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Citation for published version:
Tsiamadis, V, Valergakis, GE, Soufleri, A, Arsenos, G, Banos, G & Karamanlis, X 2023, 'Identification of temporal patterns of environmental heat stress of Holstein dairy heifers raised in Mediterranean climate during their in-utero and post-natal life periods and modelling their effects on age at first calving', *Journal of Thermal Biology*, vol. 117, 103717, pp. 1-9. https://doi.org/10.1016/j.jtherbio.2023.103717

Digital Object Identifier (DOI):
10.1016/j.jtherbio.2023.103717

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Journal of Thermal Biology

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Download date: 16. Feb. 2025

Identification of temporal patterns of environmental heat stress of Holstein dairy heifers raised in Mediterranean climate during their in-utero and post-natal life periods and modelling their effects on age at first calving

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Simple Summary

Age at first calving is an important reproductive trait of dairy cows, with long-term implications on milk yield, health, reproduction, and profitability of dairy herds. Heat stress can adversely affect the welfare and productivity of cattle. Temperature-Humidity-Index values that quantifies the degree of heat stress in dairy cattle can be easily calculated based on climatic data. However, the effect of heat stress imposed during fetal and early life on age at first calving has not been examined, yet. In the present study the temporal patterns of environmental heat stress imposed on future Holstein dairy heifers during their in-uterus development and 3-month post-natal period and the association of those patterns with age at first calving were investigated. Results offer a deeper understanding in the future impacts of heat stress in subsequent reproductive life of young heifers. Exposure of heifers to heat stress during their very early stages of life can later increase age at first calving from one up to three weeks. This important finding warrants caution in evaluating the long-term implications of heat stress in reproduction. Heat stress mitigation practices should be adopted by dairy farmers.

Abstract

A retrospective study was conducted to evaluate temporal patterns of environmental heat stress during the in-uterus period of development (IUP) and the 3-month post-natal (PN) period of dairy heifers, and to estimate their association with the age at first calving (AFC). Data from 30 dairy herds in Northern Greece including 9,098 heifers were extracted from National Cattle Database. Data (2005 – 2019) regarding 230,100 farm-specific ambient daily temperature and relative humidity records, were obtained from ERA5-Land. Average monthly Temperature-Humidity-Index values (THI; low≤68, and high>68) were calculated and matched for each heifer to their IUP and PN. Subsequently, Cluster Analysis was used with monthly THIs as predictors to allocate heifers to THI clusters. The association of clusters with AFC was assessed with Generalized Linear Mixed Model analysis, an extended form of multiple linear regression.

Finally, 8 Heat Stress Clusters (HSC; namely HSC-1 to HSC-8) were identified. Compared to HSC-8 (8th-9th IUP months and 1st PN month) heifers of HSC-5 (4th-7th IUP months) and HSC-6 (6th-8th IUP months) calved 13.8 and 17.8 days later, respectively ($P<0.01-0.001$). Moreover, when AFC was treated as a binary variable, heifers of HSC-5 and HSC-6 had 1.15 and 1.34 ($P<0.01-0.001$) higher risk of calving for the first time later than 787 days compared to HSC-8, respectively.

Introduction

Global warming refers to the rise of average air temperature over time. Under various future scenarios this increase has been averagely estimated at 0.2°C per decade and by 2100, temperature will rise by 1.4-5.8°C, relative to the 1986-2005 period [1,2]. Global warming is mainly a result of anthropogenic activities leading to the accumulation of greenhouse gasses, namely CO_2 and methane, and it is the primary and strongest phenomenon associated with climatic change [3]. Due to climatic change, warm areas are recording increasingly high temperatures for extended periods of time, and larger parts of the globe are experiencing continuously elevated temperature and relative humidity levels [2]. Heat stress in animals occurs when their core temperature increases beyond their ability to maintain thermal equilibrium and dissipate body heat effectively [4]. Dairy cows have a thermoneutral zone between 5 and 26 °C; beyond this range, cows need to adapt their metabolism in order to support their core temperature within normal levels (38.2-39.2 °C) [5–7]. The tolerance of an animal to elevated air temperatures is affected by the relative humidity in the air, which determines the rate of heat abatement through evaporate cooling. Thom, (1959), firstly introduced the term "*discomfort index*" that describes the level of heat stress experienced by humans. Later, this index has been evolved to the Temperature-Humidity-Index (THI), that quantifies the degree of heat stress in dairy cattle [8–10]. Various THI thresholds have been proposed for dairy cows, beyond which heat stress becomes a serious issue; a value of 72 units proposed following research conducted in subtropical regions [10,11]. A threshold of 68 may be more appropriate for high yielding dairy cows raised in temperate climates [12,13].

Heat stress negatively influences animal health and compromises welfare and productivity [4,14]. Increased environmental temperature has negative impacts on the reproduction of mammalian species: embryogenesis, oogenesis and spermatogenesis are affected [15–18], conception rates are reduced [19,20], and puberty becomes suppressed [21]. Moreover, long-term effects on the offspring may arise as a result of maternal heat stress. Dam exposure to high THI during the dry period (late gestation) compromises passive transfer of immunity and lowers calf weaning weight and height [22–24], compared to calves born of cooled dams. Recce et al., (2021), recently demonstrated the long-term effects of high THI values on calving to conception interval of heifers whose dams had been exposed to heat stress during heifers' intrauterine development. Age at first calving (AFC) is also an important performance trait that is recorded for all cows, with long-term implications on milk yield, health, reproduction, and profitability of dairy herds. Optimum AFC for Holstein dairy cows is considered to be between 25 and 26 months [26–28], while other researchers estimate it between 18 and 23 months [29–31]. However, the effect of heat stress imposed during fetal and early life on AFC has not been investigated, yet.

Central Macedonia is Greece's primary dairy-producing region. It has a Mediterranean climate with continental characteristics. From April to September (spring to summer) average THI values are usually above the comfort levels for cattle (THIs>68) [32]. Moreover, according to the latest Intergovernmental Panel on Climate Change report, climate change is anticipated to escalate in the Mediterranean basin [2]. Greece is expected to experience in the near future (2025-2049) longer periods of drought and extreme maximum temperatures [33].

This study aimed: a) to identify temporal patterns of environmental heat stress imposed on future Holstein dairy heifers during their in-uterus period of development and 3-month post-natal period, raised in Central Macedonia (Northern Greece), and b) to investigate the association of heat stress patterns with AFC.

Materials and methods

Herds and animals

Data from dairy farms of Thessaloniki Prefecture (Central Macedonia), Greece's major area of dairy production, were used in the study. The inclusion criteria for herds and heifers were:

I) Herds should have had a minimum yearly average number of dairy cows ≥ 100, and the geographical latitude and longitude of the premises should have been recorded in the Greek National Cattle Database.

II) Regarding heifers, information recorded in the Greek National Cattle Database should have included: (a) heifer birth date, (b) dam birth date, (c) date of heifer's 1st calving, and (d) heifers and their dams should have been both born in Thessaloniki Prefecture.

Data were derived from the Greek National Cattle Database throughout a 15-year period (2005 – 2019). Finally, 30 herds and 9,098 heifers, that calved for the first time between 2005 – 2019, fulfilled the inclusion criteria (Suppl. Table 1).

Based on the heifers birth date, for each heifer the time-length of her in-utero period (IUP) of development and the 3-month post-natal period (PN) were estimated. Both periods were partitioned in monthly increments, resulting in 9 IUP "months" and 3 PN "months" (IUPM and PNM, respectively).

The number of cows on each herd ranged from 100 to 500. During the last 5 years (2015 - 2020), the daily milk production per cow was: a) 25-27 kg, n= 3 herds, b) 28-30 kg, n= 16 herds, c) 31-33 kg, n= 5 herds, and d) 34-35 kg, n=6 herds. All cows were milked twice daily. All premises had barns with corrugated thermo-insulated roof panels. Heifers were housed in single-slope barns with large front openings and natural ventilation; barns were orientated so as to provide shade during the summer months and equipped with curtain side-walls for the winter. Dry cows were housed in single-slope barns with large front openings and natural ventilation, as well heifers (n=8) or in double-pitched roof barns with ridge openings and mechanical ventilation with fans to provide air exchange (n=22). Dry cows and heifer housing facilities consisted of traditional bedded packs. Straw was added to the bedded area (≈10 kg/cow/day) and removed every 4-6 weeks. Milking cows were housed in single-slope barns with large front openings and natural ventilation and conventional bedded pack pens (n=3) or in double-pitched roof free-stall barns, usually with 2 or 3 row pens, with ridge openings and mechanical ventilation with fans to provide air exchange (n=27). Only three farms, with double-pitched roof free-stall barns, used sprinklers in the feed alley.

Total mixed rations for heifers, dry and fresh cows were formulated to meet or exceed net energy and metabolizable protein requirements, according to National Research Council recommendations (NRC, 2001). All farms used artificial insemination for the entire study period.

Climate dataset

The historical farm-specific average daily air temperature and relative humidity data were derived from ERA5-Land, a European Centre for Medium-Range Weather Forecasts, formed within the Copernicus Climate Change Service Program of the European Commission [34]. ERA5-Land uses weather prediction models to produce a total of 50 climate variables at a spatial resolution of 9 km and with a temporal coverage from January 1950 to present.

Temperature and humidity index (THI)

In total, 230,100 average daily temperature and relative humidity records were derived from ERA5-Land. From this dataset average daily THI values were calculated based on the equation: THI = $(1.8 \times T + 32) - [(0.55 - 0.0055 \times RH) \times (1.8 \times T - 26)]$

Where:

T: temperature (in °C), and

RH: relative humidity (NRC, 1971).

Subsequently, 9 and 3 average monthly THI values, were estimated and matched for each heifer to their IUP and PN, respectively. Monthly THIs were categorised as low (THI_1≤68) and high (THI_2>68).

Thereafter, an overall 3-level categorical THI trait was established (needed for the subsequent cluster analysis, see further below) for each of the two periods (IUP and PN), defined as follows:

1. For **IUP**:
    a. IUP_1: represented heifers with 0 or 1 months of high THI values during their entire in-utero development period,
    b. IUP_2: represented heifers with 2 or 3 months of high THI values during their entire in-utero period,
    c. IUP_3: represented heifers with at least 4 months of high THI values during their entire in-utero period.
2. For **PN**:
    a. PN_1: represented heifers with 0 or 1 months of high THI values during their entire post-natal period,
    b. PN_2: represented heifers with 2 months of high THI values during their entire post-natal period,
    c. PN_3: represented heifers with 3 months of high THI values during their entire post-natal period.

Statistical analyses

1. <u>Cluster analysis</u>

Cluster analysis (CA) is a method that identifies homogenous groups of cases that form a cluster. [35,36].

a) Selection of clustering variables

In the present study, the aim of CA was to identify groups of heifers that were very similar regarding the THI load during their in-utero and post-natal period. Therefore, the average monthly THI values in the corresponding periods were selected as clustering variables (12 variables in total).

b) Decision for the number of clusters

Hierarchical CA method was selected to decide the optimal number of clusters [36]. In order to achieve low levels of collinearity among the 12 clustering variables highly correlated variables (correlation coefficients >0.90) had to be identified [36]. Therefore, a Bivariate Correlation analysis, with Kendall's tau-b test for estimation of correlation coefficients, was performed. During this preliminary analysis, moderate correlations were identified between the average monthly THI values of the 1st and the 7th month of the in-utero period of development and between the average monthly THI values of the 1st month of the in-utero period of development and the 3rd month of the post-natal period. Therefore, the removal of the average monthly THI values of the 1st month of the in-utero period of development from the clustering procedure was decided. Finally, 11 remaining monthly THIs were used in the HCA. The Kolmogorov-Smirnov test of normality revealed the non-normal distribution of those THIs (*P<0.001*). Therefore, THI values were standardized to their z scores and then inserted into the HCA as continuous variables. Euclidian distance and Ward's method were used during the HCA. The optimum number of clusters was decided using the elbow rule on the agglomeration schedule derived from HCA (Suppl. Table 2). According to this rule, the optimum number of clusters (n=8) was estimated by subtracting the number of stages (n=9,083) from the number of observations (n-1=9,097). These were used in the subsequent Two-Step cluster analysis (TSCA).

c) Selection and execution of the clustering analysis

The Two-Step Cluster Analysis (TSCA) of SPSS was used to allocate heifers to clusters [35,37]. The same 11 average monthly THI values of each heifer from HCA were considered. Moreover, the overall categorical THI traits were additionally inserted into the TSCA procedure to allow the clustering algorithm to distinguish heifers of low- and high-THI load during the two periods. All clustering predictors were equally used by the TSCA algorithm to heifer classification among the 8 clusters (predictor importance=1.00). The Log-likelihood function was used as a distance measure among clusters.

d) Validation of the clustering solution

The stability of the clustering solution was displayed by manually performing 25 iterations. Each time, the cluster membership of individual heifers was the same, indicating stability of the results. Moreover, results from the Kaiser-Meyer-Olkin (KMO) measure of sampling adequacy (0.904) and the Bartlett's test of sphericity (statistically significant at *P<0.001*), indicated that cluster analysis was appropriate for our data.

2. Regression Analysis

The association of THI clusters with AFC (in days) was assessed with Generalized Linear Mixed Model (GLMM) analysis. The model included AFC as dependent (response) variable and the following independent (explanatory) variables: the random effects of herd and the fixed effects of age of the dam of the heifer subjected to analysis at conception (4 levels, age quartile 1 [AQ1]: 292 – 537 days, n=2,247; AQ2: 537 – 880 days, n=2,247; AQ3: 881 – 1375 days, n=2,269 and AQ4: ≥ 1,376 days, n=2,335), the respective THI cluster category of each heifer, and the year that the heifer was conceived (16 levels: 2005 – 2020). Pairwise comparisons of estimated marginal THI cluster means were assessed using Least Square Adjustment. Regarding the *"herd"* random effect, there are many factors that affect both the dam and the fetus/heifer during the in-utero and the post-natal periods. These include methods of mitigating heat stress (provision of shade, sprinkler and fan systems etc.), dam and calf housing, and calf/heifer health and nutrition management practices. Those factors were considered in the statistical model as random ones within the random "*herd*" factor, as described and applied by Recce et al. 2021 [25]. The validity of the model was assessed by evaluating the normality of the residuals with Q-Q plots. The reliability of the model was assessed through split-sample analysis and subsequent cross-validation correlation, as described in Dohoo et al. 2009 [38].

Moreover, the association of THI clusters with the risk of having her 1st calving beyond the median AFC in our data, 787 days of age, was assessed with binary linear regression (LR) analysis by fitting a logit function to the above model. Age at first calving was treated as a binary variable with values of zero for AFC<787 and one for AFC>787 days. The probability derived from the binary LR analysis was treated as test variable in a subsequent Receiver Operating Characteristic (ROC) analysis aimed to evaluate the reliability of the model.

All statistical analyses were performed using the SPSS 25.0. software package (IBM SPSS, Version 25.0, Armonk, NY: IBM Corp.). Statistical significance was set at $P \leq 0.05$, in all cases.

Results

Regarding the GLMM, the correlation between the predicted and actual values for the randomly selected subset of data (n= 5,421) was 0.439 (R^2= 0.193), while for the remaining 3,677 data it was 0.479 (R^2= 0.229). The shrinkage on cross-validation was <0.1, suggesting that the selected predictors formed a reasonably reliable model. Further, regarding the binary procedure of the GLMM, the estimated model Area Under the Curve (AUC) was 0.74 (95% CI: 0.73 – 0.75, $P<0.001$), indicating adequate model reliability.

During the study period, the number of days per year with THI values > 68 increased from 95 in 2005 to 110 in 2019, representing a rise of 14.6% (Figure 1). Average monthly and yearly THI values are depicted in Figures 2 and 3, respectively. All eight clusters (namely HSC-1 to -8) were identified in each herd (Suppl. Table 3) and are described in detail below. Details regarding the distribution of low and high THIs for each month during the in-utero and the post-natal periods in all clusters are depicted in Figure 4 and 5. Details regarding the distribution of dam conception months for each cluster are presented in Suppl. Table 4.

Cluster description

HSC-1: Entire post-natal period heat stress (n = 782, 8.6%). All heifers classified in this cluster experienced average monthly THI values >68 during each of the three months of the post-natal period. During the first 2-8 months of their in-utero period of development heifers did not experience almost any average monthly THI>68. It was only until the 9th month of the in-utero period of development that about 25% of heifers experienced heat stress. Dams of heifers classified in this cluster conceived mostly during August and September and gave birth mostly during May and June of the following year.

HSC-2: 2nd month of the in-utero period of development and 2nd to 3rd month of the post-natal period heat stress (n=596, 6.6%). Almost all heifers classified in this cluster experienced heat stress during the 2nd and the 3rd month of the post-natal period (96.1% and 99.8%, respectively). Most of them (60.1%) also experienced heat stress during the 2nd month of the in-utero period of development. Dams of heifers classified in this cluster conceived mostly during summer (July and August), and gave birth during the spring months of the following year (April and May).

HSC-3: 2nd and 3rd month of the in-utero period of development and 3rd month of the post-natal period heat stress (n=860, 9.5%). Heifers classified in this cluster experienced heat stress during the 2nd and the 3rd month of the in-utero period of development (100% and 75.3%, respectively). It was only at the 3rd month of the post-natal period that 79% of heifers experienced heat stress again. Dams of heifers classified in this cluster conceived during May and mid-summer (June and July), and gave birth during March and April of the following year.

HSC-4: 2nd to 5th month of the in-utero period of development heat stress (n=1,594, 17.5%). Heifers classified in this cluster experienced heat stress during their 2nd to 5th IUPM (75.4%, 98.2%, 98.1%, and 48.9%, respectively). It was only at the 3rd month of the post-natal period that a minor percentage (4.4%) of heifers experienced heat stress. Dams of heifers classified in this cluster conceived mostly during April to June and gave birth mostly during the winter months (Jan to Feb) and in March of the following year.

HSC-5: Mostly 2nd trimester of the in-utero period of development and prolonged heat stress (n=1,620, 17.8%). Heifers classified in this cluster started to experience heat stress during the 3rd month (24.4%), mainly during the 4th to 6th (76.4%, 99.1%, and 96.3%), and to a lesser extent (49.4%) during the 7th month of the in-utero period of development. Dams of heifers classified in this cluster conceived mostly during February and March, and gave birth during the late autumn months and January of the following year.

HSC-6: 2nd and 3rd trimester of the in-utero period of development and prolonged heat stress (n=1,267, 13.9%). Heifers classified in this cluster started to experience heat stress during the 5th (31.6%), mainly during the 6th to 8th (90.1%, 100%, and 95.6%), and to a lesser extend (27.8%) during the 9th month of the in-utero period of development. Dams of heifers classified in this cluster conceived mostly during the mid-winter (Dec and Jan), and gave birth mostly during September to October of the following year.

HSC-7: Last month of the in-utero period of development/1st and 2nd month of post-natal period heat stress (n=1,044, 11.5%). Heifers classified in this cluster started to experience heat stress during the 8th (15.4%) and mostly during the 9th month of the in-utero period of development (87.5%) and the 1st and 2nd month of post-natal period (99.4 and 88.1%, respectively). Dams of heifers classified in this cluster conceived on Autumn (mostly during

Oct), and gave birth during the summer months of the following year (mostly during Jul and Aug).

HSC-8: Mostly 3rd trimester of in-utero period of development heat stress (n=1,335, 14.7%). It was only at the 7th month of the in-utero period of development that half of heifers experienced HS (52%), and thereafter during the 8th and 9th months (100% and 99.9%, respectively); half of them (53.6%) experienced HS during the 1st month of the post-natal period, as well. Dams of heifers classified in this cluster conceived mostly during late autumn (Oct and Nov) and December, and gave birth mostly during the August and September of the following year.

Association of HSC with AFC

Details regarding the association of environmental HS clusters with AFC are presented in Table 1. Cluster HSC-8 was set as the reference cluster. Compared to HSC-8, heifers classified in clusters HSC-2, HSC-3, HSC-4, HSC-5 and HSC-6 calved from 6.4 to 17.8 days later ($P<0.05 – P<0.001$). When AFC was treated as a binary variable, heifers classified in clusters HSC-5 and HSC-6 had 1.15 ($P<0.01$) and 1.34 ($P<0.001$) higher risk of performing their 1st calving beyond 787 days compared to HSC-8, respectively.

Herd was a significant risk factor for AFC. Heifers gestated by dams of AQ2 tended to calve 4.7 days later, compared to those gestated by dams of AQ1 ($P=0.059$). When AFC was treated as a binary variable, heifers gestated by dams of AQ2 and AQ4 had 1.17 and 1.14 ($P<0.05$) higher risk of performing their first calving beyond 787 days compared to heifers gestated by AQ1 dams; a similar tendency (OR=1.14, $P=0.059$) existed for AQ3 dams.

Discussion

We have conducted a retrospective study aimed to evaluate the temporal patterns of heat stress in Holstein heifers, and to estimate whether heat stress during their in-utero period of development and first months of life was associated with the AFC. To that aim, we have used cluster analysis to distinguish heifers that had similar temporal heat stress patterns during their entire gestation and post-natal period from those that differ at the same period. One of the main advantages of cluster analysis is that it is performed on raw data without any a priori knowledge of cluster characteristics [37]. Recently it has been applied in disease description [39–41]. One drawback of this method is the instability of the cluster membership (here the allocation of the subjects in different clusters every time the algorithm is executed). Thus, we have performed a series of manual iterations; every time the clustering solution was the same, suggesting the reliability of the method. Moreover, correlated predictor variables were excluded during the previous preliminary steps of the analysis to avoid collinearity complications. To our knowledge this is the first study reporting temporal patterns of environmental heat stress in Holstein dairy heifers using this method.

Further, we have used the Generalized Linear Mixed Model analysis of SPSS, an extended linear model, that enables the non-normal distribution of the dependent variable and the integration of random effects to the analysis [42]. The normal distribution of the residuals indicated the validity of the model. The reliability of the model has been assessed through the cross-validation procedure that indicated the appropriateness of the proposed analysis. Also, the relatively high AUC that was derived after fitting a logit function to the binary linear regression of GLMM culminated to the adequacy of the proposed analysis. Provided the large number of herds and animals together with the robust statistical analyses, we are confident that our initial research hypothesis that heat stress was associated with age at first calving was confirmed and that results from the present study lie within our current understanding of heat stress effect on the dairy cow population.

Heifers that had not experienced, at any time during the in-utero and the post-natal periods, average monthly THIs above 68, did not exist in this dataset and therefore, a non-heat stress cluster could not be formed. Thus, the selection of a cluster as a referent-one was a compromise. Based on cluster characteristics, heifers of HSC-8 seemed the best option, based on the fact that they experienced heat stress mostly during the 3rd trimester of their in-utero life, a period where no major differentiation and maturation of reproductive organs occurs [43].

Clusters HSC-1 and HSC-7 were not associated with AFC. The heat load of the entire post-natal period was the distinct characteristic of HCS-1; on the other hand, HSC-7 seemed to be a "*transition cluster*" between HSC-6 and HSC-8. However, they both included the last month of gestation and the first two months of the post-natal period. Similarly, the last month of gestation and the first month of the post-natal period were also included in HSC-8. These three clusters represented the heat load accumulated during the end of the in-utero life and the post-natal period. Do these findings imply a favorable effect of head load on AFC or do these clusters of heifers were more heat-resistant compared to the other clusters? Caution is warranted when interpreting these findings. High ambient temperature and extreme heat waves are risk factors for calf mortality [44,45] and therefore it is possible that heifers of these clusters represent a rather "heat-tolerant" fraction of the population. Moreover, as heat stress during late pregnancy and early life has been associated with detrimental effects on immunity, growth rate and feed utilization [46–49], the apparent lack of association with AFC, must not preclude these animals from the benefits of heat stress mitigating practices (heat-protected accommodation, proper ventilation and shading, etc.) which are of paramount importance for their welfare, especially when an increase in temperature is expected in the coming years [33].

Heifers classified in HSC-2 to HSC-6 calved from one to almost three weeks later, compared to those classified in the reference cluster; those in HSC-5 and HSC-6 were also associated with increased risk of calving beyond 787 days (≈ 26 months). From the early stages of the embryological development and during the first trimester of gestation the primordial germ cells (PGCs) starts to migrate from the epiblast towards the genital ridges, the structures destined to become the undifferentiated gonads [43]. During this period of migration, the PGCs proliferate and differentiate to oogonia. Between the end of the first and the beginning of the second trimester of gestation the assemblance of the primordial follicles is initiated. The transition of primordial to primary follicles is initiated at the beginning of the second trimester of gestation [47,50]. Consequently, any potentially factor that disrupts this well-orchestrated procedure of production, transition and differentiation of primordial follicles might eventually result in ovarian reserve depletion at birth, and on the long-term, inevitably compromise fertility [51–53]. The above timeframes coincide with those of HSC-2 to HSC-5 (2nd to 4th months of in-utero development).

Recently, Recce et al., (2021), using multivariate regression analyses, reported that cow exposure to severe THI (≥72) during the first trimester of their in-uterus life was associated with greater calving to conception interval of their progeny. Moreover, Akbarinejad et al., (2017) demonstrated that exposure of dam to heat stress could delay calving to conception interval of their progeny, with the second and third trimesters of gestation being the most critical periods. Interestingly, these researchers also hypothesized that there may be distinct temporal patterns of prenatal heat stress, given the asymmetric development of the various fetal organs throughout the consecutive gestational stages.

Of course, one might argue that HSC-5 and especially HSC-6 do not fit to the previous description. Other mechanisms during the 5th to 7th months of the in-utero period of development that negatively affect future reproductive capacity may be involved and prolonged heat stress exposure may also play a role. Heat stress can lead to placental insufficiency and thus hamper fetal development. The reduced placental size and function restricts the maternal-fetal oxygen and nutrition exchange. Many adverse and detrimental heat stress effects to placental function have been reported: reduced placental weight [54], impaired placental and umbilical blood flow and placental vascularization [55,56], induced fetus hypoxia by impeding transplacental oxygen diffusion [55], depleted glucose provision to placental tissue [57], limited supply of amino acids [38]. Moreover, heat stress compromises maternal dry matter intake [58], which is essential for the ovarian function of the offspring. Mossa et al., (2013), provided evidence that the restriction of nutrients in the dam during the first trimester of gestation resulted in decreased ovarian reserves of female offspring as a consequence of high serum maternal testosterone concentration during gestation. This issue merits further research. In any case, the increase in AFC represents a considerable cost to dairy farmers; it was recently estimated that £2.87 (€3.24) are needed for each extra day of heifer rearing to first calving [60].

In the present study, average monthly THI values indicative of heat stress (> 68) were mostly present from June to September. Moreover, since 2005, average yearly THI values have continuously increased, as well as the number days per year with THI values above 68. This increase is expected to deteriorate in the Mediterranean basin during the coming decades [33]. This could lead to more frequent and severe heat waves, with negative impacts on health, welfare and production of dairy cows. Proactive managerial strategies must be adopted towards the mitigation of these effects. Traditionally, farmers prioritize high production groups when implementing heat abatement practices and more recently, following consultants' advice, dry period cow groups. However, a careful look at HSC-5 and HSC-6 reveals that the dams of these heifers experience most of the heat stress while residing in low production groups during their lactation cycle. Therefore, adequate ventilation and relevant equipment (shades, fans, sprinklers etc.) must be available to all cows, not only to high-end groups.

In addition of exposure to high THIs during their in-utero development and early life, heifers may be subjected to other stressors until breeding; while herd was included in the logistic regression model, the authors acknowledge that it cannot completely account for differences in other variables associated with AFC beyond the 3rd month of life, namely nutrition, housing/overstocking, health status, farmer perceptions, economic conditions etc. This is undoubtedly a limitation of the present retrospective study which is based on records of official data-bases. Moreover, in prospective studies, microclimate records should be used, in order to adequately capture the association of THI truly perceived by animals with reproductive indices.

Conclusions

Temporal patterns of heat stress during the in-utero development and the 3-month post-natal period that are associated with AFC do exist for Holstein heifers. Temperature-Humidity Index values >68 between the 2nd and the 7th month of in-utero development and especially for prolonged time, increase AFC from one up to three weeks compared to exposure to heat stress during the last trimester of gestation. Heat stress mitigation practices should be applied for all animals on dairy farms.

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