Study of potential environmental factors predisposing ewes to subclinical mastitis in Greece

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ABSTRACT

Objectives of the work presented herewith were to investigate association of prevalence of subclinical mastitis with environmental (climatic and topographic) factors and to identify factors potentially predisposing ewes to the disease. Milk samples were collected from 2198 sheep in 111 farms, in all 13 administrative regions of Greece, for bacteriological and cytological examination. Data on farm location were collected in the field using hand-held Global Positioning System Garmin units. The geo-references were resolved to specific farm level. Prevalence of subclinical mastitis was 0.260. Main aetiological agents were staphylococci (*Staphylococcus aureus* and coagulase-negative species), which accounted for 0.699 of all isolates recovered. In a multivariable mixed-effects analysis, the two environmental variables found to be associated with increased prevalence of subclinical mastitis were the minimum temperature of coldest month (coefficient: -0.084 ± 0.033, $P = 0.014$) and the mean temperature for 30 days prior to sampling date (coefficient: 0.031 ± 0.014, $P = 0.029$).

1. Introduction

Climatic factors are of particular significance in development of diseases, as they can contribute to transmission of pathogens or may exert effects on hosts. In diseases caused by organisms that are enzootic in farms, they may be of lesser significance, but, nevertheless, still important in contributing to development of a disease. For example, increased rainfall would lead to muddy pastures, which limit grazing of animals and thus modify feeding patterns, or low temperatures would lead to increased energy requirements of animals, which can affect their immune status [1]. In general, climate is influenced by the combination of geography and geomorphology.

In sheep, mastitis is a multi-factorial disease, with many bacteria identified as causal agents and many factors accounting for potential predisposition to the disease [2,3]. Staphylococci, *Staphylococcus aureus* and coagulase-negative species, are the most frequent aetiological agents of the disease [2]. The disease adversely affects production and causes financial problems, especially in dairy flocks, and has also been recognised as the most important cause of welfare concerns in sheep [4]. Possible role of climatic conditions in development of mastitis has never been investigated. Further, Clark [5] has mentioned that mastitis was occurring more frequently after gusts of cold winds. Another

possible association between weather and mastitis in sheep has also been reported in the 1980s [6].

This paper presents results of an extensive, countrywide study on subclinical mastitis in sheep farms throughout Greece. The investigation included 111 flocks located in all 13 administrative regions of Greece; total ewe population in these flocks was approximately 35,000 animals. In Greece, sheep production is the predominant form of agriculture, with over 95% of ewes farmed for dairy production. Subclinical mastitis is of particular concern, because it is rarely diagnosed and thus remains untreated. Objectives of the work presented herewith were to investigate association of prevalence of subclinical mastitis with environmental (climatic and topographic) factors and to identify factors potentially predisposing ewes to the disease.

2. Materials and methods

2.1. Sheep farms and geographical information system

In total, 111 sheep farms in the 13 administrative regions of Greece were included into the study and visited for collection of samples and information; in 2016, there were registered in Greece in total 87,109 sheep farms, i.e., 0.13% of these were visited during the study [7].
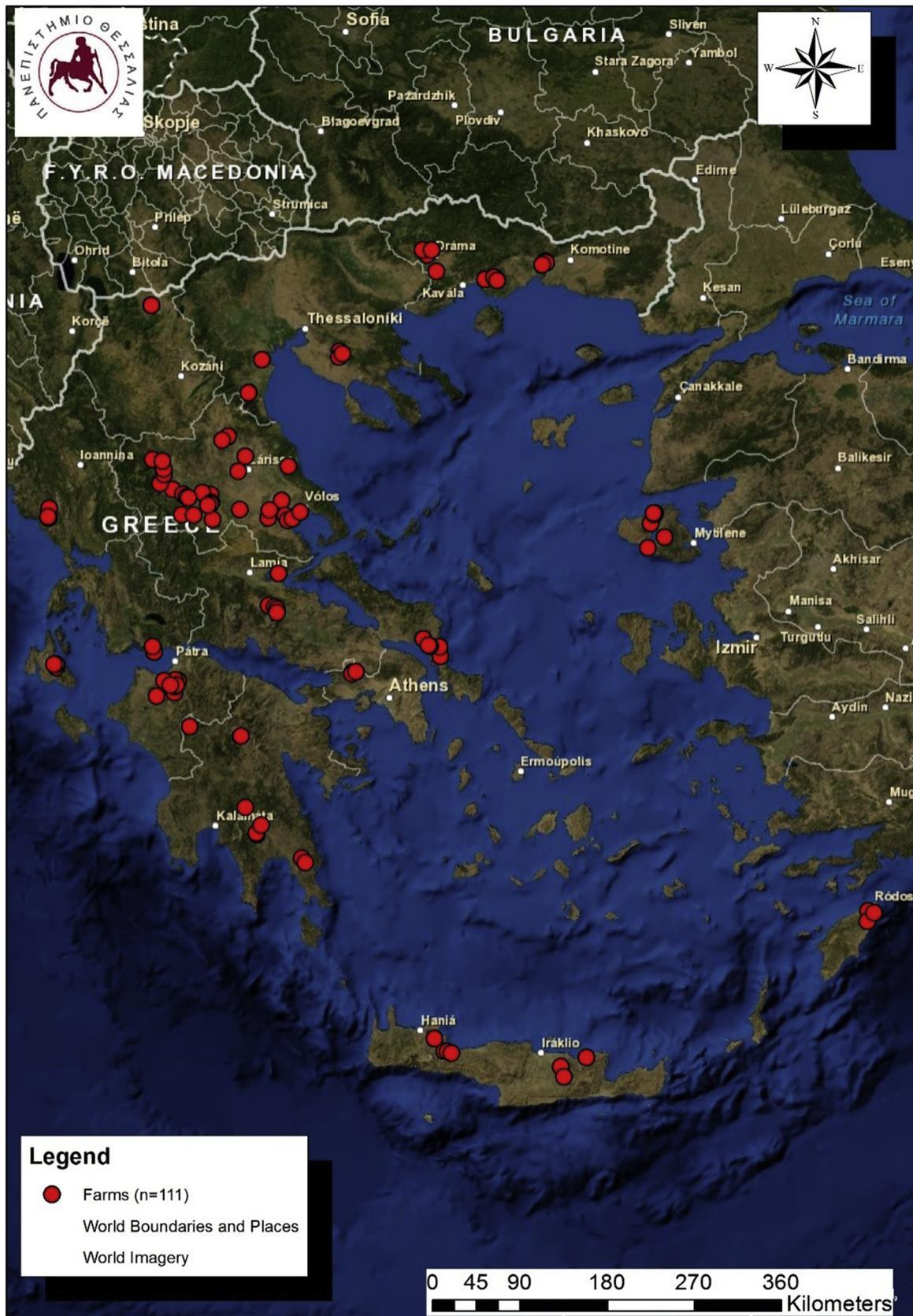
Fig. 1. Location of 111 sheep farms around Greece, which were included in an investigation on ovine subclinical mastitis.

Table 1
Continuous environmental variables used in the analysis.

Variable	Source	Mean (± standard error)	P^*
Annual mean temperature (°C)	WorldClim Database	16.0 ± 0.1	0.050
Mean diurnal temperature range (°C)	WorldClim Database	9.9 ± 0.2	0.033
Maximum temperature of warmest month (°C)	WorldClim Database	31.6 ± 0.2	0.162
Minimum temperature of coldest month (°C)	WorldClim Database	3.2 ± 0.3	0.031
Temperature annual range (°C)	WorldClim Database	28.5 ± 0.4	0.029
Mean temperature for 30 days prior to sampling date (°C)	WorldClim Database	16.3 ± 0.7	0.071
Total annual precipitation (mm)	WorldClim Database	650.5 ± 13.8	0.356
Annual mean wind speed (m s^{-1})	ArcGIS-HRAE	4.1 ± 0.1	< 0.001
Mean wind speed for 30 days prior to sampling date (m s^{-1})	ArcGIS-HRAE	7.9 ± 0.3	0.190
Altitude (m)	DEM	181.1 ± 14.4	0.081
Distance from small ruminant farms (m)	ArcGIS- GPACAP	332.6 ± 21.2	0.920
Distance from public health agencies (m)	ArcGIS- GPACAP	8288.4 ± 398.6	0.047

* P derived in the univariable model analysis.

Veterinarians active in small ruminant health management around Greece, were contacted by telephone and asked if they wished to collaborate in the investigation. In total, 25 veterinarians were contacted; of these, 23 (0.92) had agreed to collaborate. Farms were selected by the collaborating veterinarians on convenience basis (willingness of farmers to accept a visit by University personnel for sampling animals). The principal investigators (NGCV, GCF) visited all farms for sample collection. Location of farms around the country is shown in Fig. 1.

Data on farm location were collected in the field using hand-held Global Positioning System Garmin units. The geo-references were resolved to specific farm level. ArcGIS V.10.1 GIS software (ESRI; Redlands, Ca, USA) was employed for description and analysis of spatial information.

2.2. Animal sampling and processing of samples

In each farm, 20 clinically healthy ewes (*secundiparae* or older) were selected at random for sampling. For selection of animals, farmers had been asked to remove from the main flock *primiparae* ewes and ewes with known udder abnormalities. A standardised clinical examination (observation, palpation, comparison between glands) of the udder was performed, always by the principal investigator (NGCV) [8,9] and the first two squirts of secretion were drawn on the gloved hand of an assisting investigator and assessed. All investigators involved in sampling procedures wore disposable, non-sterile latex gloves. If udder abnormalities were recorded during clinical examination, the ewe was excluded from sampling. Animals found with abnormalities and excluded, were not replaced.

Standard methods for aseptic collection of milk samples were followed [8]. Then, 10 to 15 mL of secretion were collected into a sterile container; separate samples were collected from each mammary gland into separate containers. Milk samples were then drawn onto a paddle for performing the California Mastitis Test (CMT). For transportation, samples were stored into portable refrigerators with ice packs and transported by car; for samples collected in islands, airplane or boat transportation, as accompanying luggage, was also involved.

Laboratory procedures started within 24 h after collection. Milk samples (10 μL) were cultured using Columbia blood agar plates incubated aerobically at 37 °C for up to 72 h. Bacterial identifications

were performed by using standards methods [10,11].

After sample collection, at ewe-side, all samples were tested by use of the CMT. The test was performed as previously described for ewes' milk [12]; it was carried out and scored always by the same person, i.e., the principal investigator (NGCV). Five degrees of reaction ('negative', 'trace', 'l', '2', '3') were described [13]. From all samples, smears were also produced and dried. The smears were stained by the Giemsa method for estimation of leucocyte subpopulations; proportion of leucocyte types therein was calculated by observing at least 10 fields of each milk film under magnification 10 ×. Subsequently, the Microscopic cell counting method (Mccm) (IDF reference method) [14–16] was performed in 894 samples (20.3% of all samples). Milk samples for cell counting by means of the Mccm, had been selected by using an electronic random number generator (www.randomresult.com) from among all samples collected during each field trip (i.e., samples from all farms visited during that trip, considered together); at least 20% of milk samples collected during each field trip, were selected for testing.

2.3. Data management and analysis

2.3.1. Environmental parameters

Climatic variables were derived from the WorldClim website (http://www.worldclim.org), version 1.4. [17]. Wind speed was retrieved from the GeoPortal of the Hellenic Regulatory Authority for Energy (www.rae.gr). Altitude was extracted from a digital elevation model (DEM) with a spatial resolution of 1 square kilometre (http://srtm.csi.cgiar.org/Index.asp). Distances between small ruminant farms were taken from Greek public authority sources, specifically the Greek Payment Authority for Common Agricultural Policy (GPACAP; http://www.opekepe.gr)] and the Hellenic Statistical Authority (http://www.statistics.gr). Land uses and habitat types were derived from the Corine Land Cover 2000 database (European Environment Agency, Copenhagen, Denmark [http://www.eea.europa.eu/data-and-maps]). Environmental and location parameters calculated are in Tables 1 and 2.

2.3.2. Mastitis definitions

Subclinical mastitis was considered in ewes in which a bacteriologically positive milk sample ([a] > 10 colonies of the same organism and [b] no more than two different types of colonies) with

Table 2
Categorical environmental variables used in the analysis.

Variable	Source	Main categories (proportion)	P^*
Land use	ArcGIS-Corine LC (EEA)	Cultivations (0.65), shurbland (0.13), pastures (0.12)	0.132
Microhabitat type	ArcGIS-Corine LC (EEA)	Permanently irrigated land (0.20), complex cultivation patterns (0.18), non-irrigated arable land (0.14), sclerophyllous vegetation (0.09), natural grasslands (0.08), beaches, sands and dunes (0.06)	0.001

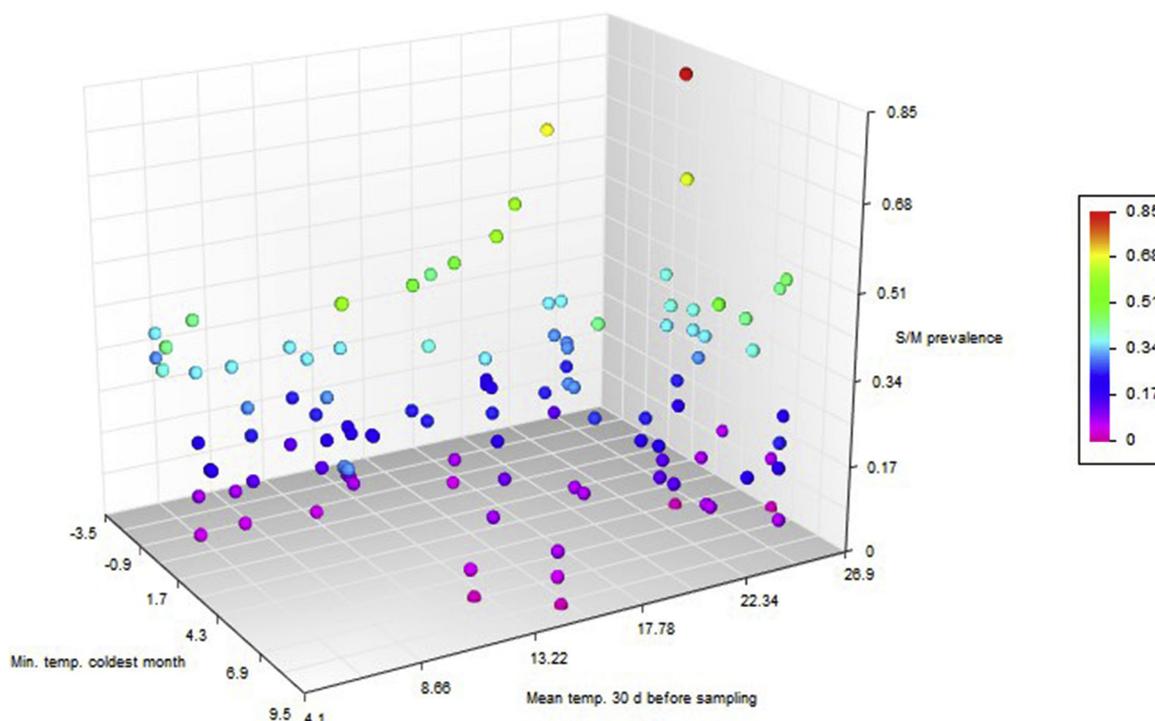* P derived in the univariable model analysis.

Fig. 2. Three-dimensional scatter plot of results of subclinical mastitis prevalence [S/M prevalence', z axis) against the two significant environmental factors: minimum temperature of coldest month (°C) ['Min. temp. coldest month', x axis] and mean temperature of 30 days prior to sampling (°C) ['Mean temp. 30 d before sampling', y axis] in 111 sheep farms in Greece.

concurrently increased CMT score (≥'l') plus neutrophil and lymphocyte proportion (≥65% of all leucocytes) was detected [18]. The definition referred to ewes (hence, animals with both glands affected were counted as one case).

Quantitative information on the cellular content of ewes' milk was obtained by using two sets of data: the CMT results and the results of the Mccm. Although it is generally established that CMT results are reliable proxy measurements for somatic cell counts (SCCs) [12,19], we further confirmed that in the present study. Following assignment of numerical values to CMT scores (value 0 to score 'negative', value 1 to score 'trace', value 2 to score '1', value 3 to score '2', and value 4 to score '3') and $\log_{10}$-transformations, correlation between CMT scores and Mccm SCCs was $r = 0.913$ (95% CI: 0.902 - 0.923) ($P < 0.001$) and the corrected $R2$ was 83.4%.

2.3.3. Significance of environmental factors

Data were entered into Microsoft Excel and analysed using STATA15 (Statacorp Inc., Texas, USA). Basic descriptive analysis was performed. The outcome of 'subclinical mastitis' was considered. Exact binomial confidence intervals were obtained.

Checks for multicollinearity revealed that the Minimum temperature of coldest month and the Annual range in temperature were very highly correlated ($r = -0.909$, $R^2 = 0.830$): the multivariable analyses for possible predictors therefore used the Minimum temperature of coldest month and excluded the Annual temperature range. A preliminary assessment of the importance of predictors was performed using by cross-tabulation with simple logistic regression without random effects. Subsequently, mixed-effects logistic regression was employed to perform the same comparisons, using the different farms (n = 111) as a 'random effect'.

A multivariable model was created using mixed-effects logistic regression with farm as the random effect, and initially offering to the model all variables which achieved a significance of $P \leq 0.2$ in the univariable analysis. Variables were removed from this initial model by backwards elimination. The P value of removal of a variable was

assessed by the likelihood ratio test, and for those with a P of > 0.2 the variable with the largest probability was removed. This process was repeated until no variable could be removed with a P value of > 0.2.

Statistical significance was defined at $P < 0.05$.

3. Results

3.1. Prevalence of subclinical mastitis

In the 111 farms, there were 35,925 ewes. In total, 2220 ewes were examined. Among these, in 9 farms, in 22 animals, which the respective farmers believed to be normal, clinically evident mammary abnormalities were detected. Then, 2198 ewes with clinically normal udders were sampled. Among these, 572 ewes were detected with subclinical mastitis; its prevalence in the population sampled was 0.260 (95% C.I.: 0.242 - 0.279). Within farms into the study, its prevalence varied from 0.000 to 0.850 (median value among farms: 0.250).

In total, 760 bacterial isolates were obtained from ewes with subclinical mastitis. The most frequently isolated bacteria from ewes with subclinical mastitis were *Staphylococcus* spp. (n = 531; *Staphylococcus aureus* (n = 77) and coagulase-negative species (n = 454) [*S. chromogenes*, *S. epidermidis*, *S. simulans*, *S. hominis*, *S. xylosus*, *S. lentus*, *S. caprae*, *S. haemolyticus*, *S. capitis*, *S. cohnii*, *S. equorum*, *S. hyicus*]). Other frequently isolated bacteria were *Streptococcus* spp. (n = 36), *Corynebacterium* spp. (n = 27), *Escherichia coli* (n = 26), *Micrococcus* spp. (n = 20), *Mannheimia haemolytica* (n = 19) and *Trueperella pyogenes* (n = 19).

3.2. Potential significance of environmental parameters for predisposing to subclinical mastitis

During univariable analysis, without consideration of farm as 'random effect', there was evidence of association of some climatic factors with mastitis prevalence; specifically, these were temperature-related parameters: annual mean temperature ($P = 0.050$), mean

diurnal temperature range (P = 0.033), minimum temperature of coldest month (P = 0.031), temperature annual range (P = 0.029), as well as the annual mean wind speed (P < 0.001). Further, a location factor was also relevant, specifically microhabitat type in the area (P = 0.001). For other factors, there was little evidence of association with prevalence of subclinical mastitis. Details are in Tables 1 and 2.

The final multivariable mixed-effects model required the following variables: (a) minimum temperature of coldest month and (b) mean temperature for 30 days prior to sampling date. From the model (P = 0.028), both the minimum temperature of coldest month (coefficient: -0.084 ± 0.033, P = 0.014) and the mean temperature for 30 days prior to sampling date (coefficient: 0.031 ± 0.014, P = 0.029) were significantly associated with prevalence of subclinical mastitis (Fig. 2).

4. Discussion

The paper describes, to the best of our knowledge for the first time, potential significance of environmental factors in predisposing to mastitis. Environmental conditions are considered to be important in the development of diseases with aerogenic transmission (e.g., Q fever [20]) or diseases caused by agents with growth dependent on weather conditions (e.g. trichostrongylid and trematode infections [21]) or by agents transmitted through vectors (e.g., bluetongue [22]). Moreover, environmental conditions may also affect susceptible hosts and thus contribute to development of a disease. A relevant example would be foot-rot, a disease in which exposure to environmental conditions devitalising the interdigital skin, is a necessary factor [23]. In this case, the environmental factors act mostly on animals, rather than on transmission of causal agents as in previous examples; their effect facilitates agent invasion and multiplication in hosts.

Environmental factors in predisposing to mastitis have attracted little attention in the past, possibly because mastitis is an endemic disease caused by organisms present in farms and on animals, with little aerogenic transmission of causal bacteria. For example, staphylococci, the principal causal agents of mastitis, are transmitted to ewes from hands of milkers, whilst *Mannheimia haemolytica*, another important pathogen of the disease, is transmitted from lambs during sucking [2].

An extensive field investigation of subclinical mastitis in ewes (one of the largest ever on worldwide basis) was carried out to evaluate potential importance of environmental factors. Sheep farms in all regions of Greece were included into the study; that way, conditions prevailing throughout the country had been taken into account and factors of regional importance weighed less. Application of consistent methodologies for examination and sampling of animals, as well as performance of all tasks always by the same investigator has contributed to minimise possible bias in selection of farms.

The results have provided evidence that temperature-related variables can play a role in predisposing animals to mastitis. Results of univariable analysis have hinted at several parameters to be important, whilst results of subsequent multivariable analysis have indicated the lowest temperature of coldest month (negative association) and the mean temperature of the period prior to sampling (positive association) as determinant environmental factors for increased prevalence of subclinical mastitis. The combination of the two factors indicated from the multivariable analysis model points out that extreme temperatures adversely affect occurrence of subclinical mastitis.

High environmental temperatures can lead in reduced leucocyte counts in sheep [24]. In such cases, leucocytes have impaired function (e.g. [25,26],). As leucocytes play a significant role in protecting ewes against mastitis, their reduced number and inefficiency would account for the increased prevalence in farms with extreme temperature measurements. Indeed, there is some evidence indicating that in increased temperatures cows develop mastitis more frequently [27]. Moreover, an indirect effect of other factors associated with increased environmental temperatures, e.g., reduced feed intake which would limit energy availability to defence systems and can predispose to mastitis [28],

should not be ruled out.

Decreased temperatures can also be implicated in favouring development of mastitis. Fox and Norell [29] and Zucali et al. [30] have reported that, in cattle, exposure to low temperatures increased staphylococcal colonization on teat skin. A possible reason for that may be that, due as the result of chapping occurring in such conditions. An increased incidence of chapped teats in cold weather has been associated with intramammary infections [31]. Leyshon [32] also reported that mastitis in ewes was particularly prevalent in cold weather. Physicochemical changes in chapped teat skin contribute to the increased susceptibility. In the epidermis, the process of drying results in a decrease in lipids (including antibacterial fatty acids), bacteriostatic salts and proteins (e.g., teat duct keratin), as well as immunoglobulins [33]. Additionally, the reduced hydration of chapped skin leads to changes in skin microflora and consequent decreased resistance to bacterial colonization [34]. Finally, chapping removes the acid mantle and, as the result of excoriation and fissuring, increases the teat surface area, thereby providing additional space for bacterial attachment [35]. In cold weather, when lambs suck with increased frequency [36] and when teats may become chapped [31], it would seem likely that lambs contribute to the transfer of bacteria to teat skin, documented [38,39] as the means of bacterial transfer leading to colonization, entry into the teat duct and mastitis.

Apart from potential effects in ewes, environmental temperatures may also have an effect on staphylococci present on hands of milkers, which can be a source of infection for the mammary gland [2]. There is some indication that high temperatures favour growth and survival of slime-producing staphylococcal strains [37] in dairy farms, thus may potentially influence virulence of these organisms and development of mastitis according to climatological circumstances. Thus, common practices, e.g., use of warm water for hand-washing when environmental temperatures are low, may support growth and multiplication of these organisms, that way increasing risk for transmission to susceptible ewes. This can explain from a different viewpoint the association of increased mastitis prevalence with environmental conditions.

Although environmental factors are outside the control of farm managers, nevertheless the results can be of value in health management of flocks. During periods of adverse climatological conditions as outlined above, when risk for mastitis would be increased, preventive measures for the disease should be applied meticulously, for example in paying particular attention in performing post-milking teat-dipping. Moreover, in farms located in areas where extreme temperatures would occur frequently, e.g., mountainous regions, long-term preventive measures should be taken, e.g., administration of anti-mastitis vaccines.

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