# Veterinarija ir Zootechnika

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2024

# Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

**Volume 82(1)**
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## Aims and Scope

An international scientific journal **"Veterinarija ir Zootechnika"** since 1924 publishes original research and review papers on all aspects of veterinary medicine, food safety and animal sciences. From 1952 to 1994 journal was published under the title: "Acta of Lithuanian Veterinary Academy". After decision of the Research Council of Lithuania under the auspices of the Government of Republic of Lithuania from year 1995 scientific journal „Veterinarija ir Zootechnika" (Vet Med Zoot) was re-established as the Official Organ of the Veterinary Academy (VA) in collaboration with Veterinary Academy, Veterinary Institute, LVA Animal Science Institute, Lithuanian University of Agriculture and Immunology Institute of Vilnius University.

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**The Guide for Authors** can be found of the journal's website https://vetzoo.lsmuni.lt/directions-to-authors

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The manuscripts are registered in the Editorial Office of LSMU VA "Veterinarija ir zootechnika" after filling the Manuscripts Registration Form (*Supplement 1*).
One copy of the manuscript should be addressed to the Editorial Office or e-mailed to the following address: evaldas.slyzius@lsmu.lt
The manuscripts are refereed (*Supplement 2*) at least by two members of the Editorial Board or appointed referees. The authors are expected to take into consideration the remarks and before the final submission make correction or give motivated explanations of discount of the remarks. The amended paper should be sent back to the Editorial Office in two weeks after the submission of reviews to the author.
If two reviews are negative the article is rejected. If one review is negative, the article should be reviewed by additional referee. If the review of the third referee is positive, the final decision is made by the Editorial board.
The final corrected manuscript should be submitted to the Editorial Board together with the signed letter that authors have approved the paper for release and in agreement with its publication (*Supplement 3*).
The paper should include reference that scientific research has been carried out in accordance with the EU and Lithuanian legal acts.

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# Factors Affecting Weight of Kits Between Birth and Weaning in Rabbits

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***Keywords:*** *cannibalism, growth, nest quality, preweaning survival, rabbits.*

***Abstract.*** *The objective of this study was to analyze the effects of season of birth, physiological status of females, parity order, nest quality, place of birth, occurrence of cannibalism in litter, and sex of kits on their body weight at birth and growth in a preweaning period. A total of 1696 rabbit kits born during 3 parities and from 82 rabbit does were used in this study. The survival rate ranged from 88.57% at birth to 74.41% at weaning. Survivor kits always presented a higher body weight than the dead kits regardless of the study period. Kits born in autumn were significantly heavier at 5 days (93.73 g vs 88.54 g;P < 0.05) and at 7 days (119.58 g vs 110.94 g; P < 0.05) than those born in summer. Except at birth, kits gestated in non-lactating females had a significantly higher body weight compared with those gestated in lactating females, over all the preweaning period (P < 0.05). Regarding parity order, at birth, kits born from nulliparous were lighter than those born from primiparous (42.52 g vs 49.80 g; P < 0.05) or multiparous (42.52 g vs 53.20 g; P < 0.05). Kits born from primiparous females maintained their superiority in terms of weight compared with those born from nulliparous females throughout the experiment period. However, kits born from primiparous females had a similar weight compared with those born from multiparous females over the entire study period. Kits born in a nest of poor quality had a higher body weight at birth and at 7 days compared with those born in a nest of intermediate (53.09 g vs 46.88 g and 126.91 vs 106.03; P < 0.05, respectively) or excellent qualities (53.09 g vs 45.56 g and 162.91 g vs 112.85 g; P < 0.05, respectively). However, at 35 days, kits born in a nest of excellent quality were heavier (P < 0.05). The kits' body weight was higher when born on cage than into nest (at birth, 52.25 g vs 44.76 g, at 5 days, 96.62 vs 85.65 g, at 7 days, 120.29 vs 110.24 g and at 14 days, 206.35 g vs 193.69 g). From 21 days, weights and weight gains became similar between both places of birth. In regard to occurrence of cannibalism in litter, the individual weight of kits was higher at birth when a kit was born in litter with cannibalism (52 g vs 45.02 g; P < 0.05). Up to the second week, the body weights of kits were higher when they were born in a litter that did not exhibit cannibalism at birth. Male kits were heavier than female kits only at birth (47.61 g vs 46.48 g; P < 0.05). In conclusion, the kits' body weight during the preweaning period was mainly affected by lactation status, parity order of the dam, and occurrence of cannibalism in litter.*

## Introduction

Breeding programs have focused on the genetic improvement of litter size, considered as the most important trait for evaluating doe productivity (García and Argente, 2020). However, increasing litter size has not been as successful as expected in increasing the total number of live weaned rabbits, due to increased mortality of kits between birth and weaning (Prayaga and Eady, 2001; Lenoir *et al.,* 2012). This mortality is mainly related to a lower body weight of kits at birth resulting from the increasing litter size, two traits negatively correlated (Belabbas *et al.,* 2023).

Several studies have reported that kit birth weight is directly related to rabbits' *in utero*, postnatal mortality and growth (Martínez-Paredes *et al.,* 2018; Agea *et al.,* 2019; Belabbas *et al.,* 2023). Therefore, the selection for the within-litter uniformity was proposed to reduce mortality related to lower birth weight (Garreau *et al.,* 2008).

In prolific species such as rabbits, the weight of a rabbit kit at birth is determined by its genotype, maternal effects (age and body weight of does, parity order, reproductive rhythm, nutritional status and uterine environment) and environmental effects (ambient temperature, food quality and breeding

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management) (Szendrö *et al.,* 2019; Farouk *et al.,* 2022). However, its growth and survival in the preweaning period are mainly related to the mothering abilities like milk yield and nest construction (Ludwiczak *et al.,* 2021; Belabbas *et al.,* 2023) and to the environmental effects (litter effects, season of birth and temperature) (Marco-Jiménez *et al.,* 2017; Pałka *et al.,* 2018; Zapletal *et al.,* 2021).

At birth, individual weight of rabbit kits is about 60–70 g, although it can range from 35–90 g (Di Meo *et al.,* 2004). Rabbit growth is linear, with an average daily gain of 11 g to 13 g per day in a litter of 10 kit rabbits during the first three weeks. From the 25th, growth accelerates to reach an average gain of 35 g to 38 g per day when the proportion of solid pelleted diet becomes significant. During lactation, the birth weight of a kit rabbit increases very quickly, from 71 g at birth to 394 g at 21 days (Ludwiczak *et al.,* 2021).

It should be noted that the factors affecting body weight and growth of kits were studied at specific dates (birth or weaning). However, few studies have analysed these factors from birth to weaning. Moreover, there are few studies on the effect of mother abilities (nest quality and cannibalism in litter) on the growth of kits during the preweaning period. This study aimed at investigating the effect of season of birth, lactation status of the dam and its parity order, nest quality, occurrence of cannibalism in litter, birth place and sex of kits on their growth during the preweaning period.

## Material and methods

*Ethics statement*

All experiment procedures involving animals were approved by the scientific council of the Biotechnology Laboratory of Animal Reproduction (code BR-001-17; date of approval 05/01/2017), part of the Institute of Veterinary Sciences at the University of Blida 1 (Blida, Algeria).

*Animals*

All animals came from the ITELV2006 line. This line was created as a part of an agreement to transfer biological material for experimental purposes between the Institute of Animal Breeding (ITELV, Algeria) and INRA (France) by means of inseminating does from a local Algerian rabbit population with the semen of bucks from the INRA2666 synthetic line (Brun *et al.,* 2004). This line has been maintained in discrete generations without selection and avoiding inbreeding. More details of this line can be found in Ezzeroug *et al.* (2020).

*Housing, feeding and management*

The females were housed individually in wired flat-deck cages (30 height × 40 width × 70 length cm) in a building equipped with a cooling system. They were submitted to a constant photoperiod with 16 hours of light and 8 hours of dark. They were fed *ad libitum* a standard commercial pelleted diet (16% crude protein, 15% crude fiber and 2.6% ether extract) and water was available *ad libitum*.

Females (n = 82) followed a semi-intensive reproductive rhythm: first mating at 20 weeks of age, with subsequent 42-day reproductive cycles. Natural mating was performed using adult males of the same line (7–10 months), within a rhythm of 2 matings per week.

Four days before the presumed date of parturition, a cleaned and disinfected nest boxes were placed. On the day of parturition, early in the morning (at 8:00 am), the nest boxes were checked and the place of kindling (inside the nest or on cage) and the presence of cannibalism in kits were noted. We considered that cannibalism had taken place when at least one kit had been devoured by its mother.

The nest quality was evaluated according to Blumetto *et al.* (2010). This evaluation consists of judging only the extent to which the female uses her hair for nest building. Three categories were considered: poor, nest contains very little or no hairs; intermediate, > 50% of the nest had material covered with hair; excellent, nest filled with hairs that occupy the entire nest box.

At birth, the litter size was recorded and kits (n = 1696) were individually identified, sexed and weighted. Afterwards, the litter size, individual body weights of alive kits, and survival rates were recorded at 0, 5, 7, 14, 21, 28, and 35 days for the three first parities. The survival rate was calculated as the number of alive kits at different dates per the total number of kits at birth. The increment of body weight was calculated as the weight difference between two given dates. The litters were not standardized and kits were reared by their dams up to weaning (35 days of age).

The study was carried out from June to November 2017. In Table 1, the temperature and relative humidity for each month are shown. Summer runs from June 1 to August 31, and autumn from September 1 to November 30.

*Statistical analysis*

The kits' body weight was analyzed using the following model:

$$Y_{ijklmnop} = \mu + S_i + Lj + P_k + N_l + CNm + Bn + SX_o + + c_{ijkmnop} + e_{ijklmnop}$$

where $\mu$ is general mean, $S_i$ is the effect of season (2 levels: summer and autumn), $L_j$ is the effect of lactation status (2 levels: lactating and non-lactating females), $P_k$ is the effect of parity order (3 levels: nulliparous, females that give birth for the first time; primiparous: females that give birth for the second time; and multiparous: females that give birth for the third time), $N_l$ is the effect of the nest quality (3 levels: poor, intermediate and excellent), $CN_m$ is the effect of cannibalism (2 levels: presence and absence), $B_n$ is the effect of birth place (2 levels: nest or cage), $SX_o$ is the effect of sex (2 levels: male and female), $c_{ijkmnop}$ is the random effect of the common litter, and

*Table 1.* Temperature and relative humidity by month

| | | Temperature Outside (°C) | | Temperature Inside (°C) | | Relative Humidity Inside (%) | |
|---|---|---|---|---|---|---|---|
| | | Minimum | Maximum | Minimum | Maximum | Minimum | Maximum |
| Summer | June | 25 | 30 | 22 | 28 | 21 | 82 |
| | July | 27 | 39 | 26 | 33 | 21 | 80 |
| | August | 30 | 35 | 28 | 36 | 23 | 80 |
| | Average | 27.3 | 34.7 | 25.3 | 32.3 | 21.7 | 80.7 |
| Autumn | September | 24 | 30 | 22 | 27 | 19 | 83 |
| | October | 21 | 27 | 20 | 23 | 21 | 79 |
| | November | 16 | 21 | 19 | 23 | 20 | 68 |
| | Average | 20.3 | 26.0 | 20.3 | 24.3 | 20.0 | 76.7 |

$e_{ijklmnop}$ is the error. The interactions between factors were not included in the statistical model because they were not significant. The body weight and the weight gain were analyzed with the number of kits total new-born and kits born alive as covariates. The mixed procedure of the SAS statistical package was used for these analyses (SAS Institute, 2024).

## Results

The survival rate ranged from 88.57% at birth to 74.41% at weaning. The weights of born alive and dead kits at birth were 52.57 g and 38.21 g, respectively. Regardless of the study period, the weight of alive kits was always greater than that of the dead kits (Fig. 1).

### *Factors affecting weight of kits*

*Effect of season*

The evolution of kit individual body weight from birth to weaning and according to the season is presented in Fig. 2. At parturition, the individual body weights of kits were not significantly affected by the season of birth. However, during their first week of life (between birth and 7 days), kits born in autumn had a significantly higher body weight compared with those born in summer (93.73 g *vs* 88.54 g and 119.58 g *vs* 110.94 g, respectively, at 5 days and 7 days of age; $P < 0.05$). These differences remained significant when the body weight was corrected by the number of total new-borns. Between 14 days and 35 days of age, no difference was found between the two studied seasons.

*Effect of lactation*

At birth, the individual body weights of kits were similar between lactating and non-lactating females (Fig. 3). Lactation affected significantly the growth of kits at 5 days and 7 days, and kits born from non-lactating females had a higher body weight and weight gain ( 94.19 g *vs* 88.07 g at 5 days and 119.85 g *vs* 110.68 g at 7 days for the body weight and 38.74 g *vs* 33.52 g from birth to 5 days and 24.75 g *vs* 22.52 g from 5 days to 7 days for weight gain; $P < 0.05$). The same observations were noted in the following weeks. The kits born from non-lactating females had higher

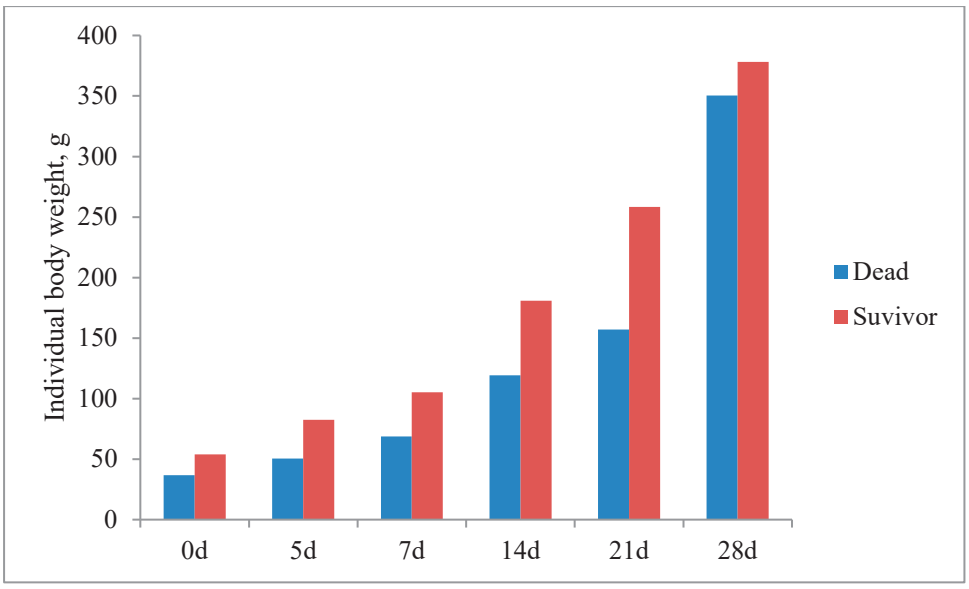


*Fig. 1.* Weight evolution for survivor and dead kits from birth to 28 days

*Table 2.* Number of observations (N), means, and standard deviations (SD) of survival, body weight and its increment from birth to weaning and for each week (Appendix)

| Traits | N | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|---|
| Survival at birth 0 d, % | 1696 | 88.57 | 23.78 | 0 | 100 |
| Survival at 5 d, % | 1431 | 83.69 | 24.74 | 0 | 100 |
| Weight increment 0–5 d (alive), g | 1431 | 27.45 | 15.77 | –32.01 | 83.49 |
| Weight increment 0–5 d of kits death at 7 d, g | 50 | 9.56 | 15.16 | –8.66 | 51.88 |
| Weight increment 0–5 d of kits alive at 7 d, g | 1381 | 28.10 | 15.42 | –32.01 | 83.49 |
| Survival at 7 d, % | 1381 | 81.07 | 26.18 | 0 | 100 |
| Weight increment 5–7 d (alive), g | 1381 | 20.89 | 12.58 | –32.17 | 124.10 |
| Weight increment 5–7 d of kits death at 14 d, g | 69 | 7.10 | 10.69 | –18.54 | 36.25 |
| Weight increment 5–7 d of kits alive at 14 d, g | 1312 | 21.61 | 12.25 | –32.17 | 124.10 |
| Survival at d 14, % | 1312 | 77.45 | 27.91 | 0 | 100 |
| Weight increment 7–14 d (alive), g | 1312 | 74.48 | 33.69 | –34.82 | 230.60 |
| Weight increment 7–14 d of kits dead at 21 d, g | 28 | 34.34 | 23.07 | –13.38 | 85.51 |
| Weight increment 7–14 d of kits alive at 21 d, g | 1284 | 75.35 | 33.36 | –34.82 | 230.60 |
| Survival at 21 d, % | 1284 | 75.80 | 28.46 | 0 | 100 |
| Weight increment 14–21 d (alive), g | 1284 | 75.96 | 39.25 | –32.80 | 290.70 |
| Weight increment 14–21 d of kits dead at 28 d, g | 18 | 13.39 | 37.04 | –32.80 | 103.85 |
| Weight increment 14–21 d of kits alive at 28, g | 1266 | 76.85 | 38.57 | 4.72 | 290.70 |
| Survival at 28 d, % | 1266 | 74.84 | 29.21 | 0 | 100 |
| Weight increment 21–28 d (alive), g | 1266 | 119.82 | 56.13 | –18.95 | 415.51 |
| Weight increment 21–28 d of kits dead at 35 d, g | 7 | 118.84 | 152.57 | –18.95 | 403.20 |
| Weight increment 21–28 d of kits alive at 35 d, g | 1259 | 119.83 | 55.29 | 8.30 | 415.51 |
| Survival at 35 d, % | 1259 | 74.41 | 29.37 | 0 | 100 |
| Weight increment 28–35 d (alive), g | 1259 | 187.53 | 91.16 | –59.20 | 535.60 |

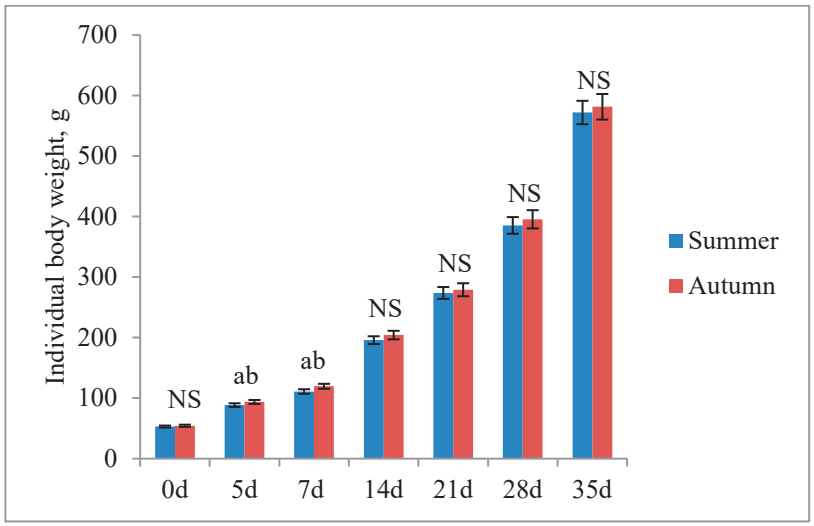


*Fig. 2.* Evolution of kits body weight according to the season of birth.
Individual body weight at 0 (birth), 5, 7, 14, 21, 28 and 35 days (weaning).
a and b: different letters indicate statistically significant differences ($P < 0.05$). NS: not significant.

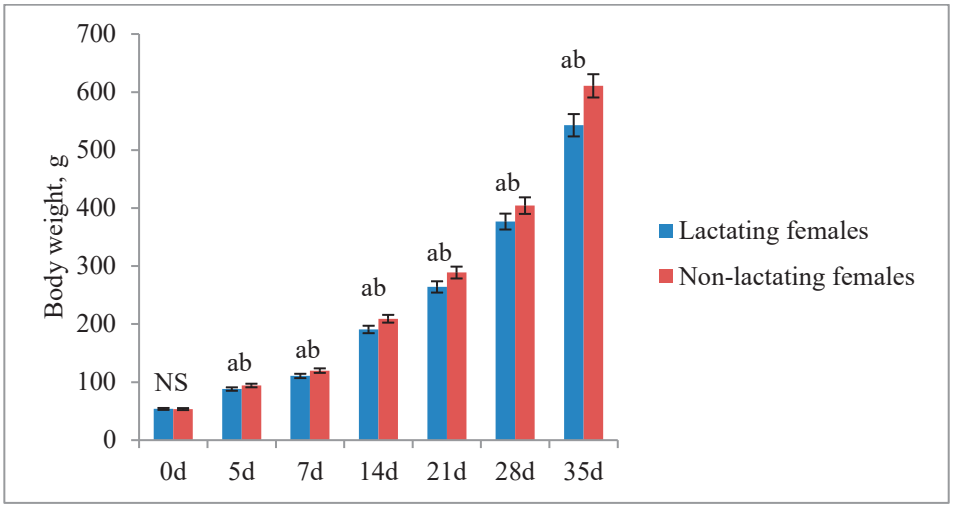


*Fig. 3.* Evolution of kits' body weight in lactating and non-lactating females.
Individual body weight at 0 (birth), 5, 7, 14, 21, 28 and 35 days (weaning).
a and b: different letters indicate statistically significant differences ($P < 0.05$). NS: not significant.

weight gains than those born form lactating females (92.27 g *vs* 81.09 g from 7 days to14 days, 79.66 g *vs* 72.11 g from 14 days to 21 days, 117.26 g *vs* 113.29 g from 21 days to 28 days and 201.73 g *vs* 172.99 g from 28 days to 35 days; $P < 0.05$). The differences noted for individual weight and weight gains lost their significance when they were corrected by the number of total new-borns.

*Effect of parity order*

At parturition, individual weight of kits born from multiparous females was significantly heavier than those born from primiparous females (Fig. 4) (53.20 g *vs* 49.80 g; $P < 0.05$) and those born from nulliparous females (53.20 g *vs* 42.52 g; $P < 0.05$). The differences did not disappear when the body weight was corrected by the number of total new-borns.

Between 5 and 7 days of age, the evolution of body weight and weight gains of the kits born from multiparous and primiparous females became comparable. However, the kits born from nulliparous females were significantly lighter than those born from primiparous females (83.01 g *vs* at 5 days and 105.04 g *vs* 118.58 g at 7 days) and those born from multiparous females (83.01 g *vs* 97.09 g at 5 days and 105.40 *vs* 121.81 g at 7 days). The same differences were noted for the weight gains between birth to 5 days and between 7 days to 14 days.

Between 14 days and 21 days of age, the differences recorded between the parities tend to persist. Kits born from nulliparous females were always lighter than those born from nulliparous and multiparous (respectively 170.86 g *vs* 211.33 g and 217.96 g at 14 days and 220.93 g *vs* 298.7 g and 311.3 g at 21 days; $P < 0.05$). However, the kit body weights were comparable between primiparous and multiparous

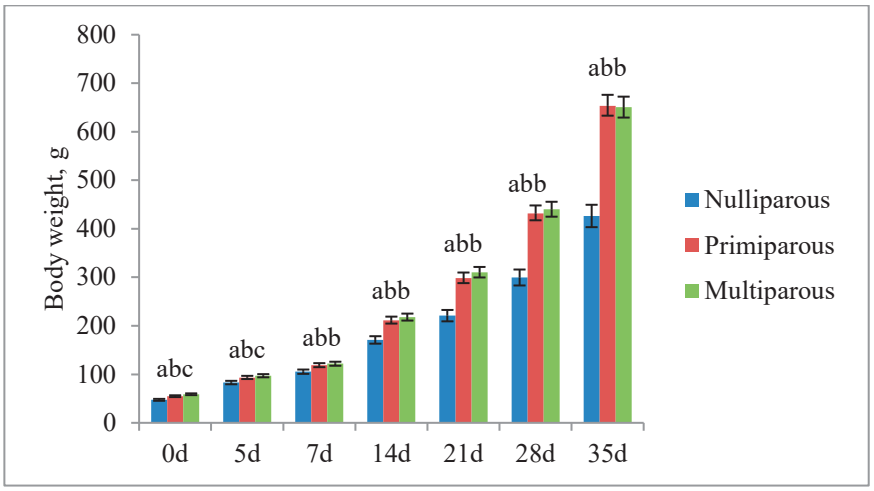


*Fig. 4.* Evolution of kits' body weight according to the parity of the dams.
Individual body weight at 0 (birth), 5, 7, 14, 21, 28 and 35 days (weaning).
a, b, c: different letters indicate statistically significant differences ($P < 0.05$).

females for the same dates. Furthermore, the kits born from nulliparous females showed significantly lower weight gains compared with those born from primiparous (68.75 g *vs* 94.82 g between 7 days and 14 days and 50.54 g *vs* 86.93 g between 14 days and 21 days) and multiparous females (68.75 g *vs* 96.45 g between 7 days and 14 days and 50.54 g *vs* 90.18 g between 14 days and 21 days). In contrast, kit weight gains were comparable between primiparous and multiparous females for the same dates. It should be noted that when the number of alive kits was used as a covariate, the differences for individual body weight and weight gain remained significant ($P > 0.05$).

*Effect of nest quality*

The evolution of individual body weight, from birth to weaning and according to the nest quality, is presented the Fig. 5. The body weight of kits was significantly higher when they were born in a nest of poor quality compared with those born in a nest of intermediate (53.09 g *vs* 46.88 g; $P < 0.05$) and excellent qualities (53.09 g *vs* 45.56 g; $P < 0.05$). This difference remained significant when the body weight was corrected by the number of total new-born kits.

At 5 days and 7 days of age, the kits born in a nest of poor quality were always significantly heavier than those born in a nest of intermediate or excellent qualities. The difference remained significant even when the body weight was corrected by the number of kits born alive. On the other hand, the body weights of kits born in a nest of excellent or intermediate qualities were comparable.

Weight gain recorded between birth and 5 days varied significantly between the three nest categories. The kits born in a nest of poor quality showed a significantly higher weight gain compared with those born in a nest of intermediate quality (43.32 g *vs* 29.47 g; $P < 0.05$) and those born in a nest of excellent quality (43.32 g *vs* 35.61 g; $P < 0.05$). No difference was found among categories of nests for weight gain measured between 5 days and 7 days.

Between 7 and 35 days of age, the evolution of individual body weight and weight gain was comparable between the 3 nest categories. However, at 35 days, kits born in a nest of excellent quality were heavier compared with those born in a nest of intermediate quality (611.88 g *vs* 561.87 g; $P < 0.05$). This difference lost its significance when the number of alive kits was used as a covariate. Weights were comparable between kits born in a nest of poor and excellent qualities on the one hand and intermediate quality on the other hand. The same results were observed for weight gain from 21 days to 28 days and 28 days to 35 days.

*Effect of birth place*

In this study, the body weight of kits born outside the nest was significantly higher than those born in the nest (52.25 g *vs* 44.76 g; $P < 0.05$) (Fig. 6). This difference remained significant when the weight was corrected by the number of total new-borns. The same results were noted for the body weight at 5 days and 7 days and also for the weight gain from birth to 5 days. However, when the number of total new-born kits was used as covariable, the difference between kits born inside a nest or in a cage disappeared.

At 14 days, kits born in a cage were always heavier than those born in the nest (206.35 g *vs* 193.69 g; $P < 0.05$). This difference remained significant when the number of alive kits was introduced as a covariate in the statistical model. The weight gain between 7 days and 14 days was comparable between kits born in a nest or in a cage. From 21 days , weights and weight gains became similar between both places of birth.

*Effect of cannibalism*

The evolution of individual body weights

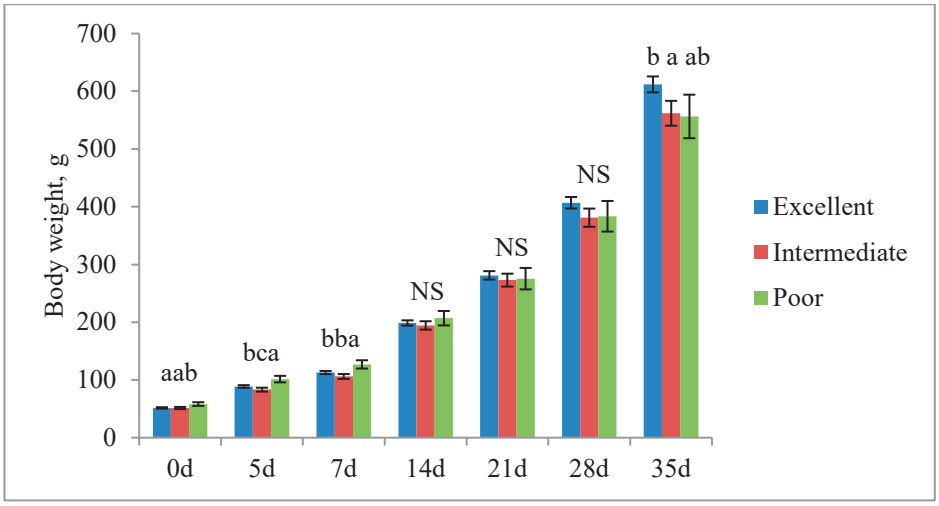


*Fig. 5.* a, b, c: different letters indicate statistically significant differences ($P < 0.05$). NS: not significant.


*Fig. 6.* Evolution of kits' body weight according to the location of birth.
Individual body weight at 0 (birth), 5, 7, 14, 21, 28 and 35 days (weaning).
a and b: different letters indicate statistically significant differences ($P < 0.05$). NS: not significant.

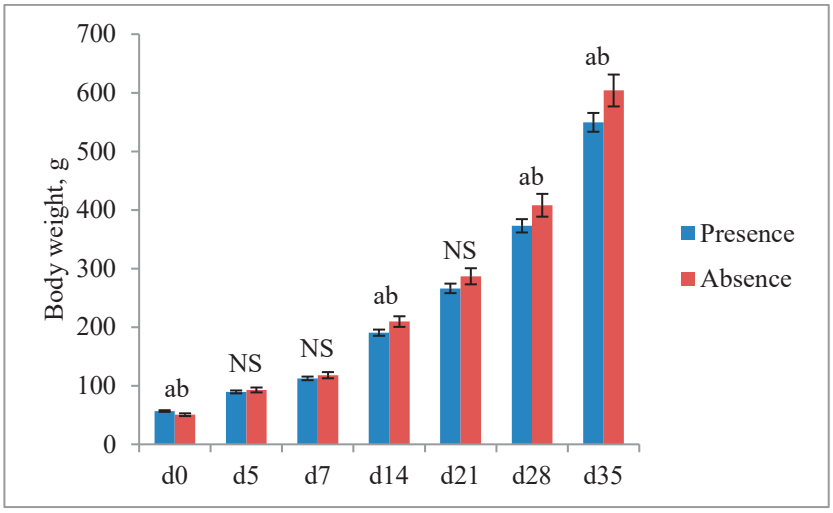


*Fig. 7.* Evolution of kits' body weight according to the occurrence of cannibalism in litter.
Individual body weight at 0 (birth), 5, 7, 14, 21, 28 and 35 days (weaning).
a and b: different letters indicate statistically significant differences ($P < 0.05$). NS: not significant.

according to the occurrence of cannibalism event in litter is presented the Fig. 7. At birth, the individual weight of kits was higher when the kit was born in litter with cannibalism (52 g *vs* 45.02 g; $P < 0.05$). These differences persisted even when the weight was corrected by the number of total new-born kits.

In contrast to the individual weight of kits at 5 days, which did not differ significantly between litters with and litters without cannibalism, weight gain between birth and 5 days was higher in litters without cannibalism phenomena (40.33 g *vs* 31.94 g; $P < 0.05$). This difference persisted even when the number of total new-borns was added as covariable to the statistical model.

The individual body weight at 7 days and weight gain between 5 days and 7 days were similar between litters with and without cannibalism. At 14 days, individual body weight and weight gain between 7 days and 14 days were significantly higher for kits born in litters that did not have cannibalism at parturition ($P < 0.05$). When the body weight was corrected by the number of alive kits, the difference measured between the two groups (absence or presence of cannibalism) disappeared for the individual body weight at 14 days, but persisted for the weight gain between 7 days and 14 days.

Individual body weight of kits at 21 days and weight gain between 14 days and 21 days of age showed no difference between litter recording occurrence of cannibalism or not.

The body weight at the age of 28 days, 35 days and the weight gain between 28 days and 35 days were significantly higher for rabbits born in a litter that did not exhibit cannibalism at birth. When these three traits were corrected by the number of alive kits, the differences noted disappeared. Furthermore, weight gain between 28 days and 35 days was noted related to the occurrence of cannibalism at birth.

*Effect of sex*

The body weight of male kits was slightly higher than that of female kits at birth (47.61 g *vs* 46.48 g; $P < 0.05$) (Fig. 8). However, for the rest of the period, the body weights and weight gains were similar between both sexes.

## Discussion

The individual body weight of kits born alive was similar to that obtained by Sid *et al.* (2018), but lower than that measured by Boudour *et al.* (2020) on the same line of rabbits. In addition, the weight of survivor kits and their weight gains were higher than those of dead kits regardless of the study period. Our results are in agreement with the literature (Belabbas *et al.,* 2023). Heavier kits are always successful to get a teat during the short nursing time, they grow better and survive longer compared with lighter kits (Agea *et al.,* 2019). It was also reported that the lightest kits are likely to die very quickly or, if they survive, they will have a lower probability of survival after weaning (Gyovai *et al.,* 2004). Moreover, the lower birth weight is associated with a higher risk of hypothermia and

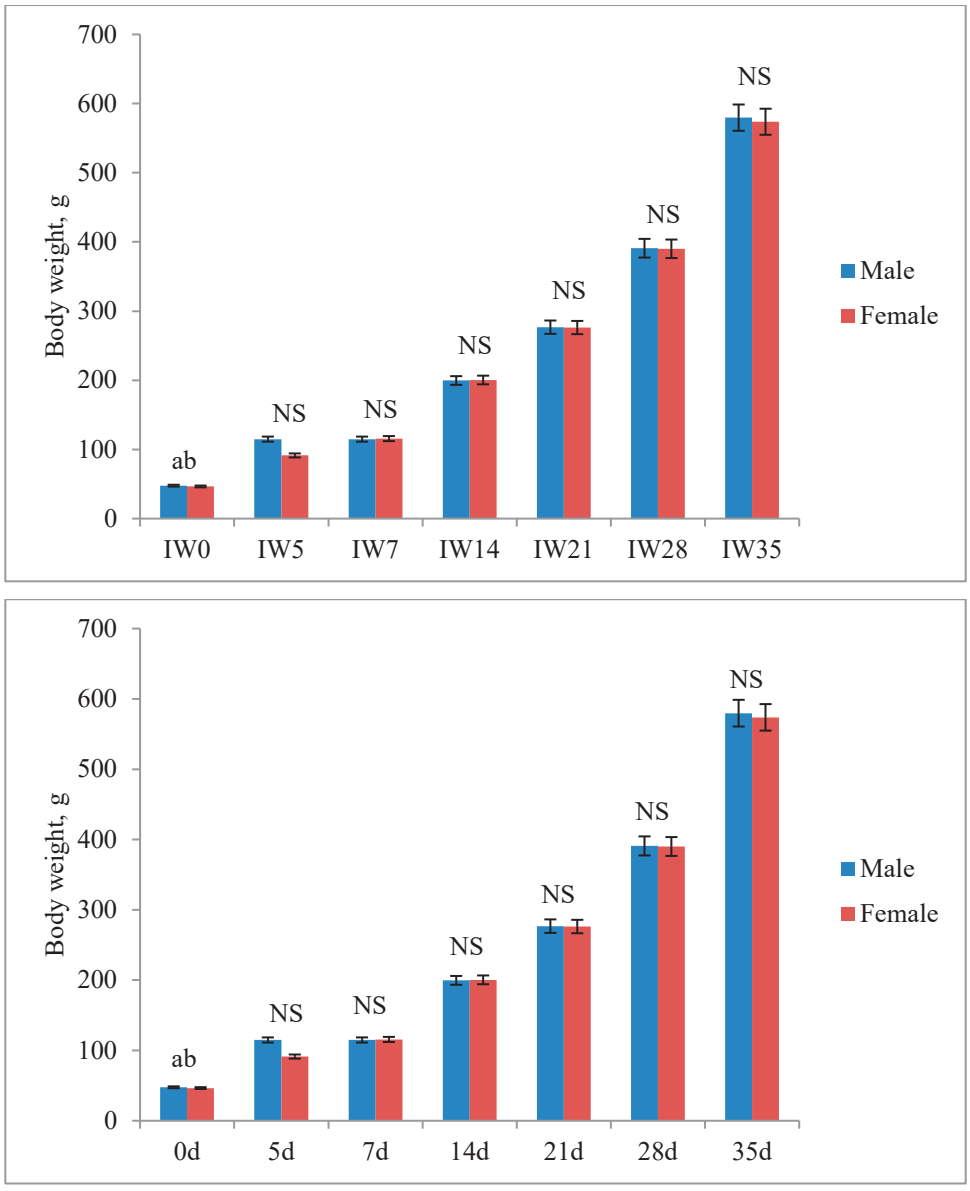


*Fig. 8.* Evolution of kits' body weight according to their sex.
Individual body weight at 0 (birth), 5, 7, 14, 21, 28 and 35 days (weaning). a and b: different letters indicate statistically significant differences ($P < 0.05$).
NS: not significant.

mortality related to a lower ratio of brown adipose tissue to body weight (Szendrö *et al.,* 2019).

The season had a significant effect on kits' individual body weight but only during the first week of life. Kits born in autumn were significantly higher than those born in summer. Several studies have shown a negative effect of the summer season on the weight and growth of kits, in agreement with our study (Sid *et al.,* 2018; Boudour *et al.,* 2020). This could be related to a lower weight of females in summer compared with autumn, which is itself linked to a low food intake, reducing *in utero* growth of kits (Nardone *et al.,* 2010). In addition, daily weight gain and feed intake of growing kits decreased during high temperature periods (Marai *et al.,* 2001). Elmaghraby *et al.* (2004) and Zerrouki *et al.* (2007) did not find a significant effect of season on weight and weight gains at either 28 days or weaning. Up to 21 days of age, kits are dependent on maternal milk, thus reducing the effect of season on growth (Ludwiczak *et al.,* 2021). The introduction of solid pelleted diet after may explain the differences in weight and weight gains observed between seasons (Elmaghraby *et al.,* 2004).

The effect of lactation on kits' growth was significant from the fifth day after birth, in favor of kits gestated in non-lactating females. Several studies agree that non-lactating females, at the time of mating, give a higher litter size, survival rate and growth of kits in the preweaning period (García-Ximénez *et al.,* 1995; Agea *et al.,* 2019). Castellini *et al.* (2010) reported that the deterioration in body condition of lactating rabbits can affect the viability and growth of their kits. On the other hand, our study shows that the difference in kit weights between lactating and non-lactating females is related to litter size because when the kits' body weight was corrected by the number of total new born or alive kits, the difference between females disappeared.

The parity order of the dam showed a significant effect on kits' growth over all the preweaning period, and kits gestated in nulliparous were the lightest. Our results agree with those reported by Zerrouki *et al.* (2007). Similarly, Elmaghraby *et al.* (2004) noted that multiparous females raised heavier kits at birth, but from 21 days of age, the weights did not differ between parities. However, multiparous females (2 parities and above) appeared to have more uniform litters, higher weight and body gain compared with females of other parities. Furthermore, Ouyed *et al.* (2007) indicated that only the average weight of kits at birth and at weaning varied according to the parity order. Kits weighed 13 g and 171 g more at birth and at weaning, respectively in multiparous females compared with nulliparous. This could be due to changes in the mother's physiological efficiency, in particular, those related to food and to the intrauterine environment provided during gestation with advancing of parity (Afifi *et al.,* 1988). In addition, milk production in rabbits increases with the number of parities, which is reflected on body weight gain of kits (Bakr *et al.,* 2015). Courreaud *et al.* (2000) have shown that primiparous females can react more aggressively to stimuli from their kits. This may affect their willingness to position themselves adequately over their kits to nurse them. These authors explained that undergoing a first cycle of reproduction might stimulate the function of the chemo emission system involved in the production and emission of pheromonal signals which direct teat seeking and suckling behavior in the offspring. Moreover, Bakr *et al.* (2015) have shown that milk production and maternal behavior improve with advancing of the parity number.

At the first week of age, kits born in a nest of poor quality were heavier compared with those born in intermediate or excellent nest quality. Our findings are in contradiction with those noted by Canali *et al.* (1991) who reported that kits born in a nest of excellent quality showed a higher body weight at 5 days of age. A lower weight of kits born in a nest of excellent quality would be linked to factors other than nest quality. Indeed, birth weight is affected by a numerous *in utero* factors, in particular, the number of blood vessels reaching each implantation site (Belabbas *et al.,* 2012), position and available uterine space for each fetus (Bautista *et al.,* 2015), female weight before mating (Jimoh *et al.,* 2017), parity order (Apori *et al.,* 2014) and litter size (Lenoir *et al.,* 2012). Moreover, birth weight plays an important role in growth and survival of kits in the preweaning period (Bautista *et al.,* 2005). Mucino *et al.* (2009) have reported that heavier kits compete more effectively for maternal milk, occupy a proper warm site in the middle of a nest to maintain body temperature and grow faster. These findings might suggest that the higher weight of kits has compensated the unfavorable effect of poor nest quality on kits' development during the first 5 days of life. It should be noted that the absence of nest construction in rabbits varies between 2.3% and 9% (Szendrô *et al.,* 1996). This phenomenon is not related to a lack of experience of the female, but may be due to specific stimuli such as endocrine changes, environmental factors like the season of kindling (González-Redondo, 2010), and genetic factors including breed which appears to be linked to hormonal sensitivities to estrogen and prolactin (González-Mariscal *et al.,* 2007). From 14 days, the body weights and weight gains became comparable between the three nest categories, then higher for the kits born in a nest of excellent quality, which is in agreement with Canali *et al.* (1991).

In our study, heavier kits were found in a cage at birth, which corroborates the findings of Briens (2011). This author noted a higher weight when kits were born outside of the nest. However, when the number of total kits born was used as a covariate, we did not find any significant difference between weights of kits born in a cage or in a nest. This indicates that

the difference in weights may be linked to the litter size, which is in agreement with literature (Olateju and Chineke, 2022).

During the first two weeks of age, the differences in body weights initially noted persisted. This is related to the higher birth weight of kits born in a cage, which favors better growth. Similarly, weight gains between birth and 5 days were higher in kits born outside the nest. However, from 7 days onwards, the weight gains measured between the different dates became comparable, as a consequence of a reduced effect of birth weight on growth. In fact, regardless of the place of birth, when litter is placed in the nest, the kits are subjected to the same environmental conditions, their growth depends on their mother's milk production and their ability to ingest a sufficient quantity of milk during the short suckling period (Martínez-Paredes *et al.,* 2018).

The occurrence of cannibalism in litter was associated with higher body weights of kits at birth, which could be related to the different factors affecting body weight at birth rather than to the occurrence of cannibalism in litter. It should be noted that cannibalism is often reported in litters with heavy kits, responsible of dystocia and stress for the female at kindling (González-Redondo, 2010). Despite their higher birth weight, at 5 days and 7 days, kits born in litter that showed cannibalism had a similar weight compared with those born in litter without cannibalism. However, the weight gains from birth to 5 days were higher for kits born in litter that presented cannibalism. From t 14 days of age, weights and weight gains recorded at different dates were higher for kits born in litter without cannibalism at birth. To the best of our knowledge, there are no researches studying the effect of cannibalism at parturition on the subsequent development of the litter. Our results suggest that rabbits surviving after cannibalism, in spite of their higher weight, did not have higher weight gain. This seems to be related to the stress caused on the day of birth. In fact, stress in rabbits can cause anorexia. Cannibalism is also linked to the lack of experience of females, primiparous rabbits being the most affected (González-Redondo *et al.,* 2010). The inhibition of cannibalistic behavior with mutilation and consumption of protruding parts or the whole body of the kits is part of the development of the maternal instinct in domestic or wild rabbits. González-Redondo *et al.* (2010) reported that in certain lines of rabbits, females are very nervous or have a poorly developed maternal instinct with a tendency to ignore or devour their kits. In these cases, the construction of the nest for the new-borns is often neglected and contains little or no straw or hair.

Males were heavier than female kits only at birth. Bolet *et al.* (1996) indicated that males were significantly heavier than females at birth (4 g). They maintained their superiority at weaning (19.3 g), although their growth rate was only significantly greater during the first two weeks. In contrast, Agea *et al.* (2019) reported that weight at 4 days of age was comparable between the two sexes. Also, Szendro *et al.* (1996) showed that differences in weight between males and females only became significant at 12 weeks of age in favor of females.

## Conclusion

In conclusion, the weight of kits during the preweaning period was affected mainly by lactation. Indeed, lactating females had heavier kits than those of non-lactating females during all the lactation period. Besides, the parity order of the dam affected the kits' body weight, and multiparous females always had heavier kits compared with nulliparous. Finally, occurrence of cannibalism in litter reduced the body weight of kits at 28 days and at weaning. Increased delivering-mating interval and reducing stress in the *peripartum* period could reduce the effect of pregnancy-lactation overlap and the occurrence of cannibalism and litter abandonment in cages.

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## Conflict of interests

Authors declare no conflict of interest.

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# Robustness of *Mycobacterium Bovis* Determination Using Fast and Simple qPCR Assay

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***Keywords:*** *food safety; Mycobacterium bovis; qPCR; Assay robustness*

***Abstract.*** *Most of the acute intestinal diseases are caused by food-borne pathogens. The robustness of qPCR-based fast and simple procedure for food safety detection of Mycobacterium bovis (M. bovis) DNA using EvaGreen real-time PCR for LightCycler was evaluated. Tm calling and Cp were used for analysis of PCR products. Tm calling showed better performance than Cp-based calculations for near limit of detection (LoD) positive samples. The studied qPCR M. bovis assay showed good sensitivity and excellent robustness, which allows using this assay during emergency or when this method is rarely used.*

## Introduction

The frequency of outbreaks of food-borne infection cases worldwide is still extremely high (Fleckenstein et al., 2010; Postollec et al., 2011). Food pathogens are commonly found in the intestines of healthy food-producing animals, and can be transmitted to humans through contamination of the food chain. *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis complex* pathogen group (MTBC), is responsible for both bovine tuberculosis (bTB) in cattle and zoonotic tuberculosis (TB) in humans (Collins et al., 2022). The transmission of *M. bovis* is possible in a number of different ways, involving mechanisms such as respiratory secretions, environmental contamination, and consumption of contaminated food. Historical data indicate that in Europe among all bacteriologically confirmed TB cases the median proportion of *M. Bovis* or *M. Caprae* infections was 0.4% (range 0%–21.1%) (Müller et al., 2013). Beyond direct health consequences, bTB has significant global economic implications in both developed and developing countries (Waters et al., 2011; Smith et al., 2006). The notable risk of *M. bovis* transmission through drinking contaminated milk is in places where pasteurization is not mandatory (Smith et al., 2009).

Thus, a strict control of the whole food chain aimed to enforce contamination detection measures is required. Molecular methods for the detection and identification of food pathogens have significant benefits as compared to traditional methods due to speediness, sensitivity, specificity and accuracy. On the other hand, they often require dedicated instrumentation, highly trained personnel, and higher labour costs. Emerging molecular techniques are widely using isothermal amplification instead of temperature cycling like PCR but PCR still remains the most used one. All of these claims are valid for both beneficial and pathogenic bacteria (Severgnini et al., 2011; Jošić et al., 2016). The advantage of molecular methods is their universal character, i.e., most of them can be used on different platforms – capillary instruments, plate instruments or micro-arrays or can be easily adapted to them (Fukushima et al, 2007, 2010). There are two mainstreams in development of new PCR-based methods. One is multiplex qPCR with a simultaneous amplification of several microbes in a single reaction (Karus et al., 2017; Cremonesi et al., 2014). In this approach, the primer sets are designed with a similar annealing temperature but significantly differentiating melting temperatures of amplicons to distinguish between amplicons following thermal cycling (Karus et al., 2017; Zhao et al., 2014). Performing a multiplex assay instead of several singleplex analyses might reduce the total costs for testing (Binnicker, 2015). Still, multiplex methods are more complex. Thus, they are less robust and usually need well trained personnel to perform. Another approach is to identify different pathogens in different capillaries or PCR plate wells. This eliminates the possible effect of competition, and the primer concentration can be higher and sensitivity can be better. There are also mixed methods available (tandem-PCR) where the short conventional multiplex PCR is followed by nested single-well target amplification using qPCR (Ginn et al., 2017). All of these methods can be used in food safety as well as in clinical (human and veterinary medical) settings depending on sample material. Although PCR can reach high sensitivity and specificity, its introduction for routine detection has been frequently hampered by a lack of robustness (Van der Wolf et al., 2001).

The objective of the present study was to revise the analytical sensitivity and test the *Mycobacterium bovis* detection assay robustness on unexperienced users.

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Such an analysis can be used to analyse any kind of food, including but not limited to milk, cheese, meat products and any animal origin novel food (Karus and Karus, 2018). However, the outbreaks of *M. bovis* are not frequent, that means that this test is not used widely in routine and, thus, the robustness is crucial if this assay should be implemented in a limited time period. The aim of the study was to investigate whether the involvement of non-proficient users impacts the outcomes of analysis and to identify potential limitations in PCR-based assays under such circumstances.

## Materials and methods

*Samples and primer design*

The concentration of *M. bovis* genomic DNA used for reference target strains varied from 1.0 till 1.9 ng/µL. Bacterial strains and accession number of target genes of isolated genomic DNA and primer sequences are listed in Table 1. Primers for real-time PCR amplification were designed using PRIMER EXPRESS (ver. 3.0). Primer pairs were ordered from Tib-MolBiol (Germany) and tested for EvaGreen assays on LightCycler 2.0® (Roche Diagnostics GmbH, Switzerland) using 5x HOTFIREPol® EvaGreen® qPCR Mix Plus(Capillary) with 7.5 mM $MgCl_2$ ready-to use mastermix (Solis BioDyne, Estonia). Samples were prepared using isolated bacterial DNA and NA-free water from Roche Diagnostics (Germany). No real food samples were used.

*EvaGreen PCR assay*

Quantitative PCR reactions followed by melting curve analysis were performed in a final volume of 20 µL. Capillaries content is given in Table 2. The reaction mix was prepared immediately (up to 30 min) before the run. Tenfold dilutions of the target genomic DNA were tested to determine the fair amount of template DNA detected by the assay (PCR sensitivity). The used testing thermal profile and full analysis protocol are given in Table 3.

In total, 38 positive samples with initial content of MB nucleic acids from 1E-9 g to 8E-9 g and 29 negative samples were performed by 2 proficient, and in total 63 fully instructed, users, who had no hands-on (first time) experience in qPCR. Two experienced laboratory technicians analysed one positive and one negative sample in each run in parallel to ensure the comparability of results.

Tm calling was performed at λ 530 nm using LightCycler480 SW1.5.0.

## Results

*Analytical sensitivity*

The analytical sensitivity in testing was identified by analyses of tenfold dilution series of bacterial DNA. The 95% hit rate was calculated from log(g/analyse): hit rate plot at 5.6E-11 g (Figure 1) according to a hit rate calculated from positively identified amplification products by Tm calling. Tm of *M. bovis* in this assay was 84.32°C (sd 0.08°C).

*User dependent assay robustness*

To analyse the potential effect of a laboratory technician, we involved non-skilled personnel and made the analyses on different days, different runs and involved total of 63 persons for the analyses. Every non-skilled person prepared and performed only one sample and one analysis of a sample with blind content to avoid any effect of manual training or sample content concern.

The results show that all (100%) positive samples

*Table 1.* Reference target bacterial strain and primers designed using PRIMER EXPRESS **(ver. 3.0)**

| Species | Strain | Gene target | Primer forward | Tm |
|---|---|---|---|---|
| *Mycobacterium bovis* | DSM 43990 | hsp | GGGTCAAGCTCGACGTTGA | 58°C |
| | | | Primer reverse | Tm |
| | | | CGGTGGTCCGTTTGGAACT | 58°C |

DSM – strains obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (Germany)

*Table 2.* Reaction mix content

| | c | V (µL) | C final |
|---|---|---|---|
| MM | 5 x | 4 | 1 x |
| Primer f | 5 pmol/µL | 1 | 250 nM |
| Primer r | 5 pmol/µL | 1 | 250 nM |
| Sample (DNA template) | | 5 | |
| $H_2O$ total | | 9 | |
| TOTAL | | 20 | |

*Table 3.* qPCR program

| | | | | |
|---|---|---|---|---|
| Program Name | | Hotstart | | |
| Cycles | 1 | Analysis mode | None | |
| Target (°C) | Acquisition mode | Hold (hh:mm:ss) | Ramp rate (°C/s) | Acquisitions (per °C) |
| 95 | None | 00:15:00 | 20.00 | |
| | | | | |
| Program Name | | Amplification | | |
| Cycles | 45 | Analysis Mode | Quantification | |
| Target (°C) | Acquisition mode | Hold (hh:mm:ss) | Ramp rate (°C/s) | Acquisitions (per °C) |
| 95 | None | 00:00:05 | 20.00 | |
| 60 | None | 00:00:14 | 20.00 | |
| 72 | Single | 00:00:31 | 20.00 | |
| | | | | |
| Program Name | | Melting | | |
| Cycles | 1 | Analysis mode | Melting curves | |
| Target (°C) | Acquisition Mode | Hold (hh:mm:ss) | Ramp rate (°C/s) | Acquisitions (per °C) |
| 75 | None | 00:00:10 | 20.00 | |
| 95 | Continuous | | 0.15 | 5 |
| | | | | |
| Program Name | | Cooling | | |
| Cycles | 1 | Analysis mode | None | |
| Target (°C) | Acquisition mode | Hold (hh:mm:ss) | Ramp rate (°C/s) | Acquisitions (per °C) |
| 40 | None | 00:00:15 | 20.00 | |

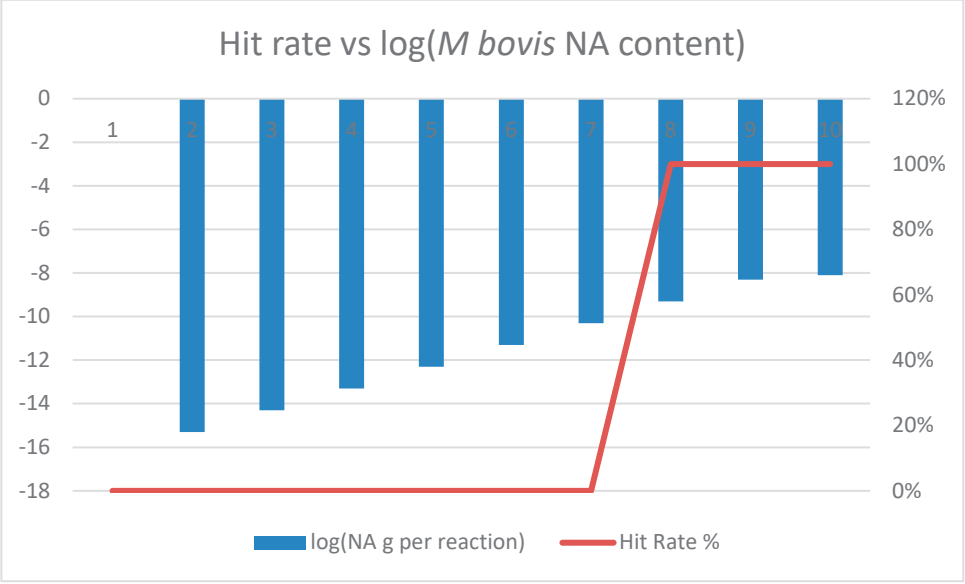



*Figure* 1. Hit rate vs log (M. bovis NA content g)

were finally identified as positive by melting curve analyses (Tm calling, Table 4). This is important, as the lowest content for analyses was near LoD, only one ng per reaction. To detect possible cross-contamination, a number of negative samples were analysed on all runs together with different concentration positive samples, and no cross-contamination was detected.

*Tm calling versus quantification (Cp calculation from an amplification curve by 2nd derivative Max)*

In robustness of the assay, we evaluated the hit rate by the 2nd derivative max calculation of an amplification curve in PCR cycling. The Tm calling

*Table 4.* Summary of robustness study based on Tm calling

| Target g | | | | |
|---|---|---|---|---|
| Reaction | N tested | N positive *M bovis* | N negative *M. bovis* | Hit rate |
| 0 | 29 | 0 | 29 | 0% |
| 1.00E-09 | 11 | 11 | 0 | 100% |
| 2.00E-09 | 11 | 11 | 0 | 100% |
| 4.00E-09 | 12 | 12 | 0 | 100% |
| 8.00E-09 | 4 | 4 | 0 | 100% |
| Total | 67 | 38 | 29 | |

*Table 5.* Summary of robustness study based on Cp value

| Target g | | | | |
|---|---|---|---|---|
| Reaction | N tested | N positive *M bovis* | N negative *M. bovis* | Hit rate |
| 0 | 29 | 0 | 29 | 0% |
| 1.00E-09 | 11 | 8 | 3 | 73% |
| 2.00E-09 | 11 | 11 | 0 | 100% |
| 4.00E-09 | 12 | 12 | 0 | 100% |
| 8.00E-09 | 4 | 4 | 0 | 100% |

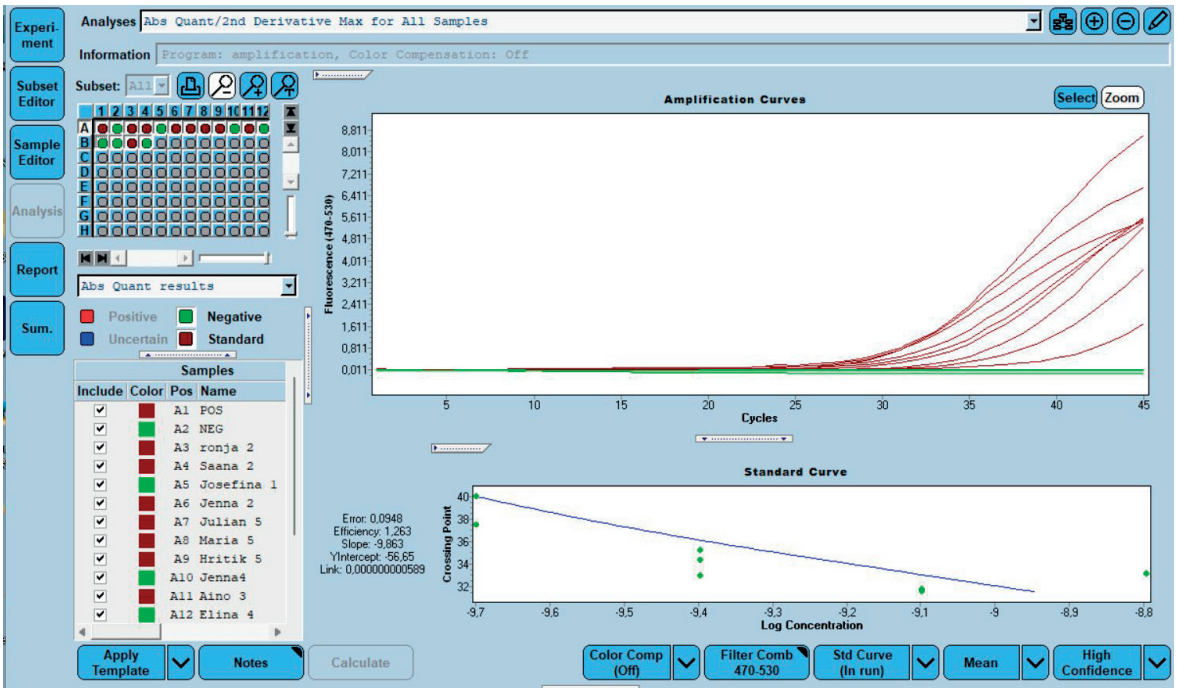
allows finding all positives by non-trained users, but not using simple Cp (quantification analyse, Table 5). This shows how important it is in such analyses not to rely only on Cp values, but also perform the melting curve analyses although this is mostly done for positive samples to prove the specificity of analysis by amplified product Tm.

Examples of quantification and melting curve analyses are given in Figure 2 and Figure 3.

The sensitivity calculations using Cp values were lower as LightCycler Software was not able to calculate Cp-values of low concentration samples and showed a status of "> - Late Cp call (last five cycles) has higher uncertainty".

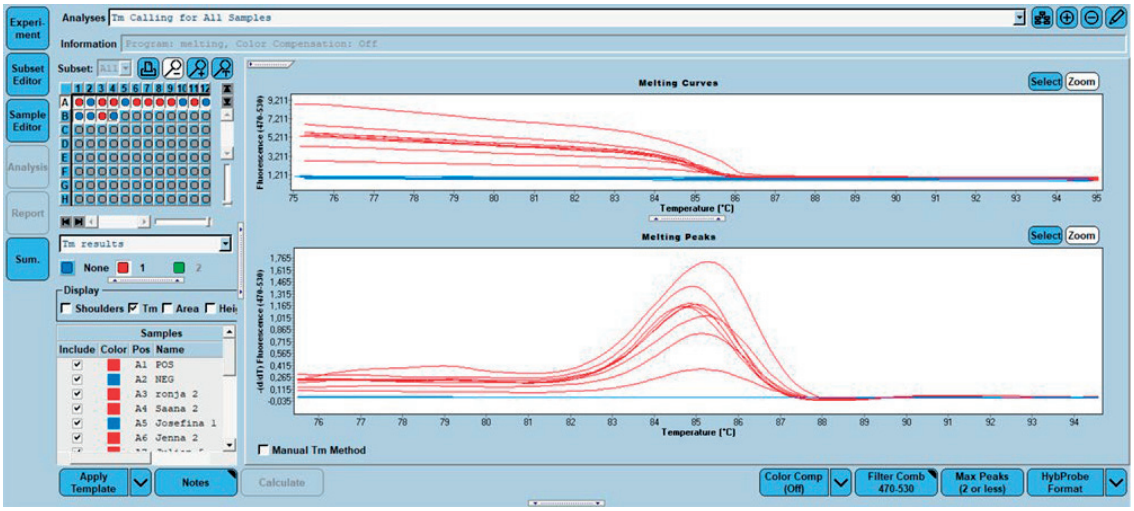
## Discussion and conclusions

It is generally accepted (Amagliani et al., 2006) that prevention of food-borne disease basically depends on surveillance and prompt identification of pathogens in food products. The major advantage of the current molecular method is simplicity, short time necessary to obtain the results and the robustness of an assay. To ensure better analytical sensitivity, we increased the sample volume to 5 µL compared to earlier protocols (Karus et al., 2017). This might be also relevant to compensate of pipetting errors in technicians. The limit of detection of our method is close to other latest methods with another target of *M. bovis* in singleplex qPCR analyses (0.01 ng *M. Bovis* BCG DNA) (Zeineldin et al., 2023). A crucial step for molecular assessment of microbial communities




Positive and/or spiked samples
Negative samples

*Figure* 2. Example of amplification curves of EvaGreen assays in a robustness test by unexperienced users




Positive and/or spiked samples
Negative samples

*Figure* 3. Example of melting curves of EvaGreen assays in a robustness test by unexperienced users

is the selection of a gene or genetic marker that can be used to differentiate a wide variety of organisms (Justé et al., 2008). Usually, the specificity of assays is ensured by using hydrolysis or hybridization probes (Severgnini et al., 2011). Indeed, any of all additional chemistries, even the most widely used TaqMan® chemistry, will increase significantly the costs of an assay. Several real-time PCR assays for a single reaction have been developed for the detection of the pathogens of our interest, and the trend has been moving towards strategies for a rapid identification of more than one pathogen through the development of multiple analysis platforms (Fukushima et al., 2010; Cremonesi et al., 2014). However, this trend does not improve the robustness of assays. Our results in *M. bovis* qPCR tests show that simple methods can be robust and have a good sensitivity when Tm calling is used. Analyses based on Cp values may cause caution due to the possibility of false negative results, but software also warns about late Cp, and, thus, prevents pathogen misdetection. Lázaro-Perona et al. (2021) showed lately, during Covid-19 pandemics, the importance of the method robustness (unexperienced user effect in nucleic acids purification), but also, that robust methods can be implemented quickly and efficiently (Lázaro-Perona et al., 2021).

There are still several concerns and limitations due to several risks in *M. bovis* analysis in real potential outbreak conditions by less experienced technicians. Efficient DNA extraction from complex matrices (such as food) can be challenging. Inhibitors present in food samples may affect PCR efficiency. Molecular methods can be highly sensitive, but false negatives can occur due to low bacterial load in samples or inhibitory substances. In our study, we did not find any cross-contamination, but when the target concentration is extremely high, there is still a danger of getting false positive results by cross-contamination of a sample itself or purified DNA by less trained technicians. Handling of live *M. bovis* poses also health risks. Proper inactivation methods are essential to protect laboratory personnel. Biosafety precautions are essential. These precautions will not diminish the importance of robustness of methods. In opposite, there is no guarantee that there is always enough laboratory capacity or no fully automated on-site testing methods available.

The real-time PCR assay described in this study has the potential to be a fast-screening assay for *M. bovis* enabling simultaneous processing of many food samples. Because the assay development does not include the sample preparation steps, the only prerequisite is to obtain good quality (good purity and sufficient concentration) purified DNA samples from various matrixes and can also be used for HACPP (analysing surfaces, etc) risk analysis, or other goals. It showed excellent performance even if used by 63 non-experienced technicians. This means that the assay does not require highly skilled molecular biology specialists to implement it, but requires only careful following of good laboratory practice. This assay may be used for accurate and rapid diagnosis of food-borne outbreaks as it has the potential to be used in routine diagnostic laboratories providing a simple, fast, cheap and sensitive alternative method to culture-based or TaqMan qPCR methods, especially if there is a need to implement this method quickly.

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# The Effect of Milking Frequency, Heat Stress and Physiological State's Interactions on Daily Milk Yield of Holstein Dairy Cows in a Saharan Zone

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***Keywords:*** *Daily milk yield, heat stress, milking frequency, Saharan environment, temperature-humidity index.*

***Abstract.*** *This study focuses on enhancing daily milk yield (DMY) in the Saharan environment by exploring the relationships among milking frequency, heat stress, and lactation stage in 187 Holstein dairy cows in the Saharan Ghardaia region over a 12-year period. Key findings indicate that milking three times daily boosts DMY by about 22.67% compared with twice daily milking, with this increase being most significant ($P < 0.0001$) in cows during their second and third lactations. The temperature-humidity index (THI) plays a pivotal role in DMY. The study shows that the best milk production occurs ($P < 0.0001$) at lower THI values (below 74), underscoring the importance of optimal environmental temperature and humidity for maximum milk yields. Cows exposed to these lower THI values and milked three times daily achieve the highest milk production (+25%). Thus, combining the right THI conditions with increased milking frequency can significantly ($P < 0.0001$) enhance milk production. Effective heat stress management is also crucial for optimal milk yields. The study recommends practical strategies like offering sufficient shade, ensuring good ventilation, and giving cows access to cool water. Adjusting their nutrition during high heat stress periods is also vital. Moreover, the timing of milking sessions, especially during the cooler parts of the day, is a key factor in milk production. In conclusion, the research highlights the intertwined roles of various factors, especially milking frequency and THI, in determining the DMY of dairy cows in Saharan areas. It supports the implementation of improved management practices to counter environmental challenges and maximize milk production in such demanding environments.*

## Introduction

The daily milk yield (DMY) of dairy cows during lactation is a crucial parameter used to assess milk production. The frequency of milking plays a significant role in determining milk yield and production efficiency. Dairy farmers often manipulate milking frequency as a management tool to enhance milk production. Increasing milking frequency has been shown to positively impact DMY and improve overall production efficiency (Erdman and Varner, 1995; Cabrera et al., 2010).

While increasing milking frequency has proven effective in boosting milk yield, it also raises operational costs and requires additional labor. Therefore, striking a balance between milking frequency and cost-effectiveness is essential. Nevertheless, studies have demonstrated that short-term increases in milking frequency, particularly in early lactation, can improve milk yield and lactation persistency (Wall and McFadden, 2012). However, the time required for additional milkings at higher frequencies may potentially interfere with important cow behaviors such as feeding, rumination, and resting. These behaviors are crucial for maintaining energy balance, promoting digestive efficiency, ensuring cow health and welfare, and meeting the cow's production demands (Hart et al., 2013).

In addition to milking frequency, heat stress poses a significant challenge to dairy farmers, particularly in regions with hot climates. Heat stress negatively affects various aspects of dairy cow productivity, including milk yield, feed intake, reproductive performance, and overall well-being. The adverse effects of heat stress result in substantial economic losses for dairy farmers, especially in tropical countries. Researchers have focused on selecting dairy cattle with improved milk production traits, often resulting in increased feed intake and subsequently higher metabolic heat production, making animals more susceptible to heat stress (Kadzere et al., 2002).

The impact of heat stress on milk production is influenced by various factors, including breed, age, sex, and physiological stage of cows. The temperature-humidity index (THI) has been established as a reliable indicator of the reduction in milk production caused by heat stress. THI considers the combined effects of

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ambient temperature and relative humidity, providing a quantifiable measure of heat stress (Ravagnolo and Misztal, 2000; Herbut and Angrecka, 2018). Furthermore, seasonal variations in dry matter intake and the activation of the negative feedback system of milk secretion contribute to the relationship between milk yield and the lactation stage (West et al., 2003; Silanikove et al., 2009).

This study aims to enhance daily milk production in Holstein dairy cows in the Saharan region by examining the interplay between key factors such as milking frequency, heat stress, and lactation stage. The research focuses on understanding how these variables influence milk yield in the Ghardaia region of the Sahara, with a particular emphasis on optimizing conditions for maximum production. Specifically, the study investigates the effects of milking frequency and environmental parameters, including the temperature-humidity index (THI), on dairy output. Additionally, it aims to propose management strategies that mitigate the adverse impacts of heat stress, providing practical solutions for dairy farmers operating in this arid environment.

## Materials and Methods

*Animal data collection*

The study was conducted on a dairy farm located in the Ghardaia region (32°41'06.7"N latitude and 4°44'10.8"E longitude), focusing on daily milk records collected over a 12-year period from 2005 to 2016. A total of 18 178 daily milk yield (DMY) records were analyzed, obtained from 187 locally-born purebred Holstein dairy cows with parity orders ranging from the first to the eighth lactation (with an average of 2.59 ± 1.61 of lactation rang), with lactation number frequencies distributed as follows (1 = 34.5%, 2 = 22.9%, 3 = 15.1%, 4 = 12.2%, 5 = 9.6%, 6 = 4%, 7 = 1.5% and 8 = 0.2%).

Throughout the study, the cows were milked either twice daily (n = 177 cows) or thrice daily (n = 10 cows). The twice-daily milking sessions occurred at 06:00 and 18:00, while the thrice-daily milking sessions were conducted at 06:00, 14:00, and 21:00. These milking sessions took place in designated milking parlors equipped with automated milk meters, which precisely measured the total milk output over a 24-hour period for each cow.

The lactation stages of the cows were categorized into four classes: early lactation (< 120 days), middle lactation (120–179 days), late lactation (180–305 days), and prolonged lactation (> 305 days).

*Environmental data collection*

The data was acquired from the local meteorological station, representing the weather conditions at the farm. This data comprises the highest daily temperatures (AT °C) and the maximum relative humidity (%RH). These variables were employed to compute the temperature-humidity index (THI), following the formula proposed by (Mader et al., 2006):

$$THI = (0.8 \times T) + [(\%RH / 100) \times (T - 14.4)] + 46.4.$$

The values of THI are divided into 4 classes, according to the classification by Nienaber et al. (2007), who evaluated the intensity of heat stress as follows: normal stress, moderate stress, severe stress, and very severe stress, corresponding to the following values: THI < 74, [75–78], [79–83], and THI > 84, respectively.

*Feed and feeding*

The dairy cows in the study were provided with a basic ration consisting of alfalfa hay and corn silage, supplemented with mixed concentrate feeds. The composition of the diets, including the dry matter percentage (DM %), dry matter intake (DMI) of the diet in kilograms (kg), and nutrient intake per cow per day, is presented in Table 1.

*Table 1.* Average daily ration composition of lactating cows during the study period (2005–2016)

| Diet | % DM | DMI of diet (kg) | Nutrient intake/cow/day | | | |
|---|---|---|---|---|---|---|
| | | | UFL[1] | PDI[2] (g) | Ca (g) | P (g) |
| Fodder sorghum | 21.36 | 5 | 3.4 | 330 | 16.5 | 17 |
| Alfalfa hay | 18.80 | 4.4 | 3.21 | 365.2 | 70.84 | 11.88 |
| Wheat bran | 10.68 | 2.5 | 2 | 187.5 | 3.5 | 24.25 |
| Flattened wheat grain | 12.82 | 3 | 3,06 | 261 | 2.1 | 10.2 |
| Maize grain | 17.09 | 4 | 4.24 | 336 | 1.6 | 10.4 |
| Date scraps | 6.41 | 1.5 | 1.27 | 42.6 | 1.5 | 1.5 |
| Soybean meal | 12.82 | 3 | 3.15 | 666 | 10.2 | 18.6 |
| Total | 100 | 23.4 | 20.33 | 2188.3 | 106.24 | 93.83 |
| Rapport PDI/UFL: 107.6 g/UFL, Energy density of diet: 0.86 UFL/kg, Hay/Concentrate ratio: 59.83/40.17%, Dry matter (%) of ration: 38.84%, $UEL^{3}_{total}$: 10.1. | | | | | | |

1: Forage unit for milk production, per kg. 2: Digestible proteins in the intestine, in g/kg. 3: Encumbrance unit for lactating females.

*Statistical Analysis*

The dataset was subjected to an extensive statistical evaluation using Minitab 18 to ascertain the influence of various factors on daily milk yield (DMY) in dairy cows. These factors included milking frequency (MF), lactation number (LN), lactation stage (LS), calving season (CS), and temperature humidity index (THI). To quantify these effects, a fixed-effect model was implemented, formulated as follows:

$$Y_{\_ijklm} = \mu + MF_{\_i} + LN_{\_j} + (MF\ x\ LN)_{\_ij} + LS_{\_k} + (MF\ x\ LS)_{\_ik} + CS_{\_l} + (MF\ x\ CS)_{\_il} + THI_{\_m} + (MF\ x\ THI)_{\_im} + e_{\_ijklm}.$$

Here, $Y_{\_ijklm}$ signifies DMY, and μ denotes the population mean. This model facilitates a comprehensive understanding of the primary effects and interactions among these variables on milk production.

## Results

According to the THI data shown in Fig. 1, thermal stress in the study area can be categorized as follows.

The months of April, May, and October experience mild to moderate thermal stress, with THI values being 72.94 ± 4.49, 77.67 ± 4.18, and 76.12 ± 4.40, respectively. In contrast, June through September undergo severe thermal stress, indicated by THI values of 82.86 ± 3.22, 85.26 ± 2.59, 85.05 ± 2.55, and 81.82 ± 3.56, respectively. Meanwhile, from November to March, there is no thermal stress, with THI values ranging from 61.22 ± 4.81, 62.63 ± 5.00, 61.22 ± 4.32, 64.23 ± 4.81 and 68.35 ± 4.81. These variations in thermal stress, especially in the Saharan regions, contribute to moderate to severe challenges that negatively affect the productivity of dairy cows.

The analysis of the results presented in Table 2 shows the effects of various factors on DMY in Holstein dairy cows.

Temperature-humidity index (THI) values significantly influence daily milk yield (DMY) ($P < 0.0001$), with bovines exposed to lower THI values (< 74) demonstrating higher mean DMY (22.88 kg) compared with those in elevated THI ranges, and an inverse relationship observed between increasing THI values and mean DMY differences. Milking frequency (MF) also significantly impacts DMY ($P < 0.0001$), with cows milked thrice daily (3X) producing a superior mean of 24.55 kg compared with 21.43 kg for twice-daily milking (2X). Calving season stress levels significantly affect DMY ($P < 0.0001$), with normal stress during parturition yielding the highest mean DMY (22.71 kg) and severe stress yielding the lowest mean DMY (20.28 kg), indicating an inverse correlation between calving stress intensity and DMY. Lactation number significantly influences DMY ($P < 0.0001$), with third-lactation cows exhibiting peak mean DMY (22.49 kg), followed by a slight decline in the fourth or greater lactations (21.70 kg), while the first and the second lactations yield substantial mean DMYs of 21.76 kg and 21.22 kg, respectively. Lactation stage also significantly impacts DMY ($P < 0.0001$), with early lactation cows demonstrating the maximum mean DMY (26.72 kg), followed by those in mid-lactation (22.75 kg), and late and extended lactation stages exhibiting progressively lower mean DMYs of 19.01 kg and 16.32 kg, respectively. The results presented in Fig. 2 demonstrate the interaction between MF and THI on the DMY of Holstein dairy cows.

The interaction between milking frequency (MF) and temperature-humidity index (THI) exhibits a significant influence on daily milk yield (DMY) ($P < 0.0001$). In twice-daily milking (2X) regimens, an inverse relationship is observed between THI and DMY. Bovines subjected to 2X milking demonstrate peak mean DMY (21.57 kg) at THI < 74, with

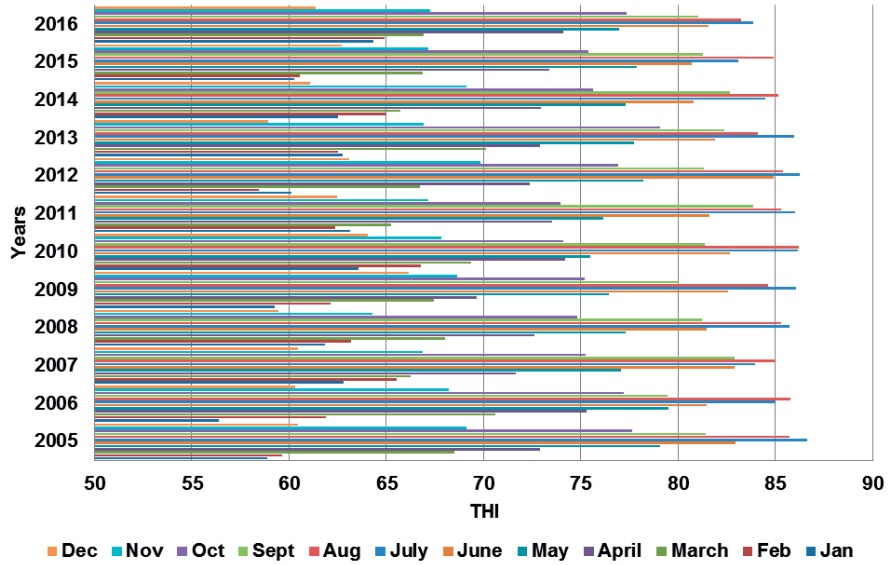


*Fig. 1.* Means of monthly distribution of THI values during the study period (2005–2016).

*Table 2.* The fixed effects of the environmental, milking frequency and animal factors on DMY (n = 18 178 records) of Holstein dairy cows

| Items | N | Mean | StDev | 95% CI | *P* value |
|---|---|---|---|---|---|
| THI values range | | | | | |
| < 74 | 7692 | 22.88 | 8.11 | (22.70; 23.05) | 0.0001 |
| [75–78] | 2769 | 22.18 | 7.83 | (21.89; 22.46) | |
| [79–83] | 5020 | 21.03 | 7.70 | (20.81; 21.24) | |
| > 84 | 2697 | 19.33 | 6.31 | (19.04; 19.62) | |
| Milking frequency | | | | | |
| 2X | 16 401 | 21.43 | 7.76 | (21.31; 21.55) | 0.0001 |
| 3X | 1777 | 24.55 | 7.75 | (24.19; 24.91) | |
| Calving season | | | | | |
| Normal stress | 9103 | 22.71 | 8.24 | (22.54; 22.86) | 0.0001 |
| Moderate stress | 2211 | 21.12 | 8.08 | (20.87; 21.52) | |
| Severe stress | 3321 | 20.98 | 7.05 | (20.72; 21.24) | |
| Very severe stress | 3543 | 20.28 | 6.07 | (20.02; 20.53) | |
| Lactation number | | | | | |
| First lactation | 6273 | 21.76 | 7.45 | (21.57; 21.96) | 0.0001 |
| Second lactation | 4171 | 21.22 | 7.99 | (20.98; 21.46) | |
| Third lactation | 2736 | 22.49 | 8.16 | (22.20; 22.78) | |
| Fourth lactation or greater | 4998 | 21.70 | 7.88 | (21.49; 21.92) | |
| Lactation stage | | | | | |
| Early lactation | 5050 | 26.72 | 7,94 | (26.53; 26.91) | 0.0001 |
| Middle lactation | 5170 | 22.75 | 6.34 | (22.56; 22.93) | |
| Later lactation | 4700 | 19.01 | 5.99 | (18.81; 19.20) | |
| Prolonged lactation | 3258 | 16.32 | 6.19 | (16.09; 16.56) | |

diminishing yields at higher THI ranges. Similarly, thrice-daily milking (3X) protocols display an inverse THI-DMY relationship. However, 3X milking yields superior mean DMY at both THI < 74 (24.95 kg) and THI 75–78 (26.42 kg). Comparative analysis reveals that 3X milking protocols result in substantial DMY improvements across various THI ranges: 15.71% (THI < 74), 28.97% (THI 75–78), 20.67% (THI 79–83), and 25.35% (THI > 84). In Fig. 3, the interaction between milking frequency (2X and 3X) and calving season on DMY of Holstein dairy cows is examined.

The findings suggest a significant difference in DMY between cows milked twice daily (2X) and those milked three times daily (3X), varying by calving season. Cows milked 3X consistently exhibit higher DMY across all levels of calving stress compared with cows milked 2X, with DMY increases ranging from 17.4% under normal stress to 29.6% under very severe stress. These results indicate that increasing milking frequency to 3X can lead to substantial gains in milk production, with more pronounced percentage increases observed under higher calving stress conditions. Fig. 4 shows the interaction between MF (2X and 3X) and lactation number on DMY Holstein dairy cows.

A significant increase in daily milk yield (DMY) was observed in cows subjected to a three-times-daily (3X) milking regimen compared with those milked twice daily (2X) across all lactations. The most pronounced difference in DMY between 3X and 2X milking was evident in the second lactation. While 3X milking consistently yielded higher DMY than 2X milking throughout the study, the magnitude of the difference between the two regimens gradually decreased with advancing lactation number. The relative increase in DMY achieved with 3X milking compared with 2X milking was 8.29%, 34.15%, 31.06%, and 16.95% for the first, second, third, and fourth or greater lactations, respectively. The results in Fig. 5 indicate the interaction between MF and lactation stage on the DMY of Holstein dairy cows.

A significant increase in daily milk yield (DMY) was observed in cows subjected to a three-times-daily (3X) milking regimen compared with those milked twice daily (2X) throughout all stages of lactation. The most pronounced effect of milking frequency (MF) on DMY was evident during early lactation. While 3X milking consistently outperformed 2X milking in terms of DMY, the magnitude of the difference between the two regimens gradually diminished as

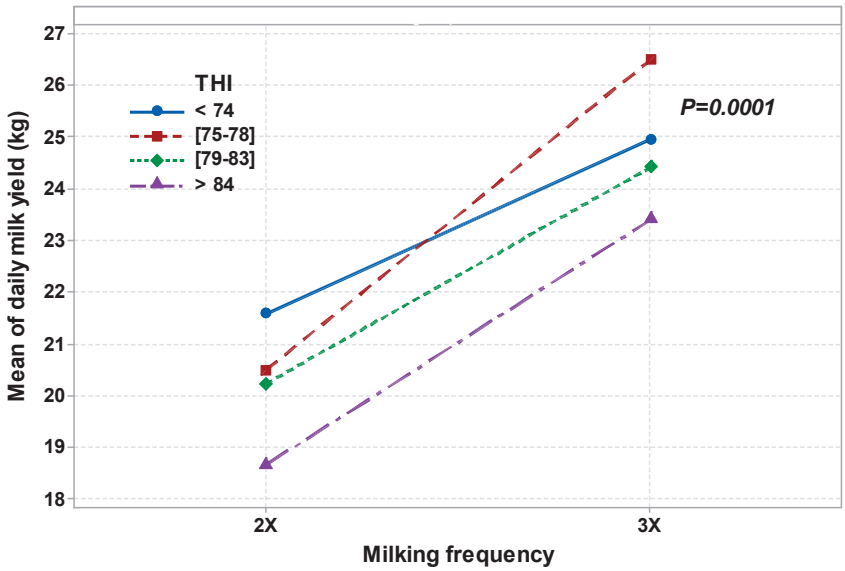


*Fig. 2.* Means of DMY (kg) depending on (MF x THI) of Holstein dairy cows

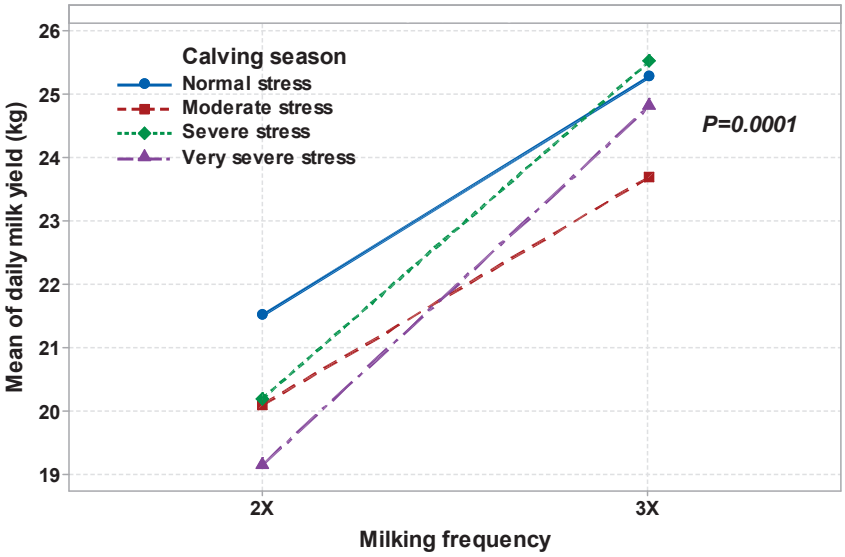


*Fig. 3.* Means of DMY (kg) depending on (MF x Calving season) of Holstein dairy cows

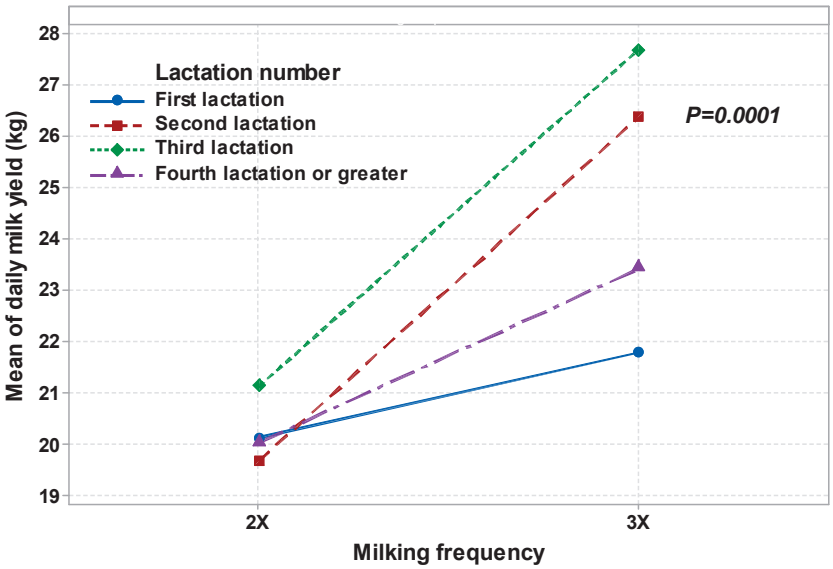


*Fig. 4.* Means of DMY (kg) depending on (MF x Lactation number) of Holstein dairy cows

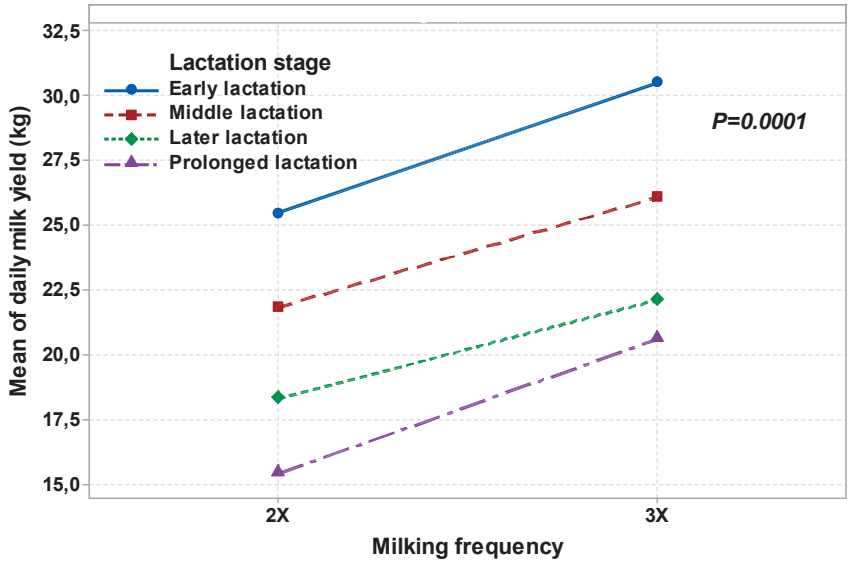


*Fig. 5.* Means of DMY (kg) depending on (MF x Lactation stage) of Holstein dairy cows

lactation progressed. Even in prolonged lactation, a significant disparity in DMY persisted between 3X and 2X milking, demonstrating the continued benefit of 3X milking. The relative increase in DMY achieved with 3X milking compared with 2X milking was 20.2%, 17.6%, 19.0%, and 29.2% for early, middle, later, and prolonged lactation stages, respectively.

## Discussion

The interaction between milking frequency (MF) and lactation stage (LS) has yielded significant insights into daily milk yield (DMY). The findings demonstrate the critical influence of LS on milk production, showing a complex interplay with MF that shapes overall DMY. Notably, heat stress negatively impacts milk production at various LS, with the most pronounced reduction occurring during mid-lactation, as highlighted by Joksimović-Todorović et al. (2011). These results emphasize the need to account for LS when assessing the relationship between MF and milk yield.

Furthermore, the concept of peak milk yield, which represents the highest milk production during a lactation cycle, has been examined, revealing that cows milked three times daily surpass those milked twice daily during this peak period (Capuco et al., 2003). This underscores the positive correlation between increased MF and enhanced DMY, especially during peak lactation. The typical lactation curve, characterized by a gradual increase in milk yield after calving, reaching a peak, followed by a decline toward late lactation, aligns with the current study's findings, which observed a steady rise in milk yield up to mid-lactation, followed by a decline.

Additionally, responses to increased MF during early lactation vary between primiparous heifers and multiparous cows. Soberon et al. (2010) reported a stronger response in heifers, while later research by Soberon et al. (2011) found comparable responses in both heifers and multiparous cows at this stage. The positive impact of increased MF on DMY is well-established, with studies reporting increases of 7% to 15% when switching from twice-daily to three-times-daily milking (Bogucki et al., 2009; Barlowska et al., 2012). However, this effect depends on factors such as LS and parity, with Bogucki et al. (2011) observing a diminishing trend in DMY with three-times-daily milking in cows beyond their first lactation.

Moreover, increasing MF during early lactation has been associated with improved milk yield and lactation persistency (Hale et al., 2003; Wall and McFadden, 2012), highlighting the long-term effects of MF manipulation during specific LS on milk production. Variations in udder anatomy, lactation persistency, and other factors may explain the differences in response to increased MF between primiparous and multiparous cows (De Vliegher et al., 2003). Therefore, a thorough investigation of the MF-LS interaction on milk yield must consider these complexities.

## Conclusion

This study underscores the significant impact of the interaction between milking frequency and the temperature-humidity index on the daily milk yield of locally-bred Holstein dairy cows. The findings reveal that cows milked three times daily exhibit a substantially higher DMY (+22.67%) compared with those milked twice daily, particularly across varying THI levels. The lowest THI range (< 74) is associated with the highest DMY for both milking frequencies, highlighting the critical role of optimal THI conditions in maximizing milk production.

Furthermore, the combination of increased milking frequency and lower THI values shows great potential for enhancing milk production in Holstein dairy cows. Dairy farmers are encouraged to carefully

manage milking frequency and maintain favorable environmental conditions, especially in terms of THI, to optimize production within their herds.

The study also confirms the significant influence of both milking frequency and lactation number on DMY. Cows milked three times daily generally produce more milk (25.11%), with this increase being most pronounced during the second and third lactations. These insights into the relationship between milking frequency, lactation number, and DMY can provide valuable guidance for dairy farmers seeking to optimize milk output in their herds.

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**Competing interests**

The authors declare that they have no potential conflict of interest.

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# An Overview on Infectious Laryngotracheitis (ILT): A Serious Threat to Chicken Intensive Production System

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***Keywords:*** *Infectious laryngotracheitis virus, susceptibility, clinical picture, diagnosis, vaccine.*

***Abstract.*** *Infectious laryngotracheitis (ILT) is a respiratory viral infection, particularly common in adult chickens. The disease widely distributed in several countries causes severe economic losses. ILT virus (ILTV) is a double-stranded DNA which belongs to Alphaherpesvirinae subfamily of Herpesviridae family. Infected and latently carrier chickens are sources of ILTV infection, while the aerosol is the main route of the virus transmission. The acute epizootic form of ILT is characterized by a sudden onset, a rapid spread, and a high mortality rate with severe conjunctivitis, dyspnoea, gasping, coughing, expectoration of blood mixed with mucus, and drop in egg production. However, chickens in the mild enzootic form show less signs with a morbidity rate up to 5% and a mortality rate between 0.1% and 2%. The most characteristic post-mortem lesions are haemorrhagic tracheitis with the presence of a yellow cheesy plug in the respiratory tract. Despite diagnostics of signs and lesions, the confirmation of ILTV infection occurs via conventional isolation, detection of the virus in the affected tissues, as well as using recent molecular techniques. Prevention of ILTV infection depends on some key lines, including biosecurity measures and vaccination. Both live attenuated and recombinant vaccines are used for the prevention and control of ILTV infection. Therefore, the present review focuses on susceptibility, transmission, clinical picture, diagnosis, differential diagnosis, immune response, and prevention and control.*

## Introduction

Infectious laryngotracheitis (ILT) is a highly contagious respiratory viral disease of chickens caused by the herpes virus (Garcia et al., 2013). The disease is associated with severe production losses as a result of mortality, decrease in the weight gain and egg production, the expenses of vaccination, biosecurity measures, and treatment of secondary infections by other avian pathogens (Guy and Garcia, 2008; Jones, 2010; Garcia et al., 2013; Parra et al., 2016). ILT shows a serious infection and causes huge economic losses particularly in high-density poultry-producing intensive production systems (Yan et al., 2016; Zorman Rojs et al., 2021). The disease was initially named as "avian diphtheria", but the name "ILT" was adopted by the Committee of Poultry Diseases of American Veterinary Medical Association by the year 1931 (Guy and Garcia, 2008). The high flock density, rearing of multiage and multipurpose chickens within the same area, short production cycles, and improper vaccination and biosecurity measures have contributed to the increased outbreaks of ILT in many countries all over the world (Blakey et al., 2019; Mishra et al., 2020; Tsiouris et al., 2021; Dodovski et al., 2022; Pajić et al., 2022; Gamal and Soliman, 2023). ILT remains a serious threat and has negative effects on the poultry industry worldwide since its first report in the mid-1920s. The disease was first described in the United States of America (USA) (May and Thittsler, 1925) and then it has been distributed in North and South America, Europe, Southeast Asia, and Australia. ILT is endemic in some countries as some regions in the same country or even multiple-age production sites (backyard flocks) are highly susceptible. Moreover, serious outbreaks are periodically observed when the strains of ILT virus (ILTV) transmit from chronically infected chickens to non-vaccinated flocks. ILTV belongs to *Alphaherpesvirinae* subfamily of *Herpesviridae* family, which has a double-stranded DNA genome of approximately 155 kb size. ILTV has a narrow host range as the main natural host is chicken. Moreover, chickens of all ages are susceptible and birds older than 4 weeks are mostly infected with ILTV (Aras et al., 2018; Tamilmaran et al., 2020). The virus shows lifelong infection by latency in the trigeminal ganglia. However, stress conditions such as transportation or reaching the peak of egg production can reactivate the latent virus to replicate and excrete (Coppo et al., 2013). Horizontal transmission of ILTV through the respiratory tract is the main route of the virus infection; however, the vertical transmission has not yet been reported (Wolfrum, 2020).

ILT is caused by a DNA virus that mostly infects the upper respiratory tract, conjunctiva, and the tracheal mucosa (Ahaduzzaman et al., 2020) causing conjunctivitis, coughing, dyspnoea, panting and stretching of the head and neck with an open beak "hunger for air", expectoration of bloody stained mucus, swelling of infraorbital sinuses, and decreasing

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egg production with a mortality rate of 10–20% (Garcia et al., 2013; Wolfrum, 2020). On post-mortem examination, bloody exudate or diphtheric membrane could be observed in the trachea (Pajić et al., 2022). The most important characteristic microscopic picture of ILTV is the presence of intranuclear eosinophilic inclusion bodies (Ou and Giambrone, 2012). The laboratory diagnosis of ILTV is based on detection and isolation, serological monitoring, and using some molecular techniques such as polymerase chain reaction (PCR) (Guy and Bagust, 2020; Carnaccini et al., 2022).

The prevention and control of ILTV infection rely on inhibiting the contact between the virus and the hosts by application of biosecurity measures and vaccination (Dufour-Zavala, 2008; Maekawa et al., 2019). Live attenuated ILTV vaccines are prepared by attenuation in a chicken embryo or in tissue culture, while recombinant vaccines are prepared by using turkey herpes virus or pox virus as a vector (Samberg et al., 1971; Coppo et al., 2013; García, 2017; Maekawa et al., 2019). Both vaccines have been used commercially. ILT is one of the listed diseases that must be reported with adoption of strict biosecurity measures during outbreaks.

Therefore, the present review focuses on ILT with respect to its incidence and distribution, aetiology, susceptibility, transmission, clinical picture, diagnosis, differential diagnosis, immune response, and prevention and control.

**Incidence and distribution**

The first report of ILT was in the USA (May and Thittsler, 1925). Then it has been detected in the United Kingdom, Australia, and Europe (Cover, 1996). Nowadays, ILTV infections have been reported as important worldwide threats. The infections with ILTV were reported in more than 100 countries during the period of 2000–2013 (Menendez et al., 2014). The disease outbreaks have been detected in Canada (Ojkic et al., 2006), Europe (Neff et al., 2008), the USA (Dormitorio et al., 2013), China (Zhuang et al., 2014), Brazil (Preis et al., 2013; Parra et al., 2015, 2016), Netherlands (Dodovski et al., 2022), Greece (Tsiouris et al., 2021), India (Gowthaman et al., 2016; Mishra et al., 2020), Ontario (Alexander et al., 1998), Australia (Agnew-Crumpton et al., 2016), Egypt (Shehata et al., 2013; Abdo et al., 2017; Magouz et al., 2018; Bayoumi et al., 2020; ElSaied et al., 2021, 2022), Serbia (Orli´c et al., 2003; Pajić et al., 2022), Namibia (Molini et al., 2019), Algeria (Salhi et al., 2021), Iran (Razmyar et al., 2021), and Iraq (Alaraji et al., 2019). The increased incidence of ILT from time to time may be due to increasing the poultry production density, decreasing the downtime of production sites, poor biosecurity, and vaccination failure.

**The aetiology**

ILTV is taxonomically identified as Gallid herpesvirus 1 of the family *Herpesviridae* in the sub-family *Alphaherpesvirinae* and genus *Iltovirus* (Roizman, 1982; Davison et al., 2009). The hexagonal nucleocapsid of ILTV is of icosahedral symmetry and is composed of 162 elongated hollow capsomeres (Watrach et al., 1963). The viral capsid is about 100 nm in diameter, while the whole viral particle size ranges from 200 to 350 nm (Granzow et al., 2001). The genome of ILTV contains 80 open reading frames (ORFs), of which 65, 9, and 6 are located in the UL, US, and IR regions, respectively (Lee et al., 2011). Besides, the virus envelope contains glycoprotein spikes or projections that surround the nucleocapsid (195–250 nm in diameter). The molecular weights of ILTV glycoproteins have been first reported as 205, 160, 115, 90, and 60 kD (York et al., 1987). The glycoproteins of ILTV envelop are able to stimulate both the humoral and cell mediate immune responses of the host (York and Fahey, 1990). Moreover, the antigens of glycoproteins gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM are key factors for the virus attachment, entry, and replication in the target cell of the host (Helferich et al., 2007; Goraya et al., 2017; Gowthaman et al., 2020). For example, gG glycoprotein of ILTV can facilitate the virus entry (Tran et al., 2000) and cell-to-cell spread (Nakamichi et al., 2002) as well as may act as a broad-spectrum viral chemokine binding protein. Moreover, glycoproteins such as gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM are encoded by highly conserved ORFs viz. UL27, UL44, US6, US8, US4, UL22, US7, US5, UL53, UL1 and UL10, respectively (Piccirillo et al., 2016). The surface gpB antigen showed a high conservancy between the different ILTV isolates in Egypt (Ali et al., 2019; Maha et al., 2020). These glycoproteins are important for the stimulation of both cell mediated and humoral immunity. Two clusters of *Iltovirus* specific genes have been identified; one is located between UL45 and UL22 which encodes 5 ORFs (ORF A-E), while the other is located between UL-1 and ICP4 and encodes UL-0 and UL-1 (Fuchs and Mettenleiter, 1996). ILTV is a linear and double stranded segmented DNA (Lieb et al., 1987). The complete genome sequence of ILTV comprises 148 kb nucleotides encoding long, short, and two inverted repeat sequences (Morales Ruiz et al., 2018). The guanine–cytosine content of ILTV is 48.2% (Lee et al., 2011).

The results of immunofluorescence, virus-neutralization, and cross-protection tests indicated that all ILTV strains are antigenically similar (Shibley et al., 1962), but they differ in their virulence in chicken embryos or in tissue culture (Izuchi and Hasegawa, 1982; Russell and Turner, 1983). Strains of ILTV vary in their virulence from highly virulent wild-type strains which cause high morbidity and mortality rates in a susceptible host to strains of low

virulence that produce mild or sub-clinical infections (Pulsford, 1963; Jordan, 1966). Many techniques, including chicken embryos inoculation (Izuchi and Hasagawa, 1982), restriction endonuclease analyses (Kotiw et al., 1982; Lieb et al., 1987; Guy et al., 1989), and DNA hybridization assays (Kotiw et al., 1986), have been adopted to differentiate ILT viruses with different virulence.

**Resistance and sensitivity of the virus**

The ILTV survives in deep litter for 3–20 days at 11–24.5°C, in the droppings of battery cages for 3 days at 11–19.5°C, and at least for 3 weeks in buried carcasses. It can survive for several months during storage at 4°C in diluents. Besides, the virus may remain viable for 10 days to 3 months at 13–23°C. The ILTV infectivity remains for months during storage at 4°C in enrichment media such as nutrient and glycerol broths. Moreover, the virus survives at 13–23°C in the tracheal exudate and chicken carcasses for days and months and at –20°C to –60°C for months and years.

On the other hand, the presence of the envelope on ILTV facilitates its inactivation by heat, organic solvents or lipolytic agents such as chloroform and ether, as well as oxidizing agents like $H_2O_2$ (Meulemans and Halen, 1978; Neighbour et al., 1994; Ou and Giambrone, 2012). ILTV may be destroyed after exposure to 55°C for 15 minutes or to 38°C for 48 hours (Jordan, 1966). Exposure to 3% cresol, 5% phenol, or 1% sodium hydroxide solution can inactivate ILTV in less than one minute (Meulemans and Halen, 1978). The complete inactivation of ILTV in contaminated poultry house equipment could be achieved via fumigation with 5% hydrogen peroxide mist (Neighbour et al., 1994). Besides, the viability of the virus in the litter could be decreased by heating at 38°C for 24 hours (Giambrone et al., 2008).

**Susceptibility**

***Host***

Despite the fact that chicken is considered as the primary host for ILTV infection (Bagust, 1986), other host species including peafowls, peacocks, pheasants, guinea fowl, and turkeys are also susceptible to the natural infections (Crawshaw and Boycott, 1982; Hanson and Bagust, 1991; Bautista, 2003; Guy and Bagust, 2003). Ducks are refractory to the infection and act as carriers (Yamada et al., 1980). Pigeons, quail, sparrows, crows, doves, and starlings are resistant to ILTV (Guy and Garcia, 2008). Regarding breeds, rare cases of ILTV infections may occur in hobby/show/game chickens, broilers, heavy breeders, and commercial leghorn hens. Despite vaccination against ILTV, sporadic cases of infections were reported in heavy breeders and leghorns due to errors in the vaccine application or adoption of improper biosecurity measures. It has been reported that a high environmental temperature (35°C) could induce a higher mortality in heavy adult breeds compared with light adult breeds that have ILTV infection (Fahey et al., 1983).

***Age***

All ages of chicken (8 days to 4 years) are susceptible to ILTV infection (Kingsbury and Jungherr, 1958; Jordan, 1966; Linares et al., 1994). Chickens more than 3 weeks of age are highly susceptible (Dufour-Zavala, 2008). Despite the fact that the disease is common in adult layer chicken flocks (Aras et al., 2018; Tamilmaran et al., 2020), it has been also recognized in 3-week-old broiler chickens (Crespo et al., 2007; Guy et al., 1990; Timurkaan et al., 2003; Sellers et al., 2004; Moreno et al., 2010; Dormitorio et al., 2013; Pitesky et al., 2014).

**Infection and transmission**

The routes of ILTV entry are the nasal, conjunctiva, oral, and infra orbital sinus of chickens. The active viral replication usually occurs in the epithelium of the trachea and larynx. So, the main routes of ILTV infection are the respiratory tract and eyes (Williams et al., 1992). Oral infection is also possible, but exposure of nasal epithelium should be present following ingestion (Robertson and Egerton, 1981). Wind-borne transmission of ILTV has been demonstrated between commercial poultry operations (Johnson et al., 2005).

The sources of ILTV include clinically infected or latently carrier chickens as well as contaminated fomites, feed and water, bedding, and equipment. Unwashed or disinfected slaughterhouses are rendering vehicles (Pajić et al., 2022).

Despite the spread of ILTV to the non-respiratory sites via leucocytes, no viremia has been detected during infection (Oldoni et al., 2009). The transmission of ILTV frequently occurs through the direct contact between acute or chronic carrier infected chickens and the susceptible ones. Birds surviving from previous outbreaks act as a chronic carrier source of ILTV infection to healthy birds. Infected birds can also shed the virus in their respiratory secretions for 10 days post-infection (Williams et al., 1992). The ILTV-infected birds may transmit the virus via oral secretions (Hughes et al., 1987). The virus may persist in the respiratory tract of sub-clinical or latent infected chickens for up to 6–8 days (Bagust et al., 1986). The long-term tracheal carriers (approximately 2%) have been detected among convalescent birds (Hanson and Hanson, 1984). ILTV can remain latent in the trigeminal ganglia, while stress factors such as egg production or transportation may reactivate it. The reactivated virus could be transmitted from one bird to another causing an increase in its virulence (Dufour-Zavala, 2008; García, 2017). Hence, live attenuated ILTV vaccines are used only in endemic areas to avoid the direct contact between vaccinated or infected chickens or non-vaccinated birds (Ou

and Giambrone, 2012). The latent ILTV infection is usually detected either via isolation on the tracheal organ culture or application of PCR (Bagust, 1986).

It has been found that ILTV may withstand in biofilms of drinking water systems and spread to susceptible birds (Ou et al., 2011). The mixing of vaccinated and non-vaccinated chickens is also important for the direct transmission. Airflow between flocks also helps in spreading ILTV (Ou and Giambrone, 2012). No vertical transmission has been reported (Wolfrum, 2020).

Backyard avian species act as a vital source of ILTV infection for commercial poultry flocks due to viral latency (Ojkic et al., 2006; Neff et al., 2008). Darkling beetles and mealworms are mechanical carriers and living ILTV has been found in them for 42 days following a disease outbreak (Ou and Giambrone, 2012). Direct and indirect contact with respiratory exudates in contaminated litter, equipment, vehicles, feed bags, feathers, dust, footwear, and clothes as well as movement of people are also other routes of ILTV infection (Kingsbury and Jungherr, 1958; Zellen et al., 1984). Dogs and cats fed on infected dead carcasses also help in the spread of the virus (Kingsbury and Jungherr, 1958).

Once ILTV enters the host via its natural portals, it rapidly replicates in the epithelium of the eye, sinuses, and larynx until it reaches the maximum virus titre on days 4 to 6 post-infection and then remains in tracheal secretions between days 6 to 10 post-infection (Hitchner et al., 1977; Robertson and Egerton, 1981; Bagust, 1986; Guy and Bagust, 2003). The virus could be detected in the trigeminal ganglion from two of cytolytic infections onwards (Bagust, 1986; Oldoni et al., 2009) causing severe damage and haemorrhages in the epithelial lining of the respiratory organs (Guy and Bagust, 2003). The replication of ILTV leads to the up-regulation of some genes which are responsible for cell growth and proliferation. With the help of up-regulated cellular proteases, the virus attaches the underlying tracheal lamina propria (Reddy et al., 2014) and then systematically disseminates to the liver, caecal tonsils, and cloaca (Oldoni et al., 2009; Coppo et al., 2013). The production of some cytokines and inflammatory mediators by the infected cells results in intensive oedema with lymphocyte infiltration (Guy and Garcia, 2008; Devlin et al., 2010). The latency of ILTV in the trigeminal ganglion depends on the induction of an effective adaptive immunity (Williams et al., 1992), while the viral reactivation is mediated by thymidine kinase and polypeptide 4 production (Schnitzlein et al., 1995; Han et al., 2002).

**Incubation period**

Under natural infection conditions, the incubation period of ILTV varies within 6–13 days (Seddon and Hart, 1935), while the intra-tracheal inoculation of the virus results in induction of signs within 4 days (Davison et al., 1989).

**Clinical picture**

The severity of the clinical signs of ILTV infection varies according to the virulence of the virus, presence of other infections, stress conditions, and the age and immune status of infected birds (Kirkpatrick et al., 2006; Gowthaman et al., 2016). Sporadic cases of ILTV infections in vaccinated flocks have been reported due to vaccination failure or improper application of biosecurity measures (Hidalgo, 2003). Following the acute infection, the virus may remain latent in the trigeminal ganglion of the central nervous system (Hughes et al., 1991; Williams et al., 1992). However, stressors such as laying, shifting, and mixing of flocks can reactivate the virus and stimulate its replication in the tracheal epithelium (Hughes et al., 1989) with a subsequent shedding and transmission to susceptible birds. The clinical course of ILTV infection ranges from 11 days to 6 weeks depending on the clinical form of the disease.

The morbidity and mortality rates of ILT are variable and depend on the virulence and load of the circulating field virus (Devlin et al., 2006; Oldoni et al., 2009), age of the flock, period of the production cycle, vaccination history, as well as concomitant other respiratory infections (Guy and Garcia, 2008) such as mycoplasmosis, colibacillosis, infectious coryza, salmonellosis, Newcastle disease, fowl pox, and other immunosuppressive diseases such as mycotoxicosis, Marek's disease, chicken infectious anaemia, and reticuloendotheliosis (Mohamed et al., 1969; Couto et al., 2016; Abdo et al., 2017; Beltran et al., 2017; Razmyar et al., 2021; Zorman Rojs et al., 2021; Pajić et al., 2022).

***Acute epizootic form***

It is characterized by a sudden onset, a rapid spread, and a high mortality rate (OIE, 2014). Sudden death has been reported in chickens with a good body condition before the appearance of any clinical disease (Preis et al., 2013). Death usually occurs within 3 days of ILTV infection (Cover, 1996).

Affected chickens with acute ILT show anorexia and severe respiratory distress in the form of dyspnoea, gasping, or rattling (Guy and Bagust, 2003). The most characteristic signs are coughing and expectoration of blood mixed with mucus due to tracheal obstruction with clotted blood and exudates. Therefore, affected chickens show long drawn-out gasps with open-mouthed breathing, high-pitched squawk, extended head and neck, and moist rales (Kernohan, 1931; Jordan, 1958). The clotted blood is found on walls of a farm as well as in cages, feed turfs, and on the floor of poultry houses. Swollen eye lids with oedema, lacrimation, and eye congestion are also common in ILTV infection. Laying chicken flocks may show a drop in egg production or even experience complete cessation of egg production, which may recover to the

normal level (Lohr, 1977). This form of the disease is characterized by a high morbidity rate (90–100%) and a sudden increase in the average daily mortality rate (5% to 70%) with an average of 10–30% for up to 15 days (Seddon and Hart, 1935; Aziz, 2010).

***Mild enzootic or chronic form***

The silence of ILT is a synonym for a milder form of the disease (Sellers et al., 2004; Garcia et al., 2013). Mild or chronic ILT is similar to other respiratory infections. It is characterized by general unthriftiness, rales, coughing, head shaking, conjunctivitis, sinusitis, drop in egg production up to 10%, and decreasing body weight gain (Hinshaw et al., 1931; Ou et al., 2012). The morbidity rate may go up to 5% and the mortality rate usually ranges between 0.1 and 2% (Bagust et al., 2000; Ou and Giambrone, 2012).

**Post-mortem lesions**

The most characteristic gross lesion of acute ILT is haemorrhagic tracheitis (Barhoom and Dalab, 2012). The tracheal mucosa and larynx could be congested or cyanotic (Zhao et al., 2013). A yellow cheesy plug of caseous material could also be observed on the larynx, syrinx, and tracheal mucosa (Gowthaman et al., 2014; OIE, 2014). Mucoid tracheitis with or without diphtheritic exudates may be noticed in the tracheal lumen of chronic or mild ILTV infected chickens (Abdo et al., 2017). A pseudo-membrane of fibrino-necrotic exudates can be observed in the upper respiratory tract (Yavuz et al., 2018). Conjunctivitis with almond-shaped eyes (Kirkpatrick et al., 2006; Ou and Giambrone, 2012) and sinusitis (Parra et al., 2016) may also be observed. The lungs and air sac lesions in ILTV infected cases are rare. Nevertheless, lung congestion and caseous air sacculitis have been noticed (Aziz, 2010). Concomitant infections of ILTV with other respiratory pathogens may result in mucoid rhinitis and sinusitis, facial swelling, and muco-fibrinous tracheitis of infected chickens (Couto et al., 2015). Rare cases have shown severe erosive esophagitis and pharyngitis as an atypical ILTV infection in backyard chickens (Sary et al., 2017).

**Histopathologic examination**

Microscopic lesions of ILTV infection in the trachea have been described as infiltration of epithelia mucosa with lymphocytes, histiocytes, and plasma cells, enlargement of cells, as well as loss of goblet cells and cilia, followed by cell destruction, necrosis, and desquamation (Timurkaan et al., 2003). Haemorrhages may be seen in the necrotic epithelium due to rupture of blood capillaries (Sary et al., 2017). Intranuclear eosinophilic inclusion bodies could be observed in epithelial cells on days 1–5 post-infection and then disappear later due to the denudation of epithelial cells (Guy et al., 1992; VanderKop, 1993; Srinivasan et al., 2012). Inclusions are clusters of viral particles, proteins, and genomes (Preis et al., 2013). Six days post-infection, regeneration with proliferation of the remaining lining epithelium could be detected in surviving chickens during the acute phase (Bagust et al., 2000). The histopathological findings in the bronchi are characterized by epithelium necrosis and degeneration as well as infiltration with mononuclear cells (Preis et al., 2013).

The conjunctiva epithelium of ILTV infected chickens could show swelling, hyperaemia, and infiltration with inflammatory cells, and could be followed by epithelial damage, sloughing, and accumulation of inflammatory exudates containing inflammatory cells and fibrinocellular debris (Aziz, 2010).

It is important to note that the histopathological findings can not differentiate between the lesions caused by ILTV field strains and those caused by the virus vaccine strains which reverted to their virulence.

**Diagnosis**

The characteristic coughing of bloody mucus, open mouth, gasping, dyspnoea, extended head respiration, conjunctivitis, haemorrhagic tracheitis, and fibrinopurulent membrane in the larynx and trachea are very suggestive for ILT. The confirmation of infection is done by conventional isolation, detection of the virus in the affected tissues, and adoption of recent molecular techniques (Humberd et al., 2002).

***Conventional isolation and detection***

Tracheal scraping or exudate is the best sample for ILTV isolation (Tripathy & Garcia, 1998). Both cell lines and egg inoculation are used for the primary isolation of ILTV; however, the cell culture cultivation is more rapid and economic than the egg inoculation method (Meulemans and Halen, 1978; Garcia and Riblet, 2001). There are different types of cell cultures that could be used for the primary isolation of ILTV such as chicken embryo liver, chicken embryo lung, and chicken kidney cell cultures (Schnitzlein et al., 1995). Other types of tissue culture including chicken embryo fibroblast, Vero cells, avian leukocyte cultures derived from a chickens' buffy coat (Chang et al., 1977), QT35 or IQ1A quail cells (Garcia et al., 2013), and Leghorn male hepatoma (Schnitzlein et al., 1995) have also been used for isolation of the virus. Both tracheal organ culture and conjunctival organ cultures have been used to study the host-pathogen interaction (Jones and Hennion, 2008). The propagation of ILTV on the tissue culture could be observed as swelling of cells, rounding of the nucleoli, and formation of syncytia. Moreover, the intranuclear inclusion bodies or syncytial cell formation could be detected following cell line inoculation (Hinshaw et al., 1931). The demonstration of specific inclusion bodies in the affected tissues has been shown to be significantly less sensitive than the virus isolation.

ILTV could also be isolated on the chorio-allantoic

membrane of embryonated chicken eggs. Two days post-inoculation, opaque plaques resulting from necrosis and proliferative tissue reactions as well as embryo's deaths could be observed.

The immunofluorescence, immuno-peroxidase, and immunohistochemistry-labeled monoclonal antibodies could be used for the detection of ILTV antigen in the affected tissues or in the trachea or conjunctiva stained smears using immunoprobes (Hitchner et al., 1977; Ide, 1978; Goodwin et al., 1991; Guy et al., 1992; Yavuz et al., 2018; Carnaccini et al., 2022). The immunohistochemistry could be useful in the detection of ILTV infection when classical histologic lesions are absent or inconclusive (Carnaccini et al., 2022). The direct electron microscopic examination has been used for the rapid detection of ILTV from the tracheal scrapings (Hughes and Jones, 1988). Monoclonal antibodies have also been applied to detect a high concentration of the virus in the tracheal scraping using enzyme-linked immunosorbent assay (ELISA) (York and Fahey, 1990).

### *Serological identification*

The agar gel immunodiffusion, virus neutralization, indirect fluorescent, and ELISA tests are used for the demonstration of specific antibodies against ILTV with variable sensitivities (Adair et al., 1985; Bauer et al., 1999).

### *Molecular detection*

The dot-blot hybridization assay and cloned DNA fragments labelled with digoxigenin are rapid techniques that could detect the ILTV DNA in acute and chronic latent infections (Keam et al., 1991; Key et al., 1994). Molecular techniques used for the detection of ILTV DNA are regarded as quick, accurate, and highly sensitive ways for the virus identification. They include dot-blot hybridization, PCR, nested PCR, real-time PCR, multiplex PCR, in situ hybridization (Nagy, 1992; Abbas et al., 1996; Clavijo & Nagy, 1997; Nielsen et al., 1998; Pang et al., 2002; Creelan et al., 2006; Ou et al., 2012), and PCR followed by restriction fragment length polymorphism (Chang et al., 1997; Kirkpatrick et al., 2006; Oldoni and Garcia, 2007; Oldoni et al., 2008). The sequencing analysis (alignment) and the phylogenetic tree of gpB, gpC, and gpG genes of the Egyptian ILTV isolates have revealed that they have a genetic stability and a high degree of identity with the wild-type viruses (Maha et al., 2020). The use of PCR method is recommended for the definitive diagnosis of ILTV infection, particularly in the absence of typical patho-morphological lesions (Shirley et al., 1990; Williams et al., 1994). The PCR is more sensitive than the virus isolation, and the real-time PCR method has been successfully used for the final diagnosis of the disease (Oldoni et al., 2008; Preis et al., 2013). Recently, a loop-mediated isothermal amplification has also been considered as a highly specific and sensitive method for the detection of ILTV DNA. This assay is suitable for the basic diagnostic laboratory detection in the field, while real-time PCR is used for further verification.

## Differential diagnosis

ILT should be differentiated from other similar viral respiratory infections such as Newcastle disease, avian influenza, infectious bronchitis, adenovirus, and fowl pox using molecular techniques (Davidson et al., 2015). Some tests are used for differential diagnosis such as the agar gel immune diffusion technique using an ILTV hyper-immune serum.

## The immune response

Following ILTV infection, different several types of immune responses could be evolved (Jordan, 1981). The neutralizing antibodies could be detected within 5–7 days post-infection, reach the peak at 21 days, and then decline to low levels over a year (Hitchner et al., 1958; York et al., 1989). In vaccinated chickens, a substantial increase in the number of immunoglobulin (Ig) A and Ig G-synthesizing cells could be detected in the trachea on day 3 post-vaccination with a significant increase in IgA-cells on day 7 (York et al., 1989). The secretory mucosal IgA protects the respiratory tract epithelium and elicits a local immune response.

The cell-mediated immune response to ILTV infection has also been demonstrated. Bursectomised chickens without specific antibodies were protected from ILTV challenge post-vaccination (Fahey et al., 1983) through the transfer of histocompatible immune lymphoid cell cells (Fahey et al., 1984). It has been reported that antibody titers against ILTV could not correlate with the resistance to the infection (Shibley et al., 1962; Jordan, 1981). Moreover, the principal mediator of ILVT resistance is the cell-mediated immunity in the trachea (Fahey and York, 1990).

The maternal derived antibodies against ILTV could not protect chicks against infection or even interfere with the vaccination (Fahey et al., 1983). Natural infection of chickens older than 2 weeks or vaccination may provide birds with a full protection against ILTV challenge (Hitchner, 1975).

## Intervention strategies for prevention and control

ILT is an important viral respiratory disease which has been included within the list E of OIE. Once ILTV infection is confirmed in a certain area, strict measures should be adopted to prevent the spread of the virus in the infected and endangered areas. For the eradication of ILT, the implementation of a strict control programme and the cooperative effort of government agencies, laboratories, poultry producers and companies, and veterinarians are the must (Dufour-Zavala, 2008). The control measures

are mainly based on the definitive diagnosis, adoption of strict biosecurity measures, and vaccination (Mallinson et al., 1981; Guy and Garcia, 2008). The application of good biosecurity measures on farms can prevent ILT. A geographic information system can provide information about biosecurity plans, quarantines, vaccinations, and ILTV outbreak sites (Dufour-Zavala, 2008). Also, a vaccination strategy is essential to prevent the spread of ILTV infection. An appropriate regulatory agency should be contacted to determine the approved vaccines and the vaccines application procedures.

***Biosecurity***

The eradication of ILTV from intensive poultry production areas appears to be an effective process due to several factors including host-specificity, fragility, and antigenic stability of the virus. The high levels of strict biosecurity measures including quarantine, restriction of workers, equipment, feed, vehicles, and bird movement, litter decontamination, thorough cleaning and disinfestation, and extension of downtime between subsequent batches should be properly adopted. Moreover, the entrance of free-living backyard and fancy birds, pet animals, and rodents to the flocks should be prohibited (Mallinson et al., 1981; Volkova et al., 2012). Further, dead carcasses should be hygienically disposed.

***Herbal treatment***

Some herbal treatments have shown efficacies against ILTV infections. For instance, a product containing Almond, Gypsum fibrosum, Herba ephedrae, Radix astragali, and Radix glycytthizae has shown an antioxidant activity and enhanced the mucosal immunity against ILTV infection through IgA production (Cheng et al., 2011). Besides, a concentration of Chinese herbal mixture has been reported to decrease the concentration of ILTV in the infected chickens' tissues with a development of mucosal immunity following 72 hours post-infection (Zhang et al., 2018).

**Vaccines**

ILT was the first viral disease of poultry in which the virulent virus vaccine was administrated via cloaca (Gibbs, 1934; Coppo et al., 2013). The ILTV strains are antigenically homogeneous, so a single vaccine can induce a cross-protective immune response to all ILTV strains.

***Live attenuated vaccines***

Since 1960s, live attenuated ILTV vaccines have been developed either from chicken embryo or tissue culture origins and they have been extensively used for controlling ILTV outbreaks all over the world (Samberg et al., 1971; García, 2017; Garcia and Zavala, 2019). These types of vaccines are used to prevent infections and also, during the outbreaks, to control the spread of the virus and shorten its duration (Lee et al., 2011; Vagnozzi et al., 2012). Moreover, they elicit the protective immune response by producing a mild tracheal infection without induction of a disease condition (Fulton et al., 2000). The tissue culture-derived vaccines are more attenuated and less immunogenic than the vaccines of chicken embryos origin or recombinant types (Andreasen et al., 1990; ElSaied et al., 2022). The tissue culture-derived vaccines are commonly used in layer and breeder chicken flocks. In the USA, the ILTV vaccines derived from chicken embryo have successfully prevented several outbreaks in broiler flocks (Vagnozzi et al., 2012).

However, ILTV in the live attenuated vaccine has the ability to spread from vaccinated to non-vaccinated chickens (Hilbink et al., 1987). The in-vivo passages of ILTV may result in a reversion to its virulence causing outbreaks of ILT (Guy et al., 1991; Dufour-Zavala, 2008; Blacker et al., 2011; Chacon et al., 2015) or a disease condition in non-vaccinated chickens due to insufficient attenuation of the virus (Perez-Contreras et al., 2021). Moreover, vaccination could induce infected chronic carriers which may be persisted as a source of ILTV infection to non-vaccinated neighbouring flocks (Bagust, 1986). Besides, the latent virus in the live vaccine may undergo reactivation, shedding, and spread to susceptible birds so that the occurrence of new outbreaks of ILT in several parts of the world may be due to the massive use of live attenuated vaccines. The direct contact between vaccinated or infected chickens and non-vaccinated chickens helps in the transmission of the virus infection (Ou and Giambrone, 2012). A prolonged ILTV infection has been reported following the extensive vaccination with a live attenuated vaccine of the chicken embryo origin (Garcia, 2016). Therefore, it is recommended to use these types of vaccines in geographic endemic areas only.

The ILTV vaccines are usually given to chickens at 6 to 8 weeks of age, followed by a booster dose in 12 to 15 week-old layers and breeders. It is recommended to use a live attenuated ILTV vaccine of chicken embryo origin at 35 weeks of age than using a tissue culture or a recombinant type to obtain better protection (Palomino-Tapia et al., 2019). The ILTV vaccination is not recommended for broilers due to economic concerns (Giambrone et al., 2008). The maximum protective level of immunity is obtained at 15 to 20 weeks post-vaccination and it may last over a year (Neff et al., 2008). There is no interference between the ILTV vaccine and the other vaccine when the interval between both is more than 2 weeks (Aston et al., 2019). The live attenuated vaccines can induce a good immune response when they have been given through the intranasal (Shibley et al., 1962) or eye drop (Sinkovic and Hunt, 1968) instillation and orally through drinking water (Samberg et al., 1971).

It is advisable to apply ILTV vaccines using the eye drop technique which is safer and gives more protection than the drinking water method (Raggi and Lee, 1965). However, the administration of ILTV vaccines through the drinking water method may result in non-homogeneity and failure of some chickens to develop protective immunity (Robertson and Egerton, 1981). In addition, the spray route of vaccination may show adverse reactions due to insufficient attenuation of the virus and the deep penetration of the small droplets size particles into the lower respiratory tract (Purcell and Surman, 1974), or using of excessive dose (Clarke et al., 1980).

***Recombinant vaccines***

Recently, recombinant vector vaccines do not transmit from chicken to chicken, decrease the severity of clinical signs, are safe and very stable, and do not revert to their virulence. However, they are not as effective as live attenuated ILTV vaccines in reducing the shedding of the virus (Garcia, 2017; Maekawa et al., 2019). Despite the safety of recombinant ILTV vaccines, they have shown a limited practical application due to their failure to stop the virus shedding as well as the neutralization of the virus vaccines by antibodies against the vector. The first application of a DNA recombinant ILTV vaccine was by Keeler et al. (1995) when intramuscularly vaccinated chickens with DNA encoding glycoprotein B showed a high level of protection in comparison with vaccinated chickens with live attenuated ILTV vaccines. In addition, the results of Gamal and Soliman (2023) have revealed that the developed ILTV DNA vaccine coding for the surface gpB could elicit potent antibody titers which are positively correlated with those of the live tissue culture-propagated vaccine as well as with an increase in the production of interferon-γ gene transcript compared with the live vaccine. Similar results have been obtained by Shahsavandi et al. (2021). Therefore, the ILTV DNA vaccines may exhibit several advantages including the long-persisted immunogenicity, induction of both humoral and cell-mediated immune responses, lack of the risks of infection or the vaccine virus replication, and absence of the possibility to revert to the virulent status with a consequence of later outbreaks (Guy et al., 1990, 1991; ElSaied et al., 2022).

In the areas with high incidences of virulent ILTV infections, it is recommended to administrate a recombinant vaccine in the hatchery accompanied by live attenuated vaccines during the production period to enhance the immune response (Maekawa et al., 2019). The immune protection can be obtained within a week following the administration of live attenuated vaccines, while this period is 4 weeks for recombinant vaccines. The role of the cell-mediated immunity against ILTV is more significant than the humoral immunity (Ou and Giambrone, 2012). The local immune response in the trachea is the principal mechanism in the defence against this infection (Garcia et al., 2013). The recombinant / subunit herpesvirus of turkey (HVT) (rHVT-LT) and the live attenuated chicken embryo origin vaccines against ILTV are commercially used (Vagnozzi et al., 2012; Maekawa et al., 2019). This type of ILTV vector vaccine has the ability to completely prevent the viral shedding after a challenge with the virulent virus strain (Catalina et al., 2021). Moreover, the produced recombinant vaccines using HVT or fowl pox virus (FPV) as a carrier for ILTV glycoproteins B and D could provoke protective immunity in vaccinated chickens (Garcia, 2017). The FPV vector vaccine carries gpB and UL32 g genes of Gallid herpesvirus 1 (FPV-LT) (Davison et al., 2006), but the HVT vector vaccine carries gpD and gpI of the virus coat that provides immunity against both Gallid herpesvirus 1 and Marek's disease (HVT-LT) (Bublot et al., 2006). A vaccination with a subunit ILTV vaccine made of a 205 kDa complex containing gpB has been reported to give a 100% protection against the development of the clinical disease and the virus replication (Chen et al., 2010, 2011)

However, the HVT vector vaccines could not reduce the virus shedding as much as the live vaccines (Johnson et al., 2010; Esaki et al., 2013). Therefore, priming vaccination with rHVT-LT followed by using a live attenuated vaccine (chicken origin) may reduce the circulation of the virus during a long term application (Maekawa et al., 2019). This combined vaccination strategy provides a safer alternative than the uninterrupted use of only living attenuated vaccines of chicken embryo origin. Both types of vector vaccines could be applied via inoculation of 18-day-old chicken embryos in-ovo or subcutaneous injection of one-day-old chicks. Moreover, the FPV vaccine can be applied though the intradermal wing web method (Menendez et al., 2014).

Many outbreaks of ILT have occurred in some regions, such as Egypt and Australia, due to the exchange of the genetic material resulting in recombination between the vaccine strains with high transmission rates. The virulent recombinant strains of Gallid herpesvirus 1 have been isolated in many Australian outbreaks (Lee et al., 2012). Some outbreaks have been reported in Egypt between 2007 and 2010, and the ILTV strains were related to the used strains of the live attenuated vaccines that revert to their virulence through bird-to-bird transmission (Shehata et al., 2013). Additionally, the gD, gG, gJ, and ICP4 gene have been characterized from 5 prototypes of ILTV strains in the Egyptian outbreaks between 2018 and 2019 (Bayoumi et al., 2020). According to phylogenetic analysis of ICP4 and gJ, these 5 strains were further genotyped into recombinant ILTV strains (3 prototype strains) and live attenuated vaccine-like ILTV strains (3 prototype strains) (Bayoumi et al., 2020; El-Saied et al., 2021). Interestingly, the outbreaks caused by

recombinant vaccine strains could be more severe than those produced by live attenuated vaccine-like strains. Besides, the pre-existence of antibodies against vectors could neutralize the virus vaccines, particularly in endemic areas (Tong et al., 2001).

## Conclusion

Despite the adoption of strict biosecurity measures and administration of different vaccines against ILT, the disease still causes serious economic losses especially in endemic areas. Therefore, the development of genetically modified/engineered protective vaccines against ILT is essential for the eradication programme and the avoidance of latent infected carrier chickens. The enhancement of DNA vaccine efficacy and the development of a practical cost-effective application of this technology will be required before its acceptance by the poultry industry.

## Conflict of Interests

The author declares that there is no conflict of interest.

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# Determination of *STAT5* and *GH* Genes Polymorphisms and Their Influence on Productivity Traits of Beef Cattle Reared in Lithuania

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**Abstract.** *The aim of this study was to investigate the prevalence of polymorphisms of STAT5 and GH genes and to determine their influence on the productivity traits in beef cattle. A total of 95 animals were genotyped, belonging to the breeds Angus, Limousin, Galloway and Simmental. Polymorphisms of STAT5 and GH locus were identified using a PCR-RFLP method. The evaluation of the STAT5 gene polymorphism (7 exon, 6853C> T) demonstrated that C allele (frequency 0.959) and CC genotype (frequency 0.918) were the most common in beef bull populations reared in Lithuania. This polymorphism had a statistically significant effect on the live weight index of animals. The examination of the GH gene polymorphism (5 exon, 2141C> G) revealed that the G gene allele (frequency 0.612) and heterozygous CG genotype (frequency 0.424) were the most common. This polymorphism had a statistically significant effect on daily bull weight and live weight. Bulls of the homozygous CC genotype exhibited better economic characteristics. In conclusion, our results demonstrated the potential of polymorphisms of GH and STAT5 genes as candidates for the investigation of quantitative traits in cattle.*

## Introduction

Carcass composition, meat quality growth and weight gain are multifactorial quantitative traits; they are influenced by both environment and genes. In particular, they are under the control of multiple genes (Keady et al., 2011; Ribeca et al., 2014; Selvaggi et al., 2015). Traditional trait improvement has centered on quantitative genetics, using statistical analysis of phenotypic data to determine animals with the highest genetic merit. This selection approach is most effectively implemented for highly heritable traits that are easily recorded before reproductive age. Genomic selection refers to the use of genome-wide genetic markers to predict the breeding value of selection candidates. This method relies on linkage disequilibrium between the markers and the polymorphisms that cause variation in important traits (Hayes et al., 2013; Odzemir et al., 2018). Molecular genetic markers in animal breeding programs could make selection precise and efficient.

Signal transducer and activator of transcription 5 (*STAT5*) is known as a main mediator of growth hormone (*GH*) action on target genes (Selvaggi et al., 2009; Dario et al., 2009). The *STAT5* transcription factors are members of the somatotropic axis. They initiate the growth process in the target cells, a process mediated by the pituitary growth hormone (Cosier et al., 2010). *STAT5* exists in two isoforms (*STAT5A* and *STAT5B*) that differ by a few amino acids in the carboxylic end of the protein molecule and are coded by two different genes (Kmiec et al., 2010; Cosier et al., 2012). These two forms of *STAT5* have been identified in sheep, mouse, human, rat and cattle cells. Owing to its mediator role in the effects of the prolactin and growth hormones, it is suggested that the *STAT5A* gene is a potential quantitative trait locus for the quantitative traits of livestock, such as meat yield and milk composition (Arslan et al., 2015).

Growth hormone (*GH*) gene acts and mediates the growth of bones and muscles. It is known that *GH* is the main regulator of postnatal somatic growth, stimulating anabolic processes and skeletal growth (Sodhi et al., 2007; Hadi et al., 2015; Omer et al., 2018). The *GH* gene is located on the 19th chromosome in the q26-qter band region. This gene is approximately 1.8 kb in size and contains 5 exons and 4 introns (Ozkan-Unal et al., 2015). The growth hormone (*GH*) gene is a candidate gene for predicting growth and meat quality traits in animal genetic improvement since it plays a fundamental role in growth regulation and development (Omer et al., 2018). The aim of this study was to investigate the prevalence of polymorphisms of *STAT5* and *GH* genes and to determine their influence on the productivity traits in beef cattle. The polymorphism of *STAT5* and *GH* genes in beef cattle raised in Lithuania has not been studied so far. The association of these genes with signs of cattle productivity has not been studied either.

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## Materials and Methods

*Samples and DNA extraction*

The study was carried out following the methodology of the Law on the Welfare of the Farm Animals of the Republic of Lithuania and complied with the Directive 2010/63/EU of the European Parliament and the Council on the Protection of Animals Used for Scientific Purposes.

Samples of cattle hair follicles were collected from 95 bulls consisting of Angus (41), Limousin (19), Galloway (19) and Simmental (16) cattle. The cattle were kept under the same rearing conditions at the bull fattening station. The data on daily weight gain records were obtained from Šilutė control bulls feeding station (Lithuania, Šilutė region, Armalėnai village). The hair samples and slaughter data were obtained from private slaughterhouses, where animals were slaughtered. Molecular genetic analysis was done at the Lithuanian University of Health Sciences, Dr. K. Janušauskas Laboratory of Animal Genetics. Bovine genomic DNA was extracted from hair follicles using Chelex DNA extraction method: 200 µL Chelex 100, 7.5 µL DTT (1M), and 10.7 µL Proteinase K (20 mg/mL). After extraction, the inactivation step was performed at 94°C, for 10 minutes. DNA samples were stored in the refrigerator at 4°C (Miceikienė et al., 2002).

*Restriction fragment length polymorphism – polymerase chain reaction (PCR-RFLP)*

The PCR was done in a reaction volume of 30 µL. The reaction consists of 2.5 µL of 10X Dream Taq Buffer, 1.5 µL each primer forward and reverse 1µM, 2.5 µL of dNTP Mix 0.2mM, 0.25 µL Dream Taq DNA Polymerase, 11.75 µL ddH2O and 10 µL genomic DNA. The reactions were done in a Thermal Cycler 2700. The primer sequences and thermal cycling programs for each SNP are represented in Table 1, respectively (Flisikowski et al., 2002; Silveira et al., 2008).

PCR product of *STAT5* gene was digested with *AvaI (Eco88I)* restriction nuclease and the *GH* gene amplified 404-bp-long DNA fragment was digested with *AluI* restriction endonuclease. Amplified DNA fragments were digested with restriction endonucleases at 37 °C for 1–16 h. The reaction volume was 20 µL consisting of 10 µL PCR product, 7.5 µL ddH2O, 2 µL 10X Buffer Tango and 0.5 µL restriction enzyme 10 U/µL. The PCR-RFLP product of each sample (8 µL) and GeneRuler 50 bp DNA Ladder (0.1 µg/µL, Thermo Fisher Scientific, Waltham, USA) were loaded in 3% (w/v, for *STAT5* gene SNP) and in 2% (w/v, for *GH* gene SNP`s) agarose gels in tris-acetate-EDTA (TAE) buffer (50X TAE Electrophoresis Buffer, staining using 10 mg/mL ethidium bromide). The electrophoresis was carried out for 60 min at 100 V. The electrophoresis gel was examined on an UV transilluminator MiniBIS Pro (Bio-Imaging Systems, Israel) and bands were visualized and photographed. Polymorphisms of *STAT5* and *GH* genes were identified based on the length of the band. The following DNA restriction fragments were obtained for locus *STAT5* (Exon 7, 6853C>T): 181 and 34 bp for the CC genotype; 215, 181, and 34 bp for the CT and 215 bp for the TT genotype. After restriction, fragments for locus GH (Exon 5, 2141C>G) were obtained: 185, 131,51 and 37 for the CC genotype; 236, 185, 131, 51 and 37 bp for the CG genotype; and 236, 131, and 37 for the GG genotype.

*Statistical analysis*

Statistical analysis was done using IBM SPSS Statistics software package and Microsoft Excel spreadsheets. The influence of genes on each indicator (bull weight and carcass weight) was calculated by using one-way ANOVA, and the influence of polymorphisms on economic traits was evaluated by calculating average means and standard errors of productivity traits. Differences between genotypes was evaluated by the *Fisher least significant difference* (LSD) test.

*Table 1.* Primer sequences and size of the amplified fragments and reaction conditions, PCR programs for each SNP (single nucleotide polymorphism)

| Genes | SNP | Sequence | PCR profile | | | PCR product size | References |
|---|---|---|---|---|---|---|---|
| *STAT5* | Exon 7, 6853C>T | F: 5`– CTG CAG GGC TGT TCT GAG AG – 3`<br>R: 5`– GGT ACC AGG ACT GTA GCA CAT – 3` | 95 °C | 2 min | | 215 | Flisikowski et al., 2002 |
| | | | 94 °C | 30 s | 35 cycles | | |
| | | | 60 °C | 60 s | | | |
| | | | 72 °C | 60 s | | | |
| | | | 72 °C | 10 min | | | |
| *GH* | Exon 5, 2141C>G | F: 5`– TAG GGG AGG GTG GAA AAT GGA– 3`<br>R: 5`– GAC ACC TAC TCA GAC AAT GCG – 3` | 94 °C | 2 min | | 404 | Silveira et al., 2008 |
| | | | 94 °C | 30 s | 40 cycles | | |
| | | | 59 °C | 80 s | | | |
| | | | 72 °C | 90 s | | | |
| | | | 72 °C | 5 min | | | |

## Results

*Polymorphism of STAT5 gene, exon 7, 6853C>T*

Both C and T alleles of the *STAT5* gene were detected in the Lithuanian beef cattle population. Frequency of C allele was found to be the highest and that of T allele – the lowest. The C and T allele frequencies were 0.961 and 0.039, respectively. The *STAT5* gene CC genotype was the most common in the studied population (92%) followed by the CT genotype (8%) while the TT genotype was not found in the analyzed population (Table 2).

The *STAT5* gene had a statistically significant effect on the live weight index of the animals. Significant differences between the CC and CT genotypes were found for live body weight at slaughter (slaughter age of cattle from 12 to 24 months) ($P < 0.05$). The animals carrying the CT genotype were 86.5 kg (632.9 ± 23.01 kg vs 546.4 ± 10.69 kg) heavier than CC homozygotes, and the difference was significant. The mean value, standard errors and influence of *STAT5* gene polymorphism on five productivity traits are shown in Table 3.

*Polymorphism of GH gene, exon 5, 2141C>G*

The C allele of the *GH* gene was found most frequently compared with the G allele. The CG genotype of the *GH* gene was the most frequent in the studied population (41%) followed by the CC genotype (36%) while the GG genotype demonstrated the lowest frequency (23%) (Table 4).

Significant differences between genotypes were found for live body weight at slaughter, hot carcass weight, carcass weight ($P < 0.01$), weight gain and average daily gain ($P < 0.05$). The live weight at slaughter was the highest for the CC homozygotes which benefited by gained 16.7 kg more than CG heterozygotes and 95.5 kg more than GG homozygotes (CC: 577.4 ± 13.29 kg, CG: 560.7 ± 15.36 kg, GG: 481.9 ± 27.83 kg). Moreover, the CC genotype was associated with a higher hot carcass weight (+16.3 kg compared with the CG genotype and +68.6 kg compared with GG genotype), and carcass weight (+15.5 kg compared with CG genotype and +66.5 kg compared with GG genotype). Average daily gain also was higher in CC genotype compared with those of GG genotype. In general, the homozygous CC genotype appeared superior in all the traits measured. The mean value and standard errors for the five productivity traits are shown in Table 5.

## Discussion

The polymorphism of *STAT5* gene was not reported previously for Lithuanian beef cattle and the frequencies of alleles obtained in this study were like those reported in other cattle breeds. Flisikowski and Zwierzchowski (2002) studied polymorphism in the bovine *STAT5* gene (6853C>T) and its association with meat production traits in beef cattle. The overall frequencies of alleles C and T were 0.82 and 0.18, respectively.Frequencies of C and T alleles obtained by Selvaggi (2009) were 0.83 and 0.17, respectively. In our study, frequencies of the *STAT5* gene alleles were quite similar (C – 0.961, T – 0.039). However, Selvaggi et al. (2015) found that the T allele was more common than the C allele in native Podolica

*Table 2.* Genotypes and allele frequencies of STAT5 gene polymorphism (6853C>T)

| Breeds | N | nCC | nCT | Alleles frequency | | Genotype frequency | |
|---|---|---|---|---|---|---|---|
| | | | | C | T | CC | CT |
| Angus | 41 | 35 | 6 | 0.927 | 0.073 | 0.854 | 0.146 |
| Limousin | 19 | 19 | – | 1 | 0 | 1 | 0 |
| Galloway | 19 | 19 | – | 1 | 0 | 1 | 0 |
| Simmental | 16 | 14 | 2 | 0.917 | 0.083 | 0.833 | 0.167 |
| Total and average | 95 | 87 | 8 | 0.961 | 0.039 | 0.921 | 0.078 |

*Table 3.* Effect of *STAT5* polymorphism (6853C>T) on productivity traits

| Trait | Influence of polymorphism | Genotype means ± standard errors | |
|---|---|---|---|
| | | CC | CT |
| Number of cattle | | 85 | 10 |
| Weight gain (kg) | 2.4% | 103.8 ± 2.83 | 118.0 ± 11.71 |
| Live weight (kg) | 6.4%* | 546.4 ± 10.69a | 632.9 ± 23.01b |
| Hot carcass weight (kg) | 3.9% | 309.7 ± 7.35 | 355.8 ± 14.81 |
| Carcass weight (kg) | 3.8% | 303.2 ± 7.19 | 347.4 ± 14.83 |
| Average daily gain (kg) | 4.5% | 0.96 ± 0.02 | 1.10 ± 0.04 |

a, b* – values with different superscript letters show statistically significant differences ($P < 0.05$) between different genotypes in the trait

*Table 4.* Genotypes and allele frequencies of *GH* gene polymorphism (2141C>G)

| Breeds | N | $n_{CC}$ | $n_{GG}$ | $n_{CG}$ | Allele frequency | | Genotype frequency | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | C | G | CC | GG | CG |
| Angus | 41 | 17 | 3 | 21 | 0.671 | 0.329 | 0.415 | 0.073 | 0.512 |
| Limousin | 19 | 13 | 1 | 5 | 0.816 | 0.184 | 0.684 | 0.053 | 0.263 |
| Galloway | 19 | 3 | 9 | 7 | 0.342 | 0.658 | 0.158 | 0.474 | 0.368 |
| Simmental | 16 | 3 | 5 | 8 | 0.417 | 0.583 | 0.167 | 0.333 | 0.500 |
| Total and average | 95 | 36 | 18 | 41 | 0.562 | 0.438 | 0.356 | 0.233 | 0.411 |

*Table 5.* Effect of *GH* polymorphism (2141C>G) on productivity traits

| Trait | Influence of polymorphism | Genotype means ± standard errors | | |
|---|---|---|---|---|
| | | CC | GG | CG |
| Number of cattle | | 36 | 18 | 41 |
| Weight gain (kg) | 7.6%* | 95.2 ± 4.69a | 70.0 ± 8.47b | 86.1 ± 5.69 |
| Live weight (kg) | 13.0%** | 577.4 ± 13.29a | 481.9 ± 27.83b | 560.7 ± 15.36c |
| Hot carcass weight (kg) | 14.2%** | 332.5 ± 9.27a | 263.9 ± 17.95b | 316.2 ± 10.31c |
| Carcass weight (kg) | 14.0%** | 325.1 ± 9.08a | 258.6 ± 17.62b | 309.6 ± 10.09c |
| Average daily gain (kg) | 7.6%* | 1.02 ± 0.03a | 0.88 ± 0.05b | 0.97 ± 0.03 |

a, b, c – values with different superscript letters show statistically significant differences ($P < 0.05$) between different genotypes in the trait.

cattle breed. The observed frequencies of C and T alleles were 0.344 and 0.656, respectively. Besides, it was found that the most frequent genotype in the Podolica breed was TT genotype (45.70%), followed by TC (39.79%) and CC (14.51%). Meanwhile, in our studies, the CC genotype was found to be the most common, and the TT genotype was not found at all. So, further studies of *STAT5* polymorphism are also needed in other cattle breeds to better clarify the role of this SNP prevalence and influence on production traits in cattle.

In our study, the influence of *STAT5* gene on the rate of live weight was found to be statistically significant. However, Flisikowski and Zwierzchowski (2002), by studying beef cattle breeds, found different associations of *STAT5* gene genotypes with productivity traits than in our study. Their study revealed a statistically significant association between *STAT5* gene polymorphism and beef production traits in cattle (Flisikowski and Zwierzchowski, 2002). They found that in the animals of the CC genotype the live body weight, weight gain and carcass weight were more favorable than in CT animals. Also, Oprzadek et al. (2005) found that the CC genotype was associated with a significantly faster growth rate from 8 to 15 months (1.04 kg daily vs 0.97 kg). However, the results of our research were opposite: we found that the CT heterozygotes were heavier than CC homozygotes. It is necessary to underline that the genotype frequencies observed in some breeds were obtained from a small sample of animals, so they cannot be considered representative for the beef breed. Only 10 animals tested had a CT genotype. Therefore, the present result can be interpreted only as an association between the marker and production trait at this time and in this population. To confirm these results, further investigations including bigger cattle populations of different beef breeds are necessary. Increasing the sample of animals could change the result; therefore, deeper investigation of this aspect may be an interesting perspective to remove all doubt.

Similar to our study according to the polymorphism of *GH* gene, Fedota et al. (2016) have also found the higher frequencies of genotype CG = 46.6% compared with CC = 8.6% and GG = 44.8% genotypes in Aberdeen-Angus cattle population. However, unlike in our study, they obtained a higher frequency of the G allele compared with the C allele. Meanwhile, the highest frequency of C allele was found in our study. Our results are consistent with those reported by Ruban et al. (2016) who studied the effects of polymorphism in GH gene (2141C>G) on growth traits in Angus cattle. They found that in the animals of the CC genotype the live weight at birth and live body weight (at slaughter) were more favorable than in CG and GG animals. Similar results were obtained in our study, CC genotype appeared superior in all traits measured. In fact, this observation is explained by the more intense secretion of the growth hormone in animals with CC genotype (Selvaggi et al., 2015). In general, due to the crucial role of GH in animal growth, the GH gene is thought to be a candidate marker for performance traits in livestock animals

such as cattle (Aytac et al., 2015). Fedota et al. (2017) have also found a statistically significant correlation between polymorphism and birth weight; cattle of the CC genotype weighed more than CG heterozygous and GG homozygous animals.

## Conclusions

In conclusion, our results demonstrate the potential of polymorphisms of *GH* and *STAT5* genes as candidates for the investigation of quantitative traits in cattle. These SNP can be used as reliable genetic markers for productivity traits in cattle breeding. Therefore, we will extend this study by increasing the number of animals analyzed and including more beef cattle breeds.

## Conflict of interest

There are no conflicts of interest involving the publication of this work, according to the authors.

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# Selection of Parameters in the Development of a Welfare Assessing System on Dairy Farms in Ukraine

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**Abstract.** *The livestock sector is of great importance in the agriculture of Ukraine, but it still does not have a system for assessing animal welfare. It is important for Ukraine to create such a system, considering legislative requirements, climatic and cultural factors, peculiarities of animal husbandry. Our goal was to select basic, practical and scientifically based parameters for the system of assessing the cow's welfare on dairy farms in Ukraine. After the first phase of our research, namely the analysis of modern global protocols, codes and evaluation systems, we determined 57 parameters. In turn, after the second phase, which consisted of expert discussions, 4 parameters were excluded due to their impracticality in Ukraine, difficulty of assessment, and assessment time. Also, as a result of expert discussions, 3 more parameters were added to the system. During the thirdphase, namely testing on two farms, we removed 8 more parameters. So, in the final phase of testing on the third farm, the expediency of using 48 parameters to assess the cows' welfare on dairy farms in Ukraine was verified and substantiated. It is appropriate to note the universality of this system in relation to different numbers of livestock and systems of keeping animals. We consider it necessary to conduct further testing of parameters on a larger number of dairy farms in Ukraine.*

## Introduction

Nowadays, the welfare of farm animals is an integral part of the sustainable development of animal husbandry. A high level of animal welfare is increasingly recognized as an important component of trade in animal products (Dunston-Clarke et al., 2020). Current research (Clark et al., 2017; Wolf et al., 2017; McKendree et al., 2014; Bejaei et al., 2011; Spooner et al., 2014; Estévez-Moreno et al., 2022; Miranda-de la Lama et al., 2017; Malek et al., 2017) proves the growing level of concern of consumers in different countries regarding the welfare of animals on farms. Modern society is increasingly interested in the conditions of keeping, feeding and treating animals whose products they consume. Therefore, worldwide social demand for quality animal products from farms with a high level of animal welfare has led to the development of various animal welfare schemes (Sapkota et al., 2020).

An important aspect of establishing welfare on farms is the development, implementation and timely updating of the legal framework for the welfare of farm animals. In this aspect, Ukraine has signed an agreement with the EU, which provides for the maximum approximation of the legislative norms of Ukraine to the EU legislation in the field of animal welfare (Petkun and Nedosekov, 2022). Despite this, there is no standardized animal welfare assessment scheme and regular assessment of dairy farms in Ukraine.

Most modern animal welfare assessment programs on dairy farms are based on the Five Freedoms model (Mellor, 2017). Grounded on this, building an evaluation system is based on direct parameters (based on animals) and indirect parameters (based on resources). Today, the vast majority of systems pay the greatest attention to direct parameters, because they reflect the direct impact of the environment on the animal and demonstrate its response to the provided resources. However, indirect parameters are also important as they are useful as predictors of potential welfare problems (Sapkota et al., 2020).

Based on the Five Freedoms model, Mellor (2017) identified four key building blocks of animal welfare management: feeding, environment, health and behaviour. Each of them needs a sufficient depth of assessment, if the welfare assessment system is aimed at a comprehensive study of both the physical and emotional states of the animal.

In turn, it is important that the assessment is appropriate for the management system in which the animal is kept (Winckler et al., 2003). Protocols suitable for one system may not be suitable for another. A similar rule can be applied to different countries, climatic zones, etc.

The purpose of our study was to select parameters for further development of a basic, practical, feasible and scientifically grounded system for assessing the cows' welfare on dairy farms in Ukraine.

## Materials and Methods

*Collection and analysis of potential parameters*

During the search and selection of parameters, the following world protocols and systems for assessing the welfare of cattle on dairy farms were taken as a basis: Welfare Quality (2009) Red Tractor, The Code of Welfare (2019) FARM (2022), Cow Comfort (Van Eerdenburg et al., 2013).

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All systems were analysed by the authors and discussed with experts in order to exclude the following: 1) parameters related exclusively to meat cattle breeding; 2) parameters related to the pasture; 3) parameters that required significantly more time than it was available to assess the farm in one day; 4) parameters that are not typical for farming in Ukraine.

The parameters that were suitable were used to create a system for assessing the dairy cows' welfare on farms in Ukraine and tested them for feasibility. The system was pre-tested for compliance with Ukrainian farm animal welfare legislation to confirm that no potentially important welfare areas covered by the legislation were missed.

*Feasibility testing of welfare assessment parameters*

The system was tested on three dairy farms of Ukraine in the period from July 2023 to October 2023. During the first stage, two farms were assessed, and after the analysis of the parameters, the final assessment took place on the third farm. All parameters were evaluated for practicality, time spent, ease of assessment, best place and period for assessment.

The first technical and economic testing was carried out in July 2023 on a farm with a tied method of keeping and a herd of 324 cows (290 milking cows). The second testing was carried out in August 2023 on a farm with a loose housed method of keeping and a herd of 1100 cows (681 milking cows).

Farms were selected specifically with different numbers of cows and husbandry methods to confirm or disprove the applicability of these welfare parameters to different dairy farms. Assessment based on farm records, as well as parameters that are difficult or impossible to investigate on the farm in a short time (mastitis records, dehorning methods, etc.), was carried out through a survey of the farmer or veterinarian (Appendix A).

All parameters (direct and indirect) were evaluated by one person at the time when the animals were in stalls or pens. When estimating animal-based parameters, we did not aim to survey the entire herd, so we applied a similar concept to the Welfare Quality protocol (Welfare Quality, 2009). In herds with up to 300 cows, 20% of animals were examined; in herds with 300–500 cows, 15% of animals were examined; and in herds with more than 500 cows, 10% of animals were examined. Cows for observation were chosen arbitrarily. Parameters such as the presence of aggressive and stereotyped behaviour and the manifestation of social contacts were studied in the entire herd during the assessment on the farm.

*Final phase*

The analysed and edited parameters of the system were tested again on a third farm with a loose housed method of keeping and a herd of 4350 cows (3800 milking cows) in September 2023. During this testing, all previous comments were taken into account and changes were made.

## Results

During the first phase of the research, after analysing world protocols, we determined 57 parameters for assessing the dairy cows' welfare on farms in Ukraine.

During the second phase (expert discussions), four parameters were deleted due to the following reasons: 1) their irrelevance in the conditions of Ukraine; 2) limited practical application; 3) spending time to conduct research on the farm (Table 1). However, three parameters were added during expert discussions (Table 2). So, after the second phase, we received 56 parameters, which were subsequently tested for the feasibility of their application on the farm.

During the first stage of third phase (2-farm testing), 8 parameters were determined to be unsuitable for a one-day, time-limited on-farm dairy cow welfare assessment with a single observer. The excluded parameters and the reasons for their exclusion are shown in Table 3. Thus, a total of 48

*Table 1* Parameters that were excluded from the evaluation system after expert discussions and rationale for their exclusion

| Rejected parameter | Reason for rejection | Rationale |
|---|---|---|
| Symptoms of heat stress | The parameter strongly depends on weather conditions. | It is impossible to evaluate this parameter objectively during one short-term visit to the farm. The assessment of this parameter was replaced by the measurement of the temperature in the barn at the moment and the presence of shelter from bad weather on the outdoor loafing area. |
| Quality behaviour assessment | Evaluation takes a lot of time. | Due to time and observer limitations, this parameter was replaced by an assessment of the presence of aggressive behaviour and stereotypical behaviour, social contacts and vocalizations. |
| Tail docking | Not typical for Ukraine | This practice is not typical for Ukraine and is carried out extremely rarely. |
| Record keeping and documentation on the farm | Difficulty of assessment | This parameter cannot be checked personally. |

parameters remained after testing the evaluation system on two different farms.

The third, final, on-farm testing confirmed that all 48 parameters were acceptable and practical. The final system with all parameters is given in Table 4.

## Discussion

The purpose of this study was to select basic, scientifically based, practical and feasible parameters for the welfare assessment system on dairy farms in Ukraine. The parameters met the requirements of limited time spent on the farm (2–3 hours depending on herd size), one person for observation and compliance with four functional domains: feeding, environment, health, and behaviour (Mellor, 2007).

### *Feeding*

Assessment of water quality and availability is an important aspect of any on-farm animal welfare assessment system. In our system, the number of water points, the serviceability and cleanliness of water points, their type and water purity were selected as measures. The latter corresponds to the Welfare Quality protocol (2009), in which water purity is an indicator of water quality.

The measurement of the water flow rate was excluded, because the evaluation of this parameter takes a lot of time. The parameter "Distance between drinking troughs" was rejected due to its feasibility for use in a pasture system of livestock keeping. For animals kept in pens, the parameters of the size of drinking bowls and the number of drinking bowls relative to the number of animals were selected.

Availability of fodder on the feed table is an important parameter for satisfying the basic food needs of cows. In turn, the presence of foreign impurities in the feed and insufficient cleanliness of the feeding table significantly reduces the quality of the feed itself. The parameter "Measurement of the length of the feed table per animal" was selected as a determination of feed availability for each animal. According to the Welfare Quality protocol (2009), the length should be 65 cm per 1 animal.

Covering the feed table is important, because the animal can be injured during feed consumption if the cover is rough enough.

### *Environment*

All parameters are indirect and demonstrate the existing conditions for the animal's life.

*Noise level.* Excessive noise negatively affects the physiological and behavioral aspects of an animal's life and its productivity. Cows do not like noise in their environment (Grandin, 1997).

Farms with a noise level of up to 70 dB are considered to be good farms in terms of noise level, and farms with a noise level of more than 70 dB are considered problematic (Dimov et al., 2023). So given that a quiet conversation = 60dB, using a noise trade-off based on how easily you can hear your interlocutor

*Table 2* Parameters that were added to the evaluation system after expert discussions

| Added parameter | Rationale |
|---|---|
| Mortality of cows per year | This parameter must be fixed on all farms. Its assessment is carried out through a survey of the farmer/veterinarian specifying the cause of death/culling. |
| Ingrown horns | These parameters are easy to estimate. They are direct indicators of management problems regarding timely and effective dehorning on the farm. |
| Eye damage | |

*Table 3* Parameters that were excluded from the evaluation system after the test evaluation of welfare on dairy farms

| Rejected parameter | Reason for rejection | Rationale |
|---|---|---|
| Width walkways | Difficulty of assessment | Due to the fact that different farms are built in different ways, this parameter loses its relevance and significance. |
| Nose discharge<br>Eye discharge<br>Vaginal discharge<br>Diarrhoea | Difficulty of assessment | In a short time, without additional research and the collection of a complete history, it is difficult to determine the pathology of these secretions. Therefore, the results of the assessment of this parameter cannot be relevant. |
| The occupancy of the stalls in the cowshed | Difficulty of assessment | Due to the different practices constantly carried out on farms, it is difficult to estimate this parameter in a single visit to the farm. |
| Slope of the floor in the stall | Difficulty of assessment | Due to the different designs of stalls and bedding, this parameter cannot be universal and its measurement is difficult. |
| Cough | Difficulty of assessment | In a short time, it is difficult to determine the pathology of the cough, its nature and intensity, without additional research and the collection of a complete history. |

*Table 4* Final set of parameters, taking into account test scores on three farms

| Welfare blocks | Parameters | Assessment type | Assessment method |
|---|---|---|---|
| Feeding | Availability of feed on the feed table | Resource-based | On the farm |
| | Feed table covering | Resource-based | On the farm |
| | The presence of foreign impurities in the feed | Resource-based | On the farm |
| | The length of the feed table for 1 animal | Resource-based | On the farm |
| | Cleanliness of the feed table | Resource-based | On the farm |
| | Number of drinking points | Resource-based | On the farm |
| | Cleanliness of drinking points | Resource-based | On the farm |
| | Water temperature | Resource-based | On the farm |
| | Water cleanliness | Resource-based | On the farm |
| | Functioning of water points | Resource-based | On the farm |
| Environmental | Technological grouping | Resource-based | Through the survey |
| | Lighting period | Resource-based | On the farm |
| | Lighting quality | Resource-based | On the farm |
| | Access to pastures | Resource-based | Through the survey |
| | Access to outdoor loafing area | Resource-based | Through the survey |
| | % of cows lying outside the "lying zone" | Animal-based | On the farm |
| | Noise level | Resource-based | On the farm |
| | Microclimate | Resource-based | On the farm |
| | Ventilation | Resource-based | On the farm |
| | Bedding (type, softness, dryness) | Resource-based | On the farm |
| | Stall (cleanliness, design) | Resource-based | On the farm |
| | Floor (cleanliness, slippery) | Resource-based | On the farm |
| | The condition of the outdoor loafing area | Resource-based | On the farm |
| | Presence of a motorized/conventional brush | Resource-based | Through the survey |
| | The presence of a maternity ward | Resource-based | Through the survey |
| Health | % of mastitis per herd per year | Record-based | Through the survey |
| | % of ketosis in the herd per year | Record-based | Through the survey |
| | Availability of necessary vaccination | Record-based | Through the survey |
| | Number of pathological births and % of birth assistance | Record-based | Through the survey |
| | Dehorning methods | Record-based | Through the survey |
| | Hoof cleaning | Record-based | Through the survey |
| | Lameness | Animal-based | On the farm |
| | BCS | Animal-based | On the farm |
| | Coat condition | Animal-based | On the farm |
| | Alopecia | Animal-based | On the farm |
| | Tails damage | Animal-based | On the farm |
| | Swelling | Animal-based | On the farm |
| | Wounds | Animal-based | On the farm |
| | Abscesses | Animal-based | On the farm |
| | Franks and hips cleanliness | Animal-based | On the farm |
| | Hind legs cleanliness | Animal-based | On the farm |
| | Udder cleanliness | Animal-based | On the farm |
| | Mortality per herd per year | Record-based | Through the survey |
| Behaviour | Human-animal relationships | Animal-based | On the farm |
| | Aggressive behaviour | Animal-based | On the farm |
| | Stereotyped behaviour | Animal-based | On the farm |
| | Social contacts | Animal-based | On the farm |
| | Vocalization | Animal-based | On the farm |

allowed us to determine the level of noise and various extraneous sounds on the farm and assess their risks to the welfare of the animals inside.

*Lighting*. The presence of high-quality lighting and a period of "darkness" is very important for the active life and rest of cows. Thus, 16 hours of light and 8 hours of darkness is ideal for animal health and welfare (Van Eerdenburg et al., 2013). We took this time ratio as the basis for our system.

*Lighting quality*. Light intensity should be at least 100 Lux (Chastain, 2000). Therefore, this parameter was measured by the ease of reading the printed text anywhere indoors (Van Eerdenburg et al., 2013).

*Temperature in stalls*. Heat stress can cause not only reduced performance, but also reproductive problems, affective states, aggressive behaviour and lameness (Polsky and von Keyserlingk, 2017). The most comfortable temperature for dairy cows is +5–

15 degrees of Celsius (Van Eerdenburg et al., 2013). Therefore, we used these temperature norms as a basis for measuring the parameter.

*Ventilation.* This parameter was measured by assessing the presence of cobwebs, condensation and fungus in the premises of the farm. High humidity causes difficulties in thermoregulation and increases the risk of spread of airborne infections (Kadreze et al., 2002).

*Bedding* provides a soft area for animals to lie down, which promotes rest, supports health and productivity (Carroll and Underwood, 2023). An ideal bedding should be dry and soft, provide thermal insulation, be easy to replace, and not be too abrasive (Anderson, 2016).

No system or code for evaluating the cows' welfare on dairy farms has specific requirements for the amount of bedding (Mc Pherson and Vasseur, 2020). In our evaluation system, we focused on such parameters as the softness and dryness of the bedding, because the main purpose is to provide a comfortable lying area for the animal.

*Stall.* Stall indicators such as cleanliness and design of the stall affect cows' welfare in stall housing systems through general indicators: lying time and comfort, the prevalence of injuries and damage to the animals' bodies, cleanliness of cows, and lameness. The cleanliness of the stall is inextricably linked to the cleanliness of the animal kept in it. This is especially noticeable in the case of tethered confinement, where the animal is unable to choose a place to lie down. In turn, longer postures reduce the risk of injuries and increase lying time (Bouffard et al., 2017; Mc Pherson and Vasseur, 2020). In order to evaluate the comfort of the stall and its conformity to the breed, we chose the parameter "Number of cows lying outside the lying area in the stall", the cleanliness of the stall and its design, namely the presence of alopecia in the withers area of the animal.

*Floor.* A floor that is too slippery or not abrasive enough increases the risk of injury. A moderate level of friction between the cow's hooves and the floor is essential for the cow's comfort and to prevent limb disease (Sharma et al., 2019). That is why floor assessment for slipperiness and cleanliness is relevant.

*Outdoor loafing areas opportunities for natural behaviour and social interactions* (Yemelianenko et al., 2022). In general, in Ukraine, the practice of organizing loafing areas is quite common, in contrast to the use of pastures. However, it is not so much the presence of a loafing area as its quality and provision that is important. Therefore, we investigated the availability of clean water, a feed table, a shelter from bad weather, and a dry elevation for rest.

### *Health*

*Lameness* interferes with the ability to express natural behaviour, change lying time, social interactions and feeding behaviour (Whay and Shearer, 2017).

To determine the level of lameness in the tied method of keeping, we studied the posture of the cow, the position of the body, how the animal carries the weight and to what position the animal returns. In order to determine the level of lameness in loose housed cows, we assess their gait and posture.

Body condition score (BCS) is an effective indicator of energy balance in the medium term (Roche et al., 2009). As a rule, extremely high or low BCS is associated with the deterioration of animal welfare, because BCS provides a general, but fairly accurate indicator of the cow's energy reserves.

The scale by which BCS is measured varies from country to country, but low values will always reflect emaciation, and high values equate to obesity. The optimal BCS for dairy cows is from 3.0 to 3.25 (on a 5-point scale). A lower BCS at calving can lead to reduced performance and reproductive performance, while a BCS greater than 3.5 is associated with reduced dry matter intake in early lactation and an increased risk of metabolic disorders. Also, weight loss or gain at calving can affect the risk of lameness (Lim et al., 2015). To determine the BCS level, we chose a scale from 1 to 5.

*Integument alterations.* Pathological changes occur as a result of contact of the animal's body with a hard floor, pressure on the stall or even blows (against elements of the stall or caused by a person). The main areas of the body affected are the hock joint, hips, neck, withers, and shoulder blade. Similarly, infections with ectoparasites lead to discomfort, itching, and pain (Winckler, 2008). During the assessment of this parameter, we paid attention to the condition of the cows' hair, alopecia, damage to the tails, the presence of swelling, wounds, and abscesses, the presence of which reflects the direct impact of the environment on the animal.

*Cleanliness.* Maintaining herd cleanliness is a key approach to ensuring good animal health, but this practice remains a challenge for intensive farms (Ramanoon et al., 2018). Dirt and faeces can compromise product quality and mammary health (Munoz et al., 2008; Lundmark Hedman et al., 2021). Wet and dirty coat loses part of its insulating ability. Urine and faeces are also known to cause burns and dermatitis (Authority EFS, 2009). Cows housed in dirty stalls are more likely to suffer from hock injuries, which can cause lameness (Kester et al., 2014).

We evaluated the parameter according to the "Cow cleanliness Assessment" by evaluating the cleanliness of the hind limbs, flanks and mammary gland using a scale from 1 to 4, where 1 is clean, and 4 is very dirty.

*Mastitis.* Despite the widespread implementation of mastitis control programs, it still remains the most common and one of the most damaging diseases in the dairy industry, with a wide range of impacts and consequences (Petersson-Wolfe et al., 2018).

During on-farm welfare assessment, it is difficult to

determine the herd's incidence of mastitis, especially with subclinical mastitis, so we chose the percentage of cows with mastitis per year as the measure of assessment. We get this parameter directly from the farmer/veterinarian during a short survey.

*Vaccination*. Timely vaccination helps to reduce the level of antibiotic use. Protecting animal health through vaccination improves animal welfare and, in turn, maintaining an appropriate level of welfare ensures the animal's successful response to vaccination. Poor welfare can lead to immunosuppression, which can affect the animal's response to vaccination (Morton, 2007). We assessed the availability of the necessary vaccination according to the recommendations.

*Dehorning* is a common practice in animal husbandry, including in Ukraine. The scientific community agrees that dehorning is a painful procedure regardless of the age of the animal and recommends the use of local anaesthetics in combination with analgesics as a means of pain relief (Marquette et al., 2023). Evaluating this parameter, we paid attention to the age of the animal when dehorning is performed, the use of anaesthetics before the procedure and analgesics after.

*Hoof cleaning*. Most cases of lameness are associated with pathological changes in the hooves (Moreira et al., 2019). Therefore, timely hoof cleaning is important for maintaining their welfare. Our assessment was based on a farmer/veterinarian survey regarding the frequency and regularity of hoof cleaning on the farm.

***Behaviour***

Assessment of animal behaviour usually involves observation of the animal in its habitat, an artificially created environment or a created situation, for example to assess the animal's reaction to a person. In terms of time, behavioural assessment can be short, if it is aimed at capturing a specific response, or long-term, if it is necessary to observe the natural behaviour of a specific animal or group of animals (Haskell and Langford, 2023).

Behavioural tests are useful because they can contribute to a better understanding of the motivational, cognitive, and emotional aspects underlying behaviour. In the evaluation system, we investigated the frequency of manifestations of complete behavioural parameters, their intensity and prevalence in the herd.

*Vocalization* is an important criterion for specific behaviour, as it is considered an "honest indicator" and a direct indicator of both positive and negative emotional states (Watts and Stookey, 2000). For example, separating a cow from a calf is a stressful stimulus for both animals, causing increased vocalization tones (Orihuela and Galina, 2019).

In our evaluation system, we paid attention to both high vocalization tones (indicators of stressful states) and low tones (indicators of positive emotional states). These parameters were recorded during the entire stay on the farm, and then their number, intensity, frequency and conditions under which vocalization was manifested were analysed.

*Human-animal relationship*. Fear of human is an important area of research for both economic and welfare issues. During the on-farm evaluation, we used an "avoidance distance" test. This test consists in the fact that the evaluator slowly moves towards the tested animal. The point at which the animal moves away, thereby avoiding contact, is the point of avoidance. This test is performed under experimental conditions to assess the quality of the relationship between animals and farm workers. The highest level of trust is the point when the animal is allowed to touch its nose (Andreasen et al., 2019).

*Aggressive behaviour*. Cattle can exhibit aggressive behaviour in the event of competition for resources such as feed, shade or comfortable places to lie down. An animal on which aggression is directed will experience fear, limited access to these resources and an increased risk of injury. As a rule, the frequency of manifestations of aggressive behaviour is greater in pen systems than in pastures.

*Stereotyped behaviour* has a fixed form and is performed repeatedly without any obvious purpose or function. Such behaviour is not as dangerous as conventionally aggressive behaviour, but its presence gives us clear signals of management problems and dissatisfaction of the animal's basic needs. In cattle, common stereotypes are tongue twisting, object licking, biting, manipulation of different body parts of same-sex individuals (Schneider et al., 2019).

*Social behaviour*. Cattle are highly social animals and have many social interactions within the herd. Also, they perceive isolation from other animals of their species very negatively. For example, allogrooming (licking) is a critically important component of forming and maintaining social relationships between animals. Social relations are an indicator of the presence of positive emotions and satisfaction of the animal's basic needs (Bouissou et al., 2001).

## Conclusions

The purpose of this research was the selection of basic and scientifically based parameters for the further development of the first Ukrainian system for assessing the cows' welfare on dairy farms. We believe that we succeeded in covering four important domains of welfare: feeding, environment, health, and behaviour.

So, the final system after 3 test evaluations had 48 parameters. Of them, 17 are direct, based on animals and, accordingly, 31 indirect, based on the resources provided to the animal. Most parameters, namely 36, are assessed directly on the farm, while the remaining 12 are assessed through a survey of the farmer/farm veterinarian.

These parameters are simple and logical in selection, easy to use, meet the requirements of a limited time spent on the farm, the number of observers conducting the assessment, and fully

meet the contemporary requirements of Ukrainian legislation in the field of veterinary medicine and animal welfare. Also, after testing these parameters, its versatility was noted for use on farms with different numbers of livestock and methods of keeping.

We consider it necessary and relevant to carry out further assessments of welfare on dairy farms of Ukraine using these parameters.

*Appendix A.* Questionnaire for owner farmers/veterinarian

| | |
|---|---|
| **1. General questions** | |
| Date | Name of the farm |
| Livestock | |
| Method of keeping | |
| Average yield of milk | |
| Breed | |
| **2. Environment** | |
| Availability of technological grouping | |
| Lighting period | |
| Availability of motorized/conventional brush on the farm | |
| Access to pasture/walking area | |
| The presence of a maternity ward | |
| **3. Health** | |
| % of mastitis in the herd per year | |
| % of ketosis in the herd per year | |
| Vaccination | |
| Number of pathological births and % of birth assistance | |
| Dehorning (animal age, use of anaesthesia) | |
| Hoof cleaning | |

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# Growth Evaluation of the Slovak National Chicken Breed Oravka Depending on the Melanocortin Receptor 4 Gene

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***Keywords:*** *MC4R gene, Oravka breed, live weight, weight gain, genotype.*

***Abstract.*** *We designed and tested the polymorphic gene MC4R in relation to live weight and weight gain of Slovak native chicken breed Oravka kept at the farm of NAFC-RIAP Nitra, which was selected for future breeding by phenotype. We monitored the weight of chickens at the age of 5, 12 and 20 weeks and calculated the weight gain for each period. The design of detection oligonucleotide primers for MC4R gene detection in DNA samples was based on the sequence available in GenBank, NCBI. Statistical analysis was done using the SAS 9.2 Stat software. We identified the GG genotype in only one hen. The number of GT heterozygotes was significantly higher in both hens (110) and roosters (16) after a long-time directive selection. The TT genotype was found in 9 hens and 5 roosters. The GG genotype was detected with a frequency of 0.71%, the GT genotype with a frequency of 89.36%, and the TT genotype with a frequency of 9.93%. The frequency of allele G was 0.454 ± 0.030; and that of allele T was 0.546 ± 0.030. At the age 12 weeks, homozygous hens were heavier (1111.33 ± 44.96 g) than heterozygous hens (1108.15 ± 13.17 g). Similarly, rooster homozygotes were on average heavier (1581.47 ± 59.11 g) than heterozygotes (1389.69 ± 34.40 g). WG 1 was significantly higher in homozygote animals TT ($P < 0.01$). The same pattern was also observed at the age of 20 weeks. Homozygous TT animals achieved a higher average weight, i.e., the weight of hens was 1947.25 ± 74.35 g, the weight of roosters was 2719.91 ± 87.75 g; meanwhile, for heterozygous animals, the weight of hens was 1888.78 ± 21.78 g, and the weight of roosters was 2525.99 ± 56.89 g. We concluded that in the selected Oravka "mutant" allele T is preferred.*

## Introduction

Current trends in food production are increasingly shifting from a quantitative to a qualitative position, i.e., towards the production of quality and safe food. This trend is mainly related to new knowledge about the rational nutrition of the population and, thus, also to the increasing interest of the population in these foods. The basic scientific approach to the production of food of animal origin is the precise characterisation and evaluation of the genetic potential of farm animals. In addition to monitoring reproduction, performance indicators and product quality, modern molecular genetics and immunological methods are used for comprehensive evaluation. These methods allow the identification of prognostically relevant biomarkers and gene expression profiles that will help to better understand the complex molecular mechanisms of cell physiology and pathology with their subsequent application in selection for higher production efficiency (higher growth rate, carcass yield, reproduction) and product quality.

A strategically important aspect in the field of animal production research is the potential to use original local breeds and farm animal lines. In Slovakia, these are currently maintained mainly by breeders organised by the Slovak Breeders' Association and specialised breeders' clubs acting under the umbrella of this organisation. Long-term one-way selection focused on the exterior characteristics of these breeds gradually leads to a significant reduction in their reproductive (low number of litters, poor viability) but also production characteristics, which belong to the typical characteristics of their breeding standards. The continuation of such an organisation of their breeding threatens the gradual extinction of already endangered populations of these breeds. The knowledge of genetic polymorphism influencing the production traits significantly contributes to streamlining the sustainability process of national breeds and lines. The Slovak native poultry breed Oravka belongs to the animal genetic resources rearing at the National Agricultural and Food Centre in Nitra (Slovak Republic). Detailed knowledge of the molecular polymorphism of the melanocortin receptor 4 (*MC4R)* gene, which significantly affects the production (growth and slaughter) properties of poultry, will make it possible to refine and objectify the breeding standard and streamline the sustainability process of the breed.

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Body weight is one of the most important economic traits in the poultry industry. It is well known that the birds' feed intake regulation and the central melanocortin system are influenced by *MC4R* gene. In addition, the regulation of melanocytic pigmentation and energy homeostasis is achieved by melanocortins that are peptide hormones derived from proopiomelanocortin (Boswell and Takeuchi, 2005). The current bibliography in animal models underlines the essential role of *MC3R* and *MC4R* in the regulation of feeding and energy homeostasis (Schwartz et al., 2000). A significant correlation between the *MC4R* genotype and body weight has been identified (Li and Li, 2006; Qiu et al., 2006; Wang et al., 2009). In humans and mice, *MC4R* has been recognised as a major regulator of food intake, body weight, and glucose homeostasis (Wardlaw, 2001; Cone, 2006). A significant association between MC4R SNPs and growth traits has been found in several farm animals, including chickens (Kim et al., 2000; Qiu et al., 2006; Wang et al., 2009; Zhang et al., 2009). In mammals, *MC4R* is associated with appetite, body weight, and energy metabolism (Andersson, 2003), and it can also mediate the function of leptin (Hoggard et al., 2004). Changes in protein structure caused by different *MC4R* genotypes are expected to affect the activity or function of proteins related to body weight in chickens (Li and Li, 2006).

The melanocortin receptor plays an important role in the central melanocortin system (CMS) and muscle cells. It is a member of the so-called G-protein receptor family, which is expressed in the hypothalamus and is involved in the control of energy homeostasis and food intake with a major impact on body weight and fat storage. The chicken *MC4R* gene is located on chromosome 2. In humans, the occurrence of heterozygous mutations in *MC4R* is associated with dominant inherited obesity and is the most common monogenic cause of human obesity. *MC4R* has been found to be involved in food intake, metabolic regulation, and body weight (Li and Li, 2006). Significant association relationships between mutations in this gene, feed intake intensity, and live weight gain have been demonstrated in many animal species (Zhang et al., 2009). Mutations in the *MC4R* gene are also associated with carcass quality in cattle (Zhang et al., 2009) and broilers (Wang et al., 2009). El-Sabrout and Aggag (2019) described a direct relationship of polymorphism in *MC4R* with live weight gain intensity and some behavioural functions of rabbits. For these reasons, it has been included as a significant candidate gene for marker-assisted selection (MAS) related to the production characteristics of rabbits and poultry, such as live weight gain, feed conversion and carcass yield.

Holsinger and Weir (2009) described a big number of single nucleotide polymorphisms in the genomes of several animal species, which allowed the study of the entire genome and the effective setting of selection by evaluating different allele frequencies between populations. Genes associated with production traits were identified using single nucleotide polymorphisms and included in the group of so-called candidate genes (Wu et al., 2015; Zhang et al., 2013).

The current economic pressure in poultry production is causing interest in specialised poultry lines focused on laying or meat performance in developed countries. Continued genetic improvement of livestock depends on the genetic variability that exists within individual breeds (Sruoga et al., 2007). Quantitative assessment of genetic variability within and between populations is an important tool in developing plans for the conservation of genetic resources. Understanding the associations between genotype and phenotype is very important in terms of faster progress in animal husbandry. Results derived from molecular genetic studies play an important role in breeding value prediction systems and in the generation of specialised lines and animal populations. The body composition, fat storage and meat quality are important indicators of poultry. The *MC4R* gene in chickens is associated with carcass yield and meat quality.

In general, two alleles-nucleotides are maintained in position 923 of the MC4R-AY545056 gene, the original G and mutant T in the chicken population. Mutations in the melanocortin-4 receptor *MC4R* gene are associated with appetite, obesity, and growth in pigs, mice, and humans. However, little is known about the function of the chicken *MC4R* gene. Qiu et al. (2006) tested F2-generation broiler chickens for *MC4R* gene polymorphisms using single-stranded conformational polymorphism PCR (PCR-SSCP) and DNA sequencing, founding four sites of single nucleotide polymorphisms (SNPs). The results provide evidence that the chicken MC4R gene can be selected as a major candidate gene for carcass traits such as body weight and growth. Molee et al. (2018) examined the association between different genotypes of the *MC4R* gene in native chickens in Thailand and found no significant relationship between this gene and live weight. Davies et al. (2002) reported that sense mutation can change the gene expression, which in turn leads to a different protein with different characteristics as a result of amino acid change. This protein may lose its function or become activated or exhibit a new function. It is possible that this variation causes a significant change of the *MC4R* function. Amino acid change may also affect the biosynthesis of other nutrients. It can stimulate the feed intake, metabolism and growth of eggs, which in turn affects the egg weight. This finding is in agreement with results obtained by El-Sabrout and Aggag (2017), who found that *MC4R* plays an area responsible for controlling feed intake behaviour, which in turn affects the body weight.

The aim of this study was to design a PCR-based

technique to detect and test specific single nucleotide polymorphisms (SNPs) in gene *MC4R* of Slovak native chicken Oravka. An innovative dimension of this study is the application of outputs and results for identifying critical populations and a breeding selection programme for the preservation of the local well-adapted hen breed. The further aim was to investigate the influence of some factors (genotype, year, sex) on the variation of live body weight and weight gain.

## Material and methods

### *Animals*

The animal genetic resources of yellow-brownish Oravka breed (hens and roosters) are kept at the farm of the National Agriculture and Food Centre (NPPC) – Research Institute for Animal Production Nitra (Slovak Republic), considered as the *ex situ* flock, and included in the experiment which was performed during three consecutive years (2018 to 2020). From all hatched chickens, only animals that had the breed standard were selected for breeding. We monitored the weight of chickens at the age of 5, 12 and 20 weeks (LW 5, LW 12, LW 20) using the BAT 1 manual poultry scale (produced by VEIT Electronics, Czech Republic). From the obtained values, we calculated the weight gain for each period (weight gain between week 5 and week 12 – WG1; weight gain between week 12 and week 20 – WG2). Health was monitored daily. At the age of 12 weeks, when the animals are selected for breeding, the wing feathers of chickens were taken for genetic analysis. Birds were kept in closed heating nurseries on deep litter until 5 weeks of age; afterwards, they were housed in a heatless hen-house with a covered yard and free range. Birds were fed (ad libitum) a complete feed (ME 2600 kcal/kg, 16% crud protein) for light laying hens (according to age categories); water was available during the whole experiment.

### *Molecular-genetics analysis*

Oligonucleotides for ARMS-PCR for *MC4R* gene detection in DNA samples were designed based on used tools like Primer-BLAST on GenBank, NCBI (Gene bank no. NC_052533, last access 15 March 2024). For ARMS primers, the 3' end of allele-specific primers was manually adjusted to align with the SNP, ensuring the specificity for either allele. Proposed diagnostic MC4R-GAL -ARMS-PCR oligonucleotide sequences were MC4R-GAL-ARMS-OMHZ-G-FOR with the sequence 5´-AATGGATCAGAAACTATTGTCATCACGCTG–3´, primer MC4R-GAL-ARMS-OMHZ-T-FOR with the sequence 5´-AATGGATCAGAAACTATTGTCATCACGCTT – 3´, and primer MC4R-GAL-ARMS-OMHZ-G+T REV with the sequence 5´-CTGGAGGGCATAAAAGATAG – 3´.

### *Processing and preparation of biological samples*

The biological samples were taken in a non-invasive manner (pulp from the growing feathers) from poultry of the yellow brownish Oravka breed. DNA isolation from biological samples was performed using commercially available automatic DNA isolation kit MagNA Pure LC DNA Isolation kit II (Tissue) and Tissue kit for manual isolation (Macherey-Nagel, NucleoSpin Tissue) following the manufacturers' instructions.

Subsequently, specific selected sections of the *MC4R* gene were amplified using specific oligonucleotides designed by our site.

### *Analysis of DNA polymorphisms of selected genes*

Molecular genetic analyses were performed at the laboratory of the Department of Nutrition and Small Farm Animals, NPPC, using a Bio-Rad T100 gradient PCR thermocycler. PCR conditions were 95°C for 2 minutes, 95°C for 30 s, 60.7°C for 30 s, 72°C for 30 s, 35 cycles, with the last extension step at 72°C for 10 minutes. The PCR reaction volume (25µL) contained a mastermix: 10 mM Tris-HCl (pH 8.6 at 25°C, 50 mM KCl, 1.5 mM MgCl2, 25 units / mL Taq DNA polymerase, 0.2 mM dNTPs, 5% glycerol, 0.08% IGEPAL® CA-630, 0.05% Tween-20 – New England Biolabs), primers (MC4R-GAL-ARMS G-FOR, MC4R-GAL-ARMS T-FOR, MC4R-GAL-ARMS G+T REV) at a concentration of 10 pmol/L. In the case of poultry, we detected a fragment with a total length of 180 bp. The polymorphism of the *MC4R* gene was determined using ARMS-PCR (PCR analysis using allele-specific oligonucleotides), followed by electrophoretic separation and visualisation using a MiniBis Pro photodocumentation device (Bio-Imaging Systems) with in situ analysis using GelQuant Express software, so that the original methodology shortened the whole analysis and made the whole process more economical. PCR products were then electrophoretically separated on a 3% agarose gel containing ethidium bromide at 80 V, 60 mA in 10 mM lithium borate buffer for 60 minutes and then visualised under UV light (Fig. 1) and photographed using MiniBis Pro documentation system (Bio-Imaging Systems).

ARMS-PCR (Amplification Refractory Mutation System-PCR) is used for genotyping SNP with the help of refractory primers. Designing primers for the mutant (with SNP) and normal (without SNP) alleles allows selective amplification, which can be easily analyzed after electrophoresis. Modification of a single base occurs at the 3' end of the primer such that one primer matches the normal allele (G) and the other matches the mutant allele (T). Both primer variants are combined in a single PCR reaction mixture so that the PCR is performed simultaneously. By running two PCR reactions (one with a mutant-specific primer and one with a wild-type-specific primer), the presence or absence of specific amplification products

can reveal whether the sample contains the wild-type, mutant, or both alleles (heterozygous condition). The modification of primers is the most crucial highlight in the mechanism of ARMS-PCR. Selective amplification happens due to the mismatch induced for primer variants. Introducing a mismatch at the 3' end of the primer alters the annealing temperature for the allele variant. Since Taq DNA polymerase is unable to perform exonuclease activity, the mismatch cannot be repaired. The general procedure of the ARMS-PCR involves four major steps: primer design, amplification, electrophoresis, and results.

For primer design, our DNA sequence, for example, features a G in the normal allele and T in place of G in the mutant allele. Designing a forward primer for the normal allele should contain G while the mutant allele contains T in place of G at the 3' end. The success of ARMS-PCR relies on the presence of an added mismatch base near to the SNP at the 3' end. The reverse primer generally remains the same. Moreover, the primer must still fulfil all the criteria for the ideal primer like in a normal PCR.

Genotype GG: at the G allele position, there is one fragment of 180 bp; the T allele position is without a fragment.

Genotype TT: at the T allele position, there is one fragment of 180 bp; the G allele position is without a fragment.

Genotype GT: at both positions of the G and T alleles, there are fragments of 180 bp.

The Sanger sequencing method (Merck KGaA, Darmstadt, Germany) was used to confirm the SNP in the observed fragment during the DNA sequencing. DNA sequence is determined by synthesising a complementary strand of DNA, a template DNA strand, and a primer. During the synthesis of the complementary strand, the DNA polymerase incorporates fluorescently labelled nucleotides at positions where they match the template DNA strand. Formed DNA strands are size separated through gel electrophoresis, with the sequence's determination being reliant on the colour of the fluorescent label at each position. Figures 2, 3 and 4 show the complementary sequences of the DNA strand to the region detected by ARMS-PCR with the target complementary base indicated.

### *Statistical analysis*

Based on molecular genetic analysis and mathematical-statistical calculations from the observed observations of live weight and live weight gain, association studies were performed in relation to the monitored growth parameters of poultry. Statistical analysis was done using the SAS 9.2 statistical software (2009). The GLM model was applied to study the influence of genotypes, year, sex and sex*genotype on live weight and weight gain of Oravka chicken.

The following model was applied:

$$Y_{ijk} = \mu + G_i + Y_j + Sk + e_{ijk},$$

where $Y_{ijk}$ – individual live body weight /weight gain parameters; $\mu$ – intercept; $G_i$ – fixed effect of genotype (GT, TT); $\Sigma_i G = 0$; $Y_j$ – fixed effect of year (18, 19, 20); $\Sigma_j Y = 0$; Sk – fixed effect of sex (F, M); $\Sigma k\ S = 0$; $e_{ijk}$ – random error.

Fixed effects included in the model were estimated using the Least Squares (LSM) method. Statistical significances of fixed effects were tested by the Fischer F test; statistical significances of individual differences between estimated levels of fixed effects were tested by the Scheffe multiple range tests. Differences were considered significant when $P < 0.05$.

## Results and discussion

In the presented study, we focused on the screening of the population of the national breed of hens – yellow brownish Oravka bred at NPPC Nitra (Slovakia). The candidate melanocortin receptor 4 gene was included for screening as a potential marker for assisted selection (MAS) related to poultry production traits such as live weight and average weight gain.

The results of genotyping of reference samples (Fig. 1) suggested that, in the selected population of standard Oravka, the *GG* genotype demonstrated a frequency of 0.71%, the *GT* genotype showed a frequency of 89.36%, and the *TT* genotype showed a frequency of 9.93%. The frequency of the original *G* allele was 0.454 ± 0.030 and the frequency of the mutant allele *T* was 0.546 ± 0.030. These results were confirmed by the Sanger sequencing method (Fig. 2, Fig. 3A, Fig. 3B, and Fig. 4) indicating the presence of selected SNP in the observed fragment during the DNA sequencing.

Similarly to this study, Molee et al. (2018) detected the higher frequency of the T allele: T = 0.668 compared with the G allele (0.332) in indigenous rapid growth rate chicken in Thailand. They investigated 510 chickens and found three genotypes with the following frequencies: *GG* = 0.24; *GT* = 0.18; *TT* = 0.58.

Table 1 shows the general linear model for live weight and weight gain of 140 animals. With respect to the coefficient of determination, the model explained the variation of evaluated variables between 39% (weight gain 2) and 62% (live weight 20). The adjustment for fixed factors (genotype, year, sex and interaction between genotype and sex) decreased the variation by 22% (live weight 5), by 37% (live weights 12 and 20), by 34% (weight gain 1) and by 80% (weight gain 2).

Least-square means and standard errors, estimated within the effects of genotype, year and sex for live weight at 5, 12 and 20 weeks of age and for weight gain between 5 and 12 weeks of age and 12 and 20 weeks of age are given in Table 2. At the beginning of the experiment, at the age of 5 weeks, average live weight was higher in heterozygote animals GT

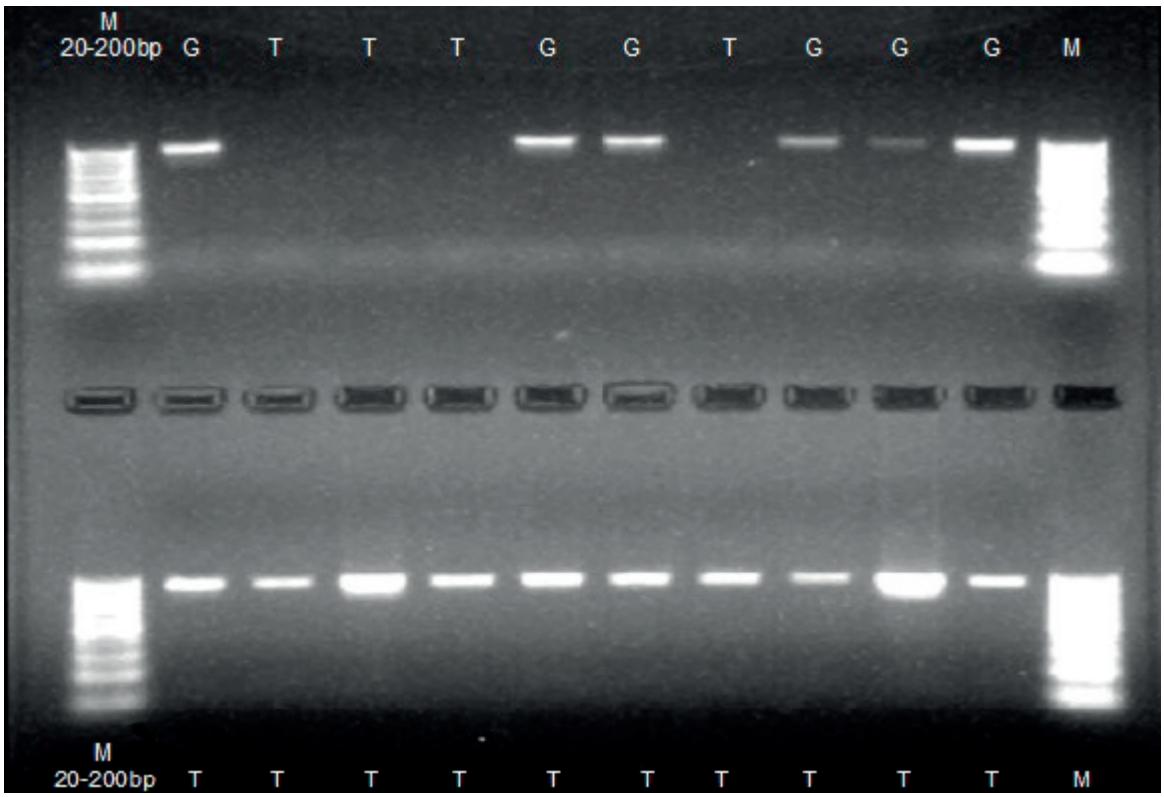


*Fig. 1.* Electrophoretogram of polymorphism with analysis of *MC4R* genotypes GT and TT of reference samples of Oravka breed.

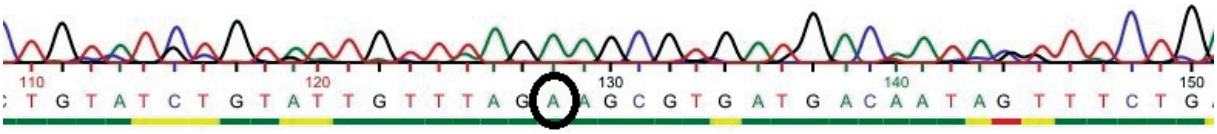


*Fig. 2.* Complementary base "A" (in the circle) in a DNA chromatogram of PCR products based on the Sanger sequencing method – *MC4R* genotype T.

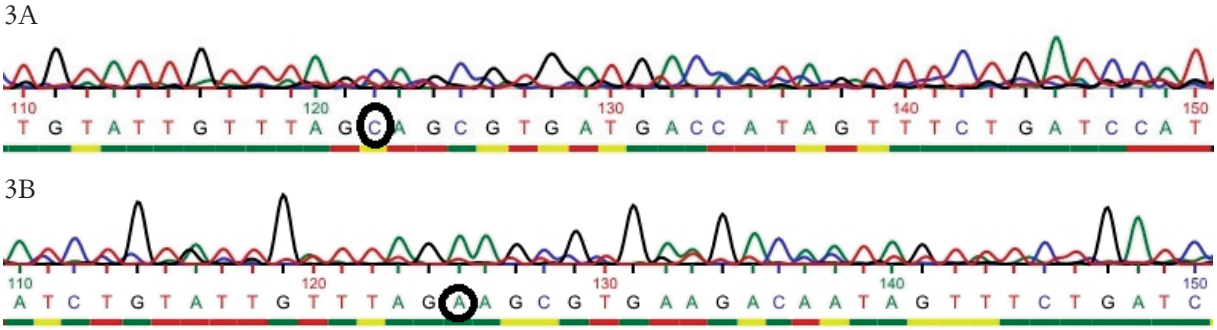


*Fig. 3A–3B.* Complementary bases "C" and "A" (in the circle) in a DNA chromatogram of PCR products based on the Sanger sequencing method – *MC4R* genotype GT.

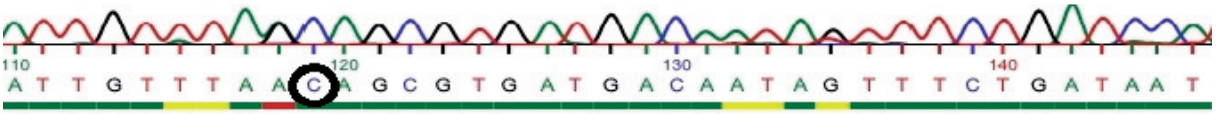


*Fig. 4.* Complementary base "C" (in the circle) in a DNA chromatogram of PCR products based on the Sanger sequencing method – *MC4R* genotype GG.

*Table 1.* Selected characteristics of the general linear model for live weight (g) and weight gain (g), N = 140

| | Live weight 5 | Live weight 12 | Live weight 20 | Weight gain 1 | Weight gain 2 |
|---|---|---|---|---|---|
| R2 | 0.419 | 0.611 | 0.617 | 0.583 | 0.385 |
| RMSE | 62.226 | 131.970 | 218.221 | 115.606 | 162.570 |
| Mean | 470.286 | 1122.929 | 1952.286 | 652.643 | 829.357 |
| STD | 80.1 | 207.9 | 346.3 | 175.8 | 203.5 |

*R*2 – coefficient of determination; RMSE – Root means squares error; STD – standard deviation.

*Table 2.* Least-squares means and standard errors for live weight and weight gain

| Effect | | LW 5 (g) | LW 12 (g) | LW 20 (g) | WG 1(g) | WG 2(g) |
|---|---|---|---|---|---|---|
| | N | μ ± sμ | μ ± sμ | μ ± sμ | μ ± sμ | μ ± sμ |
| Genotype | | | | | | |
| GT | 126 | 501.19 ± 8.88 | 1248.92 ± 18.83a | 2207.38 ± 31.14 | 747.73 ± 16.50a | 958.46 ± 23.20 |
| TT | 14 | 494.50 ± 17.56 | 1346.40 ± 37.23b | 2333.58 ± 61.57 | 851.90 ± 32.62b | 987.18 ± 45.87 |
| Year | | | | | | |
| 2018 | 43 | 564.28 ± 13.23a | 1355.03 ± 28.05a | 2340.02 ± 46.38a | 790.75 ± 24.57b | 984.99 ± 34.55 |
| 2019 | 65 | 452.97 ± 11.61c | 1132.15 ± 24.62b | 2065.90 ± 40.72b | 679.19 ± 21.57c | 933.74 ± 30.33 |
| 2020 | 32 | 476.29 ± 12.89b | 1405.80 ± 27.33c | 2405.53 ± 45.19c | 929.51 ± 23.94a | 999.73 ± 33.67 |
| Sex | | | | | | |
| Female | 119 | 455.90 ± 10.87B | 1109.74 ± 23.05B | 1918.01 ± 38.11B | 653.78 ± 20.19B | 808.28 ± 28.39B |
| Male | 21 | 539.73 ± 16.16A | 1485.58 ± 34.28A | 2622.95 ± 56.68A | 945.85 ± 30.03A | 1137.37 ± 42.23A |

LW 5 – live weight at 5 weeks of age; LW 12 – live weight at 12 weeks of age; LW 20 – live weight at 20 weeks of age; WG 1 – weight gain between 5 and 12 weeks of age, WG – weight gain between 12 and 20 weeks of age; $^{*}P < 0.05$ indicated with lowercase letters; $^{**}P < 0.01$ indicated with capital letters.

(501.19 ± 8.88 g) compared with homozygote animals TT (494.50 ± 17.56 g), but the difference was not significant. Later, at the age of 12 and 20 weeks, the homozygote animals TT had a higher live weight (LW 12 = 13.46 ± 37.23 g; LW 20 = 2333.58 ± 61.57 g) and weight gain (WG1 = 851.90 ± 32.62 g; WG 2 = 987.18 ± 45.87 g) compared with the heterozygote GT (LW 12 = 1248.92 ± 18.83 g, LW 20 = 2207.38 ± 31.14 g, WG1 = 747.73 ± 16.50 g; WG 2 = 958.46 ± 23.20 g). The animals with homozygote genotype TT at the age of 12 weeks were significantly heavier than the homozygote ones ($P < 0.05$). Also, the WG 1 between 5 and 12 weeks of age was significantly higher in homozygote animals TT ($P < 0.01$). The results demonstrated that the mutant allele T had a positive effect on body weight and weight gain. As shown in Table 2, we found a significant effect of the year on live weight and weight gain 1. We did not find a significant effect of the year on weight gain 2 from 12 to 20 weeks of age.

The obtained results also logically show a remarkably significant influence of sex on the monitored traits. Physiologically, roosters grow significantly faster and are heavier than hens in adulthood. When evaluating the effect of genotype on weight and weight gain by sex, females and males with genotype TT had a higher weight at 12 and 20 weeks of age, as well as weight gain. Females with genotype TT were heavier (1111.33 ± 44.96 g) than GT females (1108.15 ± 13.17 g). Similarly, rooster homozygotes were on average heavier (1581.47 ± 59.11 g) than heterozygotes (1389.69 ± 34.40 g). Both hens and roosters kept this trend at the age of 20 weeks. Homozygous TT animals achieved a higher average weight (hens: 1947.25 ± 74.35 g, roosters: 2719.91 ± 87.75 g) than heterozygous animals (hens: 1888.78 ± 21.78 g, roosters: 2525.99 ± 56.89 g). Similarly, weight gain was higher in homozygous animals than in heterozygous ones. Significantly higher WG 1 was observed in the homozygote TT males (1038.19 ± 51.78 g) compared with heterozygote GT (853.52 ± 30.14 g) ($P < 0.05$).

Wang et al. (2009) in their study confirmed that the *MC4R* G923T genotypes were significantly associated with live weight (LW), carcass weight (CW) and leg muscle weight (LMW). The LW of GT chickens was notably higher than that of TT ($P \geqslant 0.05$). Kubota et al. (2019) detected the significant association between the *MC4R* genotype and body weight at 2, 4, and 10 weeks of age of Korat chicken (KRC). KRC is a slow-growing chicken generated by crossbreeding male Thai indigenous chickens (Leung Hang Khao, LHK) and the female Suranaree University of Technology breeder line. The MC4R genotype had a significant

effect on body weight at 2, 4, and 10 weeks of age ($P < 0.05$); however, they could not detect any association between the *MC4R* genotype and the muscle fibre diameter.

In contrast to our results, where the higher live body weight was observed in the chicken with the TT genotype at 12 and 20 weeks of age, Molee et al. (2018) found the highest live body weight at 16 weeks of age in rapid growth rate chickens with the GG genotype (1478 ± 19.99 g). Chicken with genotype GT had LBW = 1408 ± 28.14 g, and chicken with genotype TT had LBW =1449 ± 13.19 g.

## Conclusions

We can conclude that based on population screening and subsequent association studies of the *MC4R* gene polymorphism in relation to the observed production indicators of Oravka, we proved that after a long-time direction selection for breeding based on standard phenotype, preference is given to animals that are heterozygous (GT) in the *MC4R* gene, with a preference for the mutant allele frequency "T" = 54.61%. The homozygote animals TT had a significantly higher live weight and weight gain compared with the heterozygote GT, indicating that the mutant allele T has a positive effect on body weight and weight gain.

## Acknowledgments

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# Effect of Natural Liquid Oreganum on Physiological Performance in Stressed Laying Japanese Quails Exposed to Force Molting

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***Keywords:*** *oreganum, force molt, laying quail, haematology, reproduction*

***Abstract.*** *This study aimed to study the effect of oreganum on forced molting in laying Japanese quails to improve the physiological performance and restore the reproductive capacity of females. A total of 120 female Japanese quails (Coturnix coturnix Japonica) aged 18 weeks were divided randomly into six groups: group A – pre-molting from weeks 18 to 19; group B – supplemented with oregostim at a dose of 0.3 mL\1000 mL; group C (molting from weeks 20 to 22) – fed a restricted diet; group D – fed a limited diet with oregostim at the same dose and post-molting from week 23 to week 37; group E – fed a basal diet; and group F – fed a basal diet and treated by oregostim. The results showed that the molting group caused a significant reduction in the weight of body and ovary in group C and group D, while the weights were restored in group E and group F relative to other groups. Besides, there was a significant decrease of WBCs and RBCs count in group C and group D with a significant elevation in group E and group F compared with group A and group B. The percentage of egg production and large follicle number in group C and group D decreased significantly compared with group A and group B, while they restored to normal values in group E and group F compared with group A and group B and showed a significant increase as compared with group C and group D, respectively. We concluded from this study that oregostim does not have adverse effects on the molting period. Thus, the standard diet supplemented with oregostim was considered as a better molting technique.*

## Introduction

Force molting is a procedure of inducing molting to regenerate feathers and egg production at a particular time (Maiorano et al., 2011). It also improves the economic value by temporarily delaying the broiler productive lifespan or by temporarily delaying the egg production in laying hens (Brake, 2023). Molting is a physiological process in avian species that happens naturally and includes the replacement of old feathers with new (Huss, 2008). Numerous approaches have been used to induce molting such as using a restricted diet by feeding only grain with insufficient minerals and essential vitamins such as sodium or calcium, aluminium, zinc and iodine (Khan et al., 2011). The period of the reproductive and productive lives of birds is prolonged by force molting (Heryanto et al., 2016). The biggest significance of forced molting is the regeneration of the reproductive system, which increases the tissue efficacy, gonadal growth, and loss of adipose tissue (Narinc, 2013). The common uses of force molting include either a photoperiod stage, fasting or a combination of both (Heryanto et al., 2016). The stress phase eliminating period of feeding is followed by a recovery phase when birds receive a restricted diet of protein or total feed keeping the body weight but not resuming egg laying (Huss, 2008). Oregano is a well identified essential aroma having antioxidant properties and antioxidant activity such as thymol and carvacrol (Drăgan et al., 2008). The oregano extracts contain carvacrol or thymol (Zheng et al., 2009). Oreganum is an essential oil with antibacterial activities. It acts as an antioxidant and promotes growth. It increases feed intake by stimulating the animal's sense of hunger. Furthermore, it replaces and cleans the gut lining, which boosts the animal's health by reestablishing absorbent in the gut surface (Zhang, 2021). Studies on the applications of the product in the form of powder or leaves on production parameters and metabolism are limited (Ampode et al., 2022; Bayram and Akkaya, 2018).

The aim of this study is to determine the effect of natural liquid oreganum on physiological performance in stressed laying Japanese quails exposed to force molting by resting the reproductive system for a certain period to improve its physiological performance and restore the hens' reproductive ability.

## Materials and Methods

### *Quails*

The experimental quails and laboratory analyses were supported by an animal house and laboratories of the Veterinary Medicine Department of the University of Mosul. The study included 120 female Japanese quails (Coturnix coturnix Japonica), 18 weeks old with an average body weight ranging from 180.33 ± 36 to 200 ± 32 g purchased from al Ebaa Research Centre of the Ministry of Agriculture. The hens were preserved in floor birdcages, and food and water were provided ad libitum. Food elements and nutrient levels are presented in accordance with the

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National Research Council (NRC) recommendations (Maty and Hassan, 2020).

***Experimental design***

Female laying hens were divided and distributed randomly into six equal groups A, B, C, D, E and F. All groups were fed a basal diet for one week for adaptation before inducing molting. The animals were kept under the same light 16 hours a day, and a water rule was ad libitum. The duration of the experiment was divided into three periods:

1. **Pre-molting period** – from week 18 to 19:

**(A) Control group**;

**(B) Oregostim group** was given oreganum at a dosage (0.3 mL \ 1000 mL water) in water.

*These two groups were fed a basal diet ad libitum.

2. **Molting period** – from week 20 to 22:

**(C) Control group**;

**(D) Oregostim group** was given oreganum at a dosage (0.3 mL \ 1000 mL water) in water.

*These two groups were fed a restricted diet ad libitum.

The restricted diet was given to the hen supplemented according to Younis (2019).

3. **Post-molting period** – from week 24 to 37:

**(E) Control group**;

**(F) Oregostim group was** given oreganum at a dosage (0.3 mL \ 1000 mL water) in water.

*These two groups were fed a basal diet ad libitum.

***Treated material***

Oreganum product was made from 100% of pure essential oil and manufactured by Anpario Company in the UK. Oregostem® was administered at a dosage of 300 mL per 1000 L of drinking water, as recommended by the producer.

***Weight parameters of body organs***

The weights of organs (heart, liver, gizzard, and chest muscle), where given, were all calculated by equation = (organ weight /body weight) × 100. During the trial, the body weight of the quails in all groups was calculated in gram according to the age of each group shown in the experimental design.

***Blood parameters***

Blood samples were collected during slaughter after keeping a half of the quails for the next period. The sample was divided into two parts as follows:

1. Serum samples were separated by blood centrifugation for 15 min at 3000 rpm and reserved at –26ºC until estimation (Nguyen et al., 2014).
2. Blood samples with an anticoagulant were used for complete blood count (RBCs, WBCs) using Natt and Herrick solution and for counting differential leukocyte (DLC)using Wright stain.

***Follicle parameters***

Follicles were extracted from the body and counted.

***Percentage of egg production***

Egg production was recorded daily and represented as the percentage of egg production of a hen per day and weekly during the experiment. It has been reported that egg production ceases during the entire forced molting period in all groups (Deek and Al-Harthi, 2004).

% Egg production = (Number of eggs produced per week per number of housed hens) × 100 (Deek and Al-Harthi, 2004).

***Statistical analysis of the data***

Data of body weight, reproductive organs, ovarian follicles, and blood parameters were collected and compared between control pre-molting, molting, and post-molting birds using descriptive ANOVA analyses with the Duncan multiple range test used at the probability level ($P$ 0.05). SPSS version 19.0 and MS Office Excel 2007 statistical tools were used. Data are presented as means ± SD with a significance level of $P < 0.05$ (Steel, 1997).

**Results**

At the beginning of the experiment, the mean body weight was 225 g with no significant body weight variances between the control and oregostim groups for each period in the pre-molting and the molting period, while in the post-molting period there was a significant variance between group E and group F. The oregostim group showed a restoration and a slight increase in body weight (F) as presented in Table 1. Otherwise, the results showed a significant difference between the periods. There was a decrease in body weight in group C and group D compared with the control group A and a significant increase in group E and group F compared with group B and group C, without a significant variation in the weight of other body organs (heart, muscle, gizzard and liver). There was a significant decline in ovary weight in group C and group D at the molting period and a significant increase in group E and group F compared with other groups. The birds which recovered from molting restored their weight.

Table 2 demonstrates insignificant differences in the pre-molting period in group A and group B in the numbers of red blood cells, white blood cells and differential counts of leukocytes. The analysis of variance indicated that there was a significant decrease in the molting period in group C and group D in red blood cells and white blood cells, respectively, compared with group A and group B, while there were no significant differences in other blood values, but a significant increase was observed in group E and group F in the post-molting period compared with other groups.

Table 3 demonstrates a significant decrease in the percentage of egg production and the numbers of large follicles on the ovary at the molting period compared with groups at the pre-molting period, while it is

*Table 1.* Efficiency of oregostim on body and organ weight in laying Japanese quails in pre-molting, molting and post-molting periods

| Groups | Mean ± Standard Error | | | | | |
|---|---|---|---|---|---|---|
| | Body weight (g) | Heart weight (gm /100 g.bw) | Gizzard weight (gm /100 g.bw) | Muscle weight (gm /100 g.bw) | Ovary weight (gm /100 g.bw) | Liver weight (gm /100 g.bw) |
| Pre-molting period Control (A) | 225.0 ± 2.0 b | 0.846 ± 0.163 ab | 1.92 ± 0.114 a | 25.67 ± 0.65 a | 2.808 ± 0.184 ab | 2.896 ± 0.238 ab |
| Pre-molting period Oregostim (B) | 219.4 ± 5.9 bc | 0.742 ± 0.120 ab | 1.73 ± 0.150 a | 23.90 ± 0.72 a | 3.072 ± 0.192 a | 2.842 ± 0.280 ab |
| Molting period Control (C) | 192.2 ± 4.9 d | 0.682 ± 0.017 ab | 1.66 ± 0.396 a | 22.56 ± 0.18 ab | 1.204 ± 0.041 c | 3.154 ± 0.142 a |
| Molting period Oregostim (D) | 196.6 ± 3.8 d | 0.730 ± 0.016 ab | 2.18 ± 0.089 a | 25.32 ± 0.71 a | 2.126 ± 0.150 c | 3.350 ± 0.118 a |
| Post-molting period Control (E) | 200.0 ± 10.5 c | 0.952 ± 0.040 a | 2.20 ± 0.085 a | 24.79 ± 0.13 a | 3.062 ± 0.211 a | 2.556 ± 0.167 ab |
| Post-molting period Oregostim (F) | 227.6 ± 10.1 a | 0.878 ± 0.078 a | 1.81 ± 0.132 a | 25.55 ± 0.71 a | 3.110 ± 0.147 a | 3.040 ± 0.092 ab |

The letters a, b, c and d indicate the statistically significant differences between groups; $P < 0.05$.

*Table 2.* Efficiency of oregostim on blood parameters of laying Japanese quails in pre-molting, molting and post-molting periods

| Groups | | Mean ± Standard Error | | | | | |
|---|---|---|---|---|---|---|---|
| | RBC × $10^6$ /$mm^3$ | WBC × $10^3$ / $mm^3$ | Lympho-cytes% | Heterophils % | Basophils % | Eosinophils % | Monocytes % |
| Pre-molting period Control (A) | 1.92 ± 0.58 b | 40196 ± 143 a | 70.20 ± 2.08 a | 18.20 ± 1.43 b | 0.600 ± 0.024 a | 8.40 ± 0.44 a | 9.80 ± 1.39 bc |
| Pre-molting period Oregostim (B) | 2.12 ± 1.49 b | 41796 ± 182 a | 76.00 ± 1.76 a | 10.60 ± 1.68 b | 0.600 ± 0.024 a | 1.00 ± 0.44 ab | 12.00 ± 1.30 abc |
| Molting period Control (C) | 1.60 ± 0.71 c | 33513 ± 192 b | 76.00 ± 1.76 a | 10.60 ± 1.68 ab | 0.600 ± 0.024 a | 1.00 ± 0.67 ab | 10.00 ± 1.50 bc |
| Molting period Oregostim (D) | 1.20 ± 1.01 c | 35022 ± 161 b | 71.00 ± 3.56 a | 38.20 ± 2.90 ab | 1.200 ± 0.037 a | 2.60 ± 0.37 ab | 17.00 ± 1.72 ab |
| Post-molting period Control (E) | 3.0002 ± 1.93 a | 42490 ± 247 c | 75.00 ± 3.30 a | 13.00 ± 1.75 ab | 0.400 ± 0.024 a | 0.80 ± 0.80 ab | 10.80 ± 1.59 abc |
| Post-molting period Oregostim (F) | 3.055 ± 1.80 a | 43630 ± 134 a | 79.60 ± 3.55 a | 39.20 ± 1.31 ab | 1.200 ± 0.100 a | 2.20 ± 0.34 ab | 17.80 ± 1.16 ab |

The letters a, b, c and d indicate the statistically significant differences between groups; $P < 0.05$.

demonstrated that there was a significant increase in the percentage of egg production and the numbers of large follicles in the groups at the post-molting period compared with the groups at the molting period.

The current study indicated that the ovarian large follicles during the post-molting period were somewhat bigger than the control at the pre-molting period. However, the oviducts and egg developing follicles appeared comparatively healthier and bigger in post-molted hens (Fig 1). Different sizes of eggs for each period are demonstrated in Fig. 2. Eggs of molted hens are small and have a soft shell compared with the control, while post molt hens had large size eggs, and oregano had a good effect on the

*Table 3.* Efficiency of oregostim on reproductive parameters of laying Japanese quails in pre-molting, molting and post-molting period

| Groups | Mean ± Standard Error | | |
|---|---|---|---|
| | % of egg production | Number of small follicles | Number of large follicles |
| Pre-molting period Control (A) | 52.44 ± 3.1 a | 4.41 ± 0.24 a | 25.8 ± 0.65 a |
| Pre-molting period Oregostim (B) | 56.33 ± 3.22 a | 4.83 ± 0.20 a | 26.2 ± 0.93 a |
| Molting period Control (C) | 31.55 ± 1.44 b | 4.0 ± 0.44 a | 16.2 ± 0.01 b |
| Molting period Oregostim (D) | 32.43 ± 2.12 b | 4.6 ± 0.24 a | 21.6 ± 0.50 b |
| Post-molting period Control (E) | 60.43 ± 2.54 a | 0.51 ± 0.65 a | 30.2 ± 0.33 a |
| Post-molting period Oregostim (F) | 70.65 ± 3.11 a | 0.35 ± 0.66 a | 33.2 ± 0.24 a |

The letters a, b, c and d indicate the statistically significant differences between groups; $P < 0.05$.

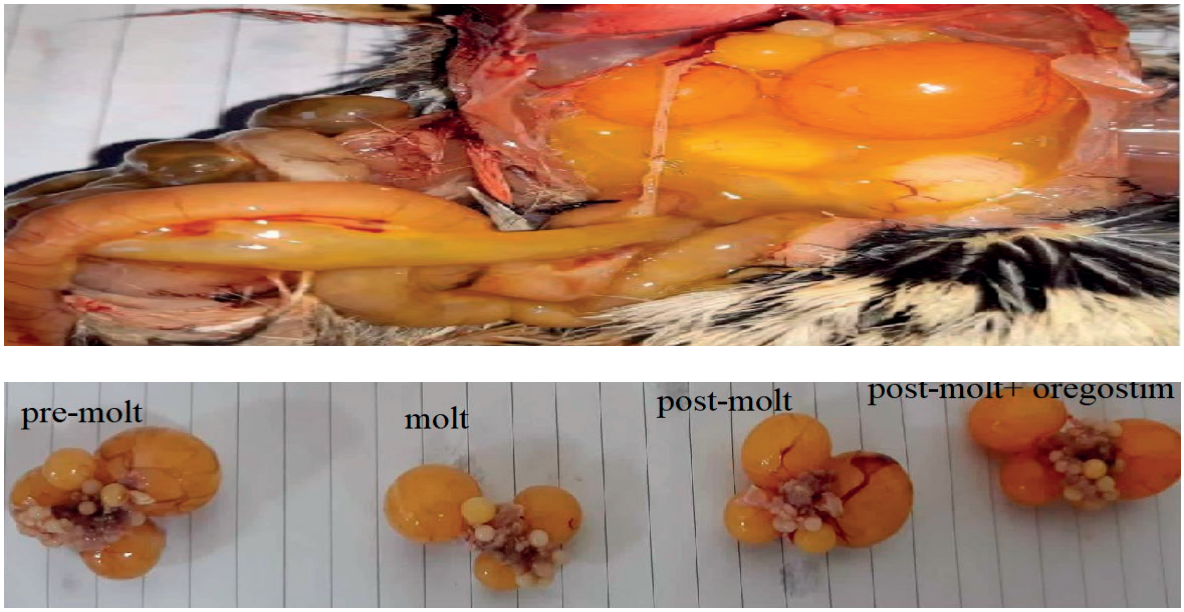


*Fig. 1.* Follicles during the molting periods.

size and shell quality compared with other groups. Fig. 3 shows the appearance of feather falling in molted hens, especially in the neck region, back and thighs. The present study showed that erythrocytes at the pre-molting period were smaller in size and round in shape. During molting, erythrocytes were larger, became more elongated and decreased in numbers, while they increased in their counts and were restored to a normal shape and size at the post-molting period (Fig. 4).

## Discussion

The results of this study demonstrate that induced molt causes a significant decrease in the body weight and ovary weight at the molting period 20$^{th}$ to 22$^{nd}$. This may be due to the stressful effect of induced molt on hens fed a restricted diet because of the lack of minerals, proteins and other supplemented substances in the diet. Otherwise, the supplementation of oregostim in drinking water in molted hens failed to restore the hen's ovary and body weight to normal values during the molting period 20$^{th}$ to 22$^{nd}$, which may be because molt is highly stressful for hens, causing loss of body weight, stagnation of organs of the reproductive tract, cessation of laying eggs, and feather loss (Lee, 1982). Other studies have summarized that the yellow corn feed intake in quails with low protein properties has a reduction effect on body weight and other body organs (Maty et al., 2020). In our study, there was a significant decline in the weight of ovary and large follicles numbers in molted hens with and without oregostim while the weight

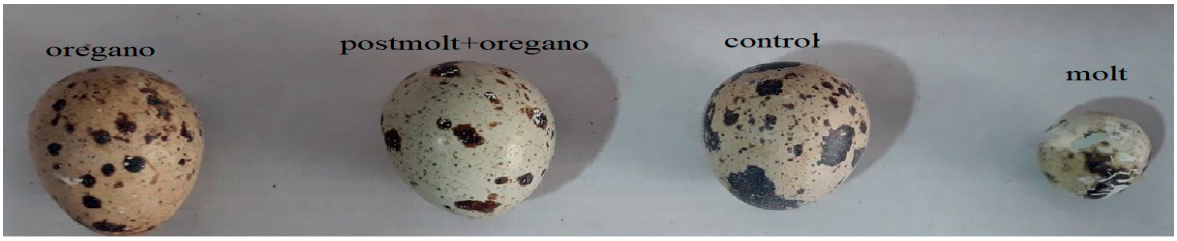


*Fig. 2.* Sizes of eggs for each period.

*Fig. 3.* The appearance of feather falling in molted hens, especially in the neck region, back and thighs.

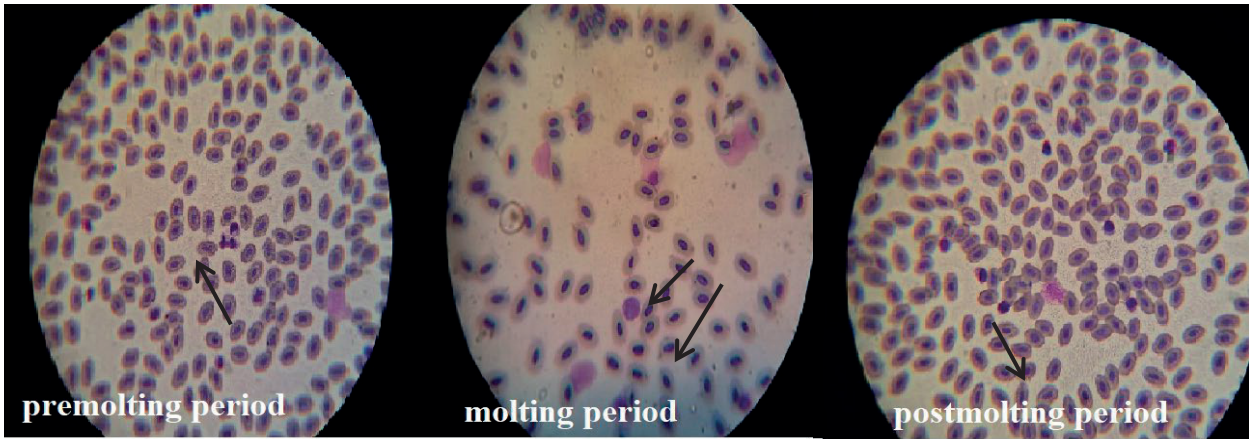


*Fig. 4.* Erythrocytes during the molting periods.

gain happened in both groups at the post-molting period compared with other groups. This indicates the positive effect of oregostim at the post-molting period 23$^{th}$ to 37$^{th}$ compared with groups without oregostim supplementation at the same period, as it caused weight gain of hens at the post-molting period. Studies have reported that oregostim promotes animal feed intake and enhances and restores the gut lining to improve the animal's health, by renewing the absorbent gut surface (Maty et al., 2020). Oregostim has been reported to cause stimulation of appetite which increases feed consumption (Wen, 2008). Oregostim has an active substance such as thymol, which activates the digestive system and feed intake (Palmer, 1972), increasing the generation of digestive enzymes and improving digestion by promoting liver activities, to stimulate feed intake (Ciftci et al., 2005). This may be the main reason of body weight gain during the post-molting period with oregostim.

Blood parameters of hens are important in acquiring necessary information on the immune status of animals (Brake, 1979). Haematological changes are used to regulate the body status and evaluate the effect of environmental and nutritional stresses (Kohn and Allen, 1955). In our study, we noticed that there were different sizes and shapes of erythrocytes. Erythrocytes at the pre-molting period were smaller and had a round shape. During molting, erythrocytes changed their size and shape. They were bigger, more elongated and decreased in numbers, while they increased in their counts and were restored to normal shape and size at the post-molting period. The difference in the size and forms of red blood cells during molt replicate the definite physiological needs of the organism. The change in the size of red blood cells is the key for perfusion of

the tissue, specifically oxygen distribution (Haas and Janiga, 2020). Metabolism increases during molt, so the structure of an erythrocyte affects its morphology, properties and consequently contributes to the ability for gas transfer and blood (Kuenzel, 2003). Changes in blood parameters at the molting period, a significant decrease in both red blood cells counts and white blood cells counts compared with control groups while they get restored to normal values at the post molting period suggest that oregano does not have adverse effects on haematological limits. The various changes in haematological and blood parameters obstruct the birds' ability several systemic functions (Arora and Vatsalya, 2011). Molting modifies hormonal levels, haematological values and blood chemicals (Groscolas and Robin, 2001). A number of theories have explained this drop-in haematocrit during the molt (Robin et al.,1988). Blood parameters might be decreased because of a decline in erythropoiesis secondary to nutritious and energetic imbalance involving some degree of fasting, potentially leading to iron deficiency (Cherel et al., 1988). Previous studies have shown that molting is associated with low concentrations of sex steroid hormones and a severe increase and subsequent decrease of thyroid hormones. These hormonal changes may impact haematological standards (Graw and Kern, 1985).

Another possible explanation for the decrease in haematocrit is hem dilution, an increase in plasma volume as an osmotic adjustment in response to the extensive vascularization of growing feathers without an accompanying increase in erythrocyte population or size (Jouraw et al., 2005). Molting decreases the number of follicles in ovaries. Fasting has been related to autophagy and apoptosis in granulosa cells (Berg and Bearse, 1947). Thus, the causes of a decline in the numbers of ovarian large follicles may be due to a follicle atresia caused by molt, and the body can resorb it to supply energy (Berg and Bearse, 1947). This may disturb the percentage of egg production in molted laying hens which leads to a decrease in the numbers of eggs at the molting period from 20$^{th}$ to 22$^{nd}$. The amount of eggs produced by laying hens increases with age, while the level of reactive oxygen species (ROS) in the ovary and the activity of antioxidant enzymes declines, resulting in an increase in follicle atresia and a decline in laying ability (Oguike, 2005). Consequently, molt causes deterioration of large yellow ovarian follicles and a decrease in the weight of an oviduct, leading to a sharp decrease in egg production (Chowdhury and Yoshimura, 2003). Less blood vessels and a decrease in the expression of FSH and FSH receptors appear in follicles (Bölükbaşı and Kaynar, 2007). In the current study, the restricted diet affected the egg production because hens needed high protein and a sufficient amount of calcium to produce eggs. On the other hand, the water supply with oregostim had no positive effect at the molting period on egg production. Our findings indicate that egg production in laying hens significantly increased at the post-molting period and restored to a normal value compared with control groups at the pre-molting period. Other studies have reported that adding thyme oil and mixed essential oils to the diet increased the total quantity of eggs produced (Han et al., 2022) and supplementing the diet with 100 mg/kg of oreganum considerably enhanced egg production, average egg weight, and food conversion rate (Wang et al., 2021). Plant extracts and spices, individually or in combination, can improve animal performance and health (Jiang et al., 2013; Yang, 2021).

The conclusion from our finding is that oregostim does not have adverse effects on the molting period. It may, therefore, be that the use of a dose of 3 mL /10 L of water is not enough for molted hens. However, it is reported that it has a positive effect at the post-molting period compared with the molting period by using restricted diet. Therefore, the normal diet supplemented with oregostim can be considered a better molting technique.

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