2026**, *54*, D1779–D1792. <https://doi.org/10.1093/nar/gkaf1292>.
43. Kanehisa, M. Toward Understanding the Origin and Evolution of Cellular Organisms. *Protein Sci.* **2019**, *28*, 1947–1951. <https://doi.org/10.1002/pro.3715>.
44. Kanehisa, M.; Furumichi, M.; Sato, Y.; Matsuura, Y.; Ishiguro-Watanabe, M. KEGG: Biological Systems Database as a Model of the Real World. *Nucleic Acids Res.* **2025**, *53*, D672–D677. <https://doi.org/10.1093/nar/gkae909>.
45. Collier, R.J.; Renquist, B.J.; Xiao, Y. A 100-Year Review: Stress Physiology Including Heat Stress. *J. Dairy Sci.* **2017**, *100*, 10367–10380. <https://doi.org/10.3168/jds.2017-13676>.
46. Dutta, G.; Alex, R.; Singh, A.; Gowane, G.R.; Vohra, V.; De, S.; Verma, A.; Ludri, A. Functional Transcriptome Analysis Revealed Upregulation of MAPK-SMAD Signalling Pathways in Chronic Heat Stress in Crossbred Cattle. *Int. J. Biometeorol.* **2024**, *68*, 1371–1385. <https://doi.org/10.1007/s00484-024-02672-y>.
47. Belhadj Slimen, I.; Najar, T.; Ghram, A.; Abdrrabba, M. Heat Stress Effects on Livestock: Molecular, Cellular and Metabolic Aspects, a Review. *J. Anim. Physiol. Anim. Nutr.* **2016**, *100*, 401–412. <https://doi.org/10.1111/jpn.12379>.
48. Kalaignazhal, G.; Sejian, V.; Velayudhan, S.M.; Mishra, C.; Rebez, E.B.; Chauhan, S.S.; DiGiacomo, K.; Lacetera, N.; Dunshea, F.R. Applications of Next-Generation Sequencing Technologies and Statistical Tools in Identifying Pathways and Biomarkers for Heat Tolerance in Livestock. *Vet. Sci.* **2024**, *11*, 616. <https://doi.org/10.3390/vetsci11120616>.

49. Nawaz, A.H.; Setthaya, P.; Feng, C. Exploring Evolutionary Adaptations and Genomic Advancements to Improve Heat Tolerance in Chickens. *Animals* **2024**, *14*, 2215. <https://doi.org/10.3390/ani14152215>.
50. Chat, V.; Ferguson, R.; Morales, L.; Kirchhoff, T. Ultra Low-Coverage Whole-Genome Sequencing as an Alternative to Genotyping Arrays in Genome-Wide Association Studies. *Front. Genet.* **2022**, *12*, 790445. <https://doi.org/10.3389/fgene.2021.790445>.
51. Gilly, A.; Southam, L.; Suveges, D.; Kuchenbaecker, K.; Moore, R.; Melloni, G.E.M.; Hatzikotoulas, K.; Farmaki, A.-E.; Ritchie, G.; Schwartzentruber, J.; et al. Very Low-Depth Whole-Genome Sequencing in Complex Trait Association Studies. *Bioinformatics* **2019**, *35*, 2555–2561. <https://doi.org/10.1093/bioinformatics/bty1032>.
52. Chen, W.; Coombes, B.J.; Larson, N.B. Recent Advances and Challenges of Rare Variant Association Analysis in the Biobank Sequencing Era. *Front. Genet.* **2022**, *13*, 1014947. <https://doi.org/10.3389/fgene.2022.1014947>.
53. Cupples, L.A.; Shadyab, A.H.; McKnight, B.; Shoemaker, B.M.; Mitchell, B.D.; et al. Assessing the Contribution of Rare Variants to Complex Trait Heritability from Whole-Genome Sequence Data. *Nat. Genet.* **2022**, *54*, 263–273. <https://doi.org/10.1038/s41588-021-00997-7>.
54. Uemoto, Y.; Pong-Wong, R.; Navarro, P.; Vitart, V.; Hayward, C.; Wilson, J.F.; Rudan, I.; Campbell, H.; Hastie, N.D.; Wright, A.F.; et al. The Power of Regional Heritability Analysis for Rare and Common Variant Detection: Simulations and Application to Eye Biometrical Traits. *Front. Genet.* **2013**, *4*, 232. <https://doi.org/10.3389/fgene.2013.00232>.
55. Mukhopadhyay, C.S.; Kaur, B. NGS-Based Biomarkers in Livestock. In *Biotechnological Interventions Augmenting Livestock Health and Production*; Mukhopadhyay, C.S.; Choudhary, R.K.; Panwar, H.; Malik, Y.S., Eds.; Springer Nature: Singapore, 2023; pp. 107–148. https://doi.org/10.1007/978-981-99-2209-3_7.
56. Sengar, G.S.; Deb, R.; Singh, U.; Raja, T.V.; Kant, R.; Sajjanar, B.; Alex, R.; Alyethodi, R.R.; Kumar, A.; Kumar, S.; et al. Differential Expression of MicroRNAs Associated with Thermal Stress in Frieswal (*Bos taurus* × *Bos indicus*) Crossbred Dairy Cattle. *Cell Stress Chaperones* **2018**, *23*, 155–170. <https://doi.org/10.1007/s12192-017-0833-6>.
57. Sengar, G.S.; Deb, R.; Singh, U.; Junghare, V.; Hazra, S.; Raja, T.V.; Alex, R.; Kumar, A.; Alyethodi, R.R.; Kant, R.; et al. Identification of Differentially Expressed MicroRNAs in Sahiwal (*Bos indicus*) Breed of Cattle during Thermal Stress. *Cell Stress Chaperones* **2018**, *23*, 1019–1032. <https://doi.org/10.1007/s12192-018-0911-4>.
58. Zheng, Y.; Chen, K.-L.; Zheng, X.-M.; Li, H.-X.; Wang, G.-L. Identification and Bioinformatics Analysis of MicroRNAs Associated with Stress and Immune Response in Serum of Heat-Stressed and Normal Holstein Cows. *Cell Stress Chaperones* **2014**, *19*, 973–981. <https://doi.org/10.1007/s12192-014-0521-8>.
59. Kosior, M.A.; Masiello, L.; Piscopo, F.; Esposito, R.; Rizzo, A.; Longobardi, V.; Lange-Consiglio, A.; Capra, E.; Lazzari, B.; Gagni, P.; et al. Impact of Heat Stress on the miRNA Cargo of Pre-Ovulatory Follicular Fluid Extracellular Vesicles and Fertility in Dairy Cattle. *Theriogenology* **2026**, *259*, 117910. <https://doi.org/10.1016/j.theriogenology.2026.117910>.
60. Lee, J.; Kim, D.; Lim, B.; Ryu, G.; Baek, H.; Kim, J.; Ha, S.; Kim, S.; Lee, S.; Choi, T.; et al. Differential Expression of Circulating microRNAs in Lactating Holstein and Jersey Cows Exposed to Heat Stress. *J. Anim. Reprod. Biotechnol.* **2024**, *39*, 221–232. <https://doi.org/10.12750/JARB.39.4.221>.
61. Zhang, J.; Long, K.; Wang, J.; Zhang, J.; Jin, L.; Tang, Q.; Li, X.; Ma, J.; Li, M.; Jiang, A. Yak miR-2285o-3p Attenuates Hypoxia-Induced Apoptosis by Targeting Caspase-3. *Anim. Genet.* **2022**, *53*, 49–57. <https://doi.org/10.1111/age.13153>.
62. Lee, J.; Lee, S.; Son, J.; Lim, H.; Kim, E.; Kim, D.; Ha, S.; Hur, T.; Lee, S.; Choi, I. Analysis of Circulating-microRNA Expression in Lactating Holstein Cows under Summer Heat Stress. *PLoS ONE* **2020**, *15*, e0231125. <https://doi.org/10.1371/journal.pone.0231125>.
63. Zhang, Y.; Labrecque, R.; Tremblay, P.; Plessis, C.; Dufour, P.; Martin, H.; Sirard, M.A. Sperm-Borne tsRNAs and miRNAs Analysis in Relation to Dairy Cattle Fertility. *Theriogenology* **2024**, *215*, 241–248. <https://doi.org/10.1016/j.theriogenology.2023.11.029>.
64. Dementieva, N.V.; Dysin, A.P.; Shcherbakov, Y.S.; Nikitkina, E.V.; Musidray, A.A.; Petrova, A.V.; Mitrofanova, O.V.; Plemmyashov, K.V.; Azovtseva, A.I.; Griffin, D.K.; et al. Risk of Sperm Disorders and Impaired Fertility in Frozen–Thawed Bull Semen: A Genome-Wide Association Study. *Animals* **2024**, *14*, 251. <https://doi.org/10.3390/ani14020251>.
65. Qassim, L.A.; Le Guillou, S.; Royo, L.J. Variation of miRNA Content in Cow Raw Milk Depending on the Dairy Production System. *Int. J. Mol. Sci.* **2022**, *23*, 11681. <https://doi.org/10.3390/ijms231911681>.
66. Luoreng, Z.-M.; Wang, X.-P.; Mei, C.-G.; Zan, L.-S. Comparison of MicroRNA Profiles between Bovine Mammary Glands Infected with *Staphylococcus aureus* and *Escherichia coli*. *Int. J. Biol. Sci.* **2018**, *14*, 87–99. <https://doi.org/10.7150/ijbs.22498>.
67. Ding, Q.; Ding, X.; Xia, S.; Zhao, F.; Chen, K.; Qian, Y.; Cao, S.; Lin, Z.; Gao, Y.; Wang, H.; et al. Bta-miR-6531 Regulates Calcium Influx in Bovine Leydig Cells and Is Associated with Sperm Motility. *Genes* **2022**, *13*, 1788. <https://doi.org/10.3390/genes13101788>.

68. Lacroix, E.; Momchilova, E.A.; Chandhok, S.; Padavu, M.; Zapf, R.; Audas, T.E. PI3K/AKT Signaling Mediates Stress-Inducible Amyloid Formation through c-Myc. *Cell Rep.* **2025**, *44*, 115617. <https://doi.org/10.1016/j.celrep.2025.115617>.
69. Liu, Y.; Cai, H.; Guo, X.; Aierken, A.; Hua, J.; Ma, B.; Peng, S. Melatonin Alleviates Heat Stress-Induced Testicular Damage in Dairy Goats by Inhibiting the PI3K/AKT Signaling Pathway. *Stress Biol.* **2022**, *2*, 47. <https://doi.org/10.1007/s44154-022-00068-9>.
70. Thompson, S.M.; Callstrom, M.R.; Jondal, D.E.; Butters, K.A.; Knudsen, B.E.; Anderson, J.L.; Lien, K.R.; Sutor, S.L.; Lee, J.-S.; Thorgeirsson, S.S.; et al. Heat Stress-Induced PI3K/mTORC2-Dependent AKT Signaling Is a Central Mediator of Hepatocellular Carcinoma Survival to Thermal Ablation-Induced Heat Stress. *PLoS ONE* **2016**, *11*, e0162634. <https://doi.org/10.1371/journal.pone.0162634>.
71. Ding, H.; Wang, Y.; Zhao, H.; Wang, J.; Huang, D. Negative Effect of Seasonal Heat Stress on Testis Morphology and Transcriptomes in Angora Rabbit. *BMC Genom.* **2025**, *26*, 478. <https://doi.org/10.1186/s12864-025-11659-x>.
72. Maeng, S.; Ko, Y.-J.; Kim, G.-B.; Jung, K.-W.; Floyd, A.; Heitman, J.; Bahn, Y.-S. Comparative Transcriptome Analysis Reveals Novel Roles of the Ras and Cyclic AMP Signaling Pathways in Environmental Stress Response and Antifungal Drug Sensitivity in *Cryptococcus neoformans*. *Eukaryot. Cell* **2010**, *9*, 360–378. <https://doi.org/10.1128/EC.00309-09>.
73. Eslamizad, M.; Albrecht, D.; Kuhla, B. The Effect of Chronic, Mild Heat Stress on Metabolic Changes of Nutrition and Adaptations in Rumen Papillae of Lactating Dairy Cows. *J. Dairy Sci.* **2020**, *103*, 8601–8614. <https://doi.org/10.3168/jds.2020-18417>.
74. Neves, L.F.M.; Gomes, M.B.; Campolina, J.P.; Campos, M.M.; Souza, E.M.B.; Diavão, J.; Silva, A.S.; Tomich, T.R.; Carvalho, W.A.; Lage, H.F.; et al. Impact of Heat Stress on Intake, Performance, Digestibility, and Health of Neonatal Dairy Calves. *Animals* **2025**, *15*, 1876. <https://doi.org/10.3390/ani15131876>.
75. Hannun, Y.A.; Obeid, L.M. Sphingolipids and Their Metabolism in Physiology and Disease. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 175–191. <https://doi.org/10.1038/nrm.2017.107>.
76. Park, W.J.; Park, J.W. The Role of Sphingolipids in Endoplasmic Reticulum Stress. *FEBS Lett.* **2020**, *594*, 3632–3651.
77. Aravindh, S.; Silpa, M.V.; Voggu, S.P.; Rebez, E.B.; Kalaignazhal, G.; Srinivas, M.V.; Dunshea, F.R.; Sejian, V. Epigenetic Mechanisms Associated with Livestock Adaptation to Heat Stress. *Biology* **2025**, *14*, 1154. <https://doi.org/10.3390/biology14091154>.
78. Bewicke-Copley, F.; Mulcahy, L.A.; Jacobs, L.A.; Samuel, P.; Akbar, N.; Pink, R.C.; Carter, D.R.F. Extracellular Vesicles Released Following Heat Stress Induce Bystander Effect in Unstressed Populations. *J. Extracell. Vesicles* **2017**, *6*, 1340746. <https://doi.org/10.1080/20013078.2017.1340746>.
79. Gebremedhn, S.; Ali, A.; Gad, A.; Prochazka, R.; Tesfaye, D. Extracellular Vesicles as Mediators of Environmental and Metabolic Stress Coping Mechanisms During Mammalian Follicular Development. *Front. Vet. Sci.* **2020**, *7*, 602043. <https://doi.org/10.3389/fvets.2020.602043>.
80. Castellani, S.; Basiricò, L.; Maggiolino, A.; Lecchi, C.; De Palo, P.; Bernabucci, U. Effects of Milk Extracellular Vesicles from Holstein Friesian and Brown Swiss Heat-Stressed Dairy Cows on Bovine Mammary Epithelial Cells. *J. Dairy Sci.* **2025**, *108*, 1978–1991. <https://doi.org/10.3168/jds.2024-25133>.

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.