Veterinarija ir Zootechnika

Volume 80(2)
2022

Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Volume 80(2)
2022

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.

Issues per Year – 4. From 2016 - 2.

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

Submission of Manuscripts

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@lsmuni.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.

Publication Information:

„VETERINARIJA ir ZOOTECHNIKA"
A scientific journal and the Official Organ of the Veterinary Academy,
Lithuanian University of Health Sciences (LSMU VA).

ISSN 1392-2130 (Print)
ISSN 2669-2511 (Online)

evaldas.slyzius@lsmu.lt

Layout Rūta Atie
Printed by LSMU Academic Publishing Department, A. Mickevičiaus 9, LT-44307 Kaunas, Lithuania. Edition of 10 copies.

Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Volume 80(2)
2022

Veterinarija ir Zootechnika

LITHUANIAN UNIVERSITY OF HEALTH SCIENCES

Volume 80(2)
Pages 1–100
2022

CONTENTS

Influence of Heat Stress on Some Physiological, Productive and Reproductive Indicators in Dairy Cows – A Review

Martin Stojnov[1], Dimo Dimov[1], Toncho Penev[1], Ivaylo Marinov[2], Jurii Mitev[1], Chonka Miteva[1], Krasimira Uzunova[3]

[1]Department of Ecology and Animal Hygiene, Faculty of Agriculture, Trakia University, 6000 Stara Zagora, Bulgaria
[2]Department of Animal husbandry – Ruminant animals and animal products technologies, Faculty of Agriculture, Trakia University, 6000 Stara Zagora, Bulgaria
[3]Department of General Livestock Breeding, Faculty of Veterinary Medicine, Trakia University, 6000 Stara Zagora, Bulgaria

Keywords: *heat stress, dairy cows, physiology, productive, reproduction.*

Abstract. *The purpose of this review is to examine the scientific literature on the effects of heat stress on some physiological, productive and reproductive parameters in dairy cows. The article analyzes the scientific papers in which the influence of heat stress and its impact on some indicators is studied. As a result of the review, it became clear that heat stress has an impact on the studied indicators, but there are no clear criteria at which values of temperature-humidity index (THI) this effect is registered. The relationship between heat stress, productivity, successive lactation and physiological and reproductive parameters in dairy cows is still controversial. This poses a challenge, through research, to solve the problems in regards to high temperature and animal welfare and productivity for specific climatic conditions.*

Introduction

Heat stress in dairy cows is considered to be a combination of environmental factors that cause an increase in body temperature and a number of other reactions.

Temperature and humidity are considered to be the main indicators of the environment. There are other additional environmental factors such as air velocity and solar radiation that affect the cooling of cows under heat stress. In order to take into account the impact of all these meteorological conditions and their impact on the formation of heat stress, indices have been developed to measure the value of this stress.

As a result of heat stress in the body of cows, a number of physiological changes are observed. The main ones are changes in body temperature, respiratory rate, heart rate, digestive changes, hormonal reactions and reactions in the acid-base balance of the body. Under the influence of heat stress and the physiological-adaptive processes in cows, there are changes in the quantity and quality of milk produced, as well as a number of reproductive changes, usually associated with deterioration of reproductive performance under heat stress.

Despite many studies on the topic of heat stress in dairy cows, research continues to this day. Data on the impact of heat stress on dairy cows varies, as they are conducted in different parts of the world, characterized by the specifics of climatic characteristics, as well as some individual characteristics of reared cows, such as breed, productivity and others.

Correspondence to Toncho Penev, Department of Ecology and Animal Hygiene, Faculty of Agriculture, Trakia University, Bulgaria. E-mail: tonchopenev@abv.bg

All this makes the issue of heat stress relevant, given the search for an adequate response and addressing its consequences.

Heat stress in modern cattle breeding

Heat stress is defined as a set of external forces that act directly on the animal's body, causing an increase in body temperature and inducing a series of adaptive responses (Dikmen and Hansen, 2009). The steady rise in temperatures and global warming (Schär et al., 2004), combined with the significant increase in the number of productive animals and the intensification of cattle breeding (Renaudeau et al., 2012), make heat stress a great challenge and a problem for modern farmers. Given the normally high heat loads in productive dairy cows caused by the large amount of energy used for milk production and other physiological needs (Chebel et al., 2004), high temperatures and humidity can significantly contribute to deterioration in the health status of animals and impairment of their comfort (West, 2003). Not surprisingly, the problem of heat stress is most common in geographical areas where the summer season is long and prolonged exposure to sunlight and high humidity is established (Schüller et al., 2014). Animals located in northern geographical areas may also be exposed to heat stress, where the summer season is shorter but hot enough and there is a minimal drop in temperature during the dark part of the day. Heat stress leads to significant economic losses for farmers, deteriorating many productive, reproductive and health indicators in cows.

Environmental parameters influencing as a risk factor for heat stress

Cows are able to adapt to changing temperature and

humidity conditions throughout the year (Kadzere et al., 2002). This is confirmed by the relatively wide range of thermo-neutral conditions found in cattle. Temperature fluctuations in the range of –0.5–20.0°C and 60–80% relative humidity (West, 2003) are generally accepted as a thermo-neutral value that does not significantly affect the normal physiology of animals. Berman et al. (1985) claim that the upper limit of the air temperature at which the basic body temperature in cattle can be maintained is 25.0–26.0°C.

Air temperature and relative humidity are considered to be the most important factors that determine the heat exchange between the animal's body and the environment. Other important elements of the microclimate – such as air movement and sunlight – also play a significant role in inducing a heat stress response in animals (West, 2003; Da Silva et al., 2010). Changes in air velocity affect convection cooling in cows (Davis and Mader, 2003). The recommended air velocity for dairy cattle in the United States during heat stress is 1.8 to 2.8 m/s (Bailey et al., 2016). Berman (2005) believes that air velocity is lower when cows move in the barn, so measurements do not always reflect real values. This is in line with the study of Herbut (2013) and Hempel (2018), which indicated the need for measurements in the entire area where the cows are housed, and not just in individual points. Kadzere et al. (2002) note that during the hot months, maintaining an air velocity above 1.0 m/s at high humidity (e.g., by means of sprayers) significantly cools the animal's body.

Solar radiation is one of the leading environmental factors affecting ruminants (Schutz et al., 2009). Radiation includes both direct radiation from the sun and diffuse radiation received from the sky and/or reflected from clouds (Da Silva et al., 2010). The effects of radiation, whether direct, diffuse or reflected, can be a major determinant of the environmental conditions in which cows are reared, mainly in grazing (Schutz et al., 2008; Tucker et al., 2008). Studies conducted in free barns show significant differences in microclimate conditions. These variations are the result of the higher air temperature and litter surface observed during the day in boxes adjacent to walls that are exposed to direct sunlight compared to those in the shade (Angrecka and Herbut, 2016).

Many environmental indices have been proposed, which are used to measure meteorological conditions. Examples are the THI, the Black Globe-Humidity Index (BGHI) and the Environmental Sustainability Index (ESI). The situation with heat stress has been shown to worsen when high relative humidity is observed at high air temperatures in the animal environment (Hill and Wall, 2015; Herbut et al., 2018 b). Over the years, two main methods have been developed to assess environmental risk factors and animal responses to changing environmental conditions. The first of them is a variety of different indices of temperature and humidity, expressed in absolute values, which determine the thermal comfort of animals. The second includes algorithms expressed in °C. The indices have undergone many modifications and include different ranges of values that determine the threshold levels of heat stress in dairy cows.

Physiological changes in the body of animals that are affected by the effects of heat stress

Body temperature

The body temperature of cows under thermo-neutral conditions is maintained by the thermoregulatory system in the range of 38 to 39.2°C (Ammer et al., 2016). Under these conditions, the heat exchange in the animal's body (through cell and vascular membranes) and the heat exchange with the environment are balanced. This process is almost always dynamic (Taylor et al., 2014).

When the ambient temperature is elevated, mechanisms are activated in the body that aim to maintain the homeostasis and temperature status of the animals or to regulate them within acceptable physiological limits (Werner et al., 2008). The mechanisms that release excess heat from the body are regulated by the hypothalamus. It receives information about fluctuations in body surface temperatures and deeper tissues from receptors located on them. Under temperature stress, a rapid response is induced, initiated by skin receptors. As a result, the central nervous system, the endocrine system and peripheral components of the autonomic system are activated (Colier et al., 2012).

When thermoregulatory mechanisms prove insufficient to dissipate external heat, body temperature rises. At values > 39.4°C, a state of hyperthermia occurs. Nowadays, temperature changes on the surface of the body as well as in the internal body temperature can be easily monitored using non-invasive methods such as thermography (Godyń et al., 2013; Hoffmann et al., 2013; Unruh et al., 2017 , Hristov et al. 2021a; Hristov et al. 2021b) or small wireless sensors (Lees et al., 2018). Cattle body temperature can be measured in various parts of the body, such as the abdomen, ear canal and vagina, but the most common method of assessing internal body temperature is to measure rectal temperature. Lemerle and Goddard (1986) found that rectal temperature began to rise at THI values > 80. Collier et al. (2006) found that cows reared in shady areas had a rectal temperature 0.5°C (38.9 to 39.4°C) lower than cows exposed to direct sunlight without shade access.

Respiratory rate

Sweating and panting are considered to be the first and main reactions of animals to temperature stress. Collier et al. (2012) reported that the body of animals is in the acute phase of response to heat stress when the skin temperature reaches 35°C and the respiratory rate is 60–70 dd/min. Lemerle and Goddard (1986)

found that the respiratory rate began to increase gradually, at THI > 73, and significantly rapidly at THI > 80. It has been proven that the value of this physiological parameter depends on the amount of shade and cooling in the area where the cows are raised, their age and the time they spend in an upright and lying position. In adult cows, a respiration rate of up to approximately 80 breaths per minute has been observed (Stevens, 1981). In addition, Collier et al. (2006) found that cows kept in shady areas had a respiratory rate of 54 breaths per minute, while cows without access to shading had 82 breaths per minute. Similar results were reported by Eigenberg et al. (2005), whose studies found about 16 rpm more in cows raised without access to shady areas. Cows reared in free barns with a cooling system showed a reduction in the number of breaths per minute from 95 to 57 (West, 2003). Based on studies conducted in the afternoon, Chaiyabutr et al. (2008) found that cows kept in refrigerated rooms made 64 breaths per minute, while cows in uncooled areas made 86 breaths per minute.

Heart rate

When the animal's body is exposed to heat, cardiac output increases. While the stroke volume is maintained or slightly increased, the heart rate accelerates significantly and is the main driving force behind this process (Johnson and Proppe, 1996). Control and regulation of the heart rate in heat stimuli may be a consequence of direct irritation of high temperature on the sinoatrial node and the sympathetic and parasympathetic nerve endings of the heart (Wilson and Crandall, 2011). Kovács et al. (2018) found that Holstein calves reared in conditions of extreme heat load without shade have a higher heart rate than calves kept in the shade. Similar results are shared by Bun et al. (2018) in a study of dairy cows. Dalcin et al. (2016) found that at a BGHI value of 72, the heart rate began to increase linearly in dairy cattle.

Digestion and absorption of nutrients

Digestion is influenced by various factors, such as the time the animals consume the food, the quality of the food, the composition of nutrients, the rate at which nutrients pass through the digestive tract and the volume of the digestive organs (Ellis et al., 1984). All these factors are affected by heat stress. At high temperatures, reduced food intake leads to increased digestive processes by slowing the movement of food in the proventriculus and increasing the volume of the rumen (Lippke et al., 1975). These physiological changes are more pronounced in animals that consume more feed.

Peripheral vasodilation and central vasoconstriction lead to a reduced blood flow to the proventriculus of ruminants (Engelhardt et al., 1977). This in turn reduces the plasma flow through the portal vein, which inhibits nutrient absorption (McGuire et al., 1989).

Influence of heat stress on hormones in dairy cows

The endocrine system, which is a major link in the coordination of metabolism, changes significantly when animals are under heat stress (Beede et al., 1986). Hormones associated with adaptation to heat stress are prolactin (PRL), growth hormone (GH), thyroid hormones, glucocorticoids, mineralocorticoids, atecholamines, and antidiuretic hormone (ADH). Prolactin is vital for mammogenesis (Buttle et al., 1979), lactogenesis (Akers et al., 1981) and to varying degrees for galactopoiesis (Wilde et al., 1996). Plasma PRL concentrations increase during heat stress in dairy cows (Wetteman et al., 1979). Collier et al. (1982) suggest that increased PRL is associated with increased water and electrolyte requirements when animals are exposed to heat stress.

Growth hormone is produced in the anterior pituitary gland. It does not perform its functions through the target gland, but exerts its effect on almost all tissues of the body. Plasma GH levels decreased from 18.2 ng/mL in thermo-neutral environments to 13.5 ng/mL in heat stress in Jersey cows (Mitra et al., 1972). Igono et al. (1988) reported that the GH content in the milk of low, medium and high productive groups of cows decreased when the THI exceeded 70. A decrease in plasma GH was not observed in cows reared in thermo-neutral conditions subjected to the same diet (McGuire et al., 1989). Decreased GH hinders the formation of energy used for heat production in the body of animals (Bauman et al., 1980). GH also promotes heat production by stimulating thyroid activity (Yousef et al., 1966). Therefore, decreased secretion of the growth hormone is more than a physiological response necessary for the survival of homothermic animals at high ambient temperatures.

The thyroid gland secretes triiodothyronine (T3) and thyroxine (T4). These hormones are essential for the regulation of metabolism and have a positive correlation with weight gain and tissue formation (Magdub et al., 1982). The response of T3 and T4 to heat stress is slow and it takes several days to reach a constant level of concentration (Silanikove, 2000). A decrease in plasma T3 concentrations from 2.2 to 1.16 ng/mL was reported by Johnson et al. (1988). This decrease in thyroid hormones together with the decreased level of GH in plasma has a synergistic effect in the body's desire to reduce heat production (Yousef et al., 1966).

Acute and chronic heat stress lead to various changes in glucocorticoid concentrations. Alvarez and Johnson (1973) reported an increase in glucocorticoid levels from 2.4 to 3.9 µg/100 mL (62%) by the second hour of heat exposure, reaching a peak of 5.4 µg/100 mL (120%) at the 4th hour, then gradually decreasing to the norm of 2.4 µg/100 mL at the 48th hour, maintaining this concentration despite the continuing thermal irritant. The initial increase in

plasma glucocorticoids is due to the activation of an adrenocorticotropin-releasing mechanism (ACTH) in the hypothalamus by skin thermoreceptors (Chowers et al., 1966), while a later decrease to normal, despite continued thermal irritation, shows negative feedback between increasing glucocorticoid concentrations and reporting a decrease in glucocorticoid-binding transortin (Lindner, 1964). Glucocorticoids act as vasodilators, promoting heat loss. They have a stimulating effect on proteolysis and lipolysis, thus providing energy to the animal, compensating for reduced food intake (Cunningham and Klein, 2007).

The relationship between heat stress, plasma aldosterone concentration and urinary electrolyte excretion has been documented by El-Nouty et al. (1980). Plasma aldosterone concentrations remained unchanged during the first few hours of heat exposure. However, with prolonged exposure, it is 40% lower and decreases rapidly in the following hours. This decrease in aldosterone levels is due to a decrease in serum K levels as a result of increased sweat excretion (El-Nouty et al., 1980) and is explained by the large difference between ruminants and non-ruminants in terms of Na and K during heat stress. Non-ruminants excrete sweat with high Na concentration and low K concentration (Lippsett et al., 1961); unlike ruminants, in which the opposite is true. The concentration of catecholamines increases in both acute and chronic heat stress. Alvarez and Johnson (1973) reported an average increase of 45% and 42% for short and 91% and 70% for long heat exposure for adrenaline and noradrenaline, respectively. Allen and Bligh (1969) reported that catecholamines activate the sweat glands and participate in the regulation of their activity.

Increased plasma osmolarity and decreased blood volume lead to secretion of ADH by the pituitary gland, which in turn acts on the kidneys, leading to water retention (Cunningham and Klein, 2007). Increased loss of water through the airways and skin of heat-stressed animals results in increased secretion of ADH, which is intended to retain water in the body and increase its intake (El-Nouty, 1980).

Acid-base balance and heat stress

Cows subjected to heat stress usually show changes in acid-base balance as a result of physiological reactions accompanying the cooling of the body. Frequent respiratory activity and sweating increase in proportion to the body's need for cooling. Accelerating respiration increases CO2 loss through pulmonary ventilation, reduces the concentration of carbonic acid in the blood and upsets the balance with bicarbonate, which changes the pH of the blood and leads to respiratory alkalosis (Benjamin, 1981). Compensation for respiratory alkalosis includes increased urinary excretion of bicarbonate (Benjamin, 1981), which leads to a decrease in its concentration in the blood.

Influence of heat stress on productive indicators

Amount of milk

Lactating cows are more sensitive to heat stress than dry cows. This is due to milk production, which significantly speeds up metabolism (Purwanto et al., 1990). In addition, due to the positive relationship between milk production and heat production, cows with higher milk yields are more prone to heat stress than animals with lower milk yield (Spiers et al., 2004). When a cow is under heat stress, adaptive mechanisms are activated that reduce the nutrients used for milk synthesis (West, 2003; Rhoads et al., 2009). At the same time, it speeds up the metabolism caused by the activation of the thermoregulatory system. Under mild to severe heat stress, the requirements for maintaining a normal metabolism can increase from 7 to 25% (NRC, 2001), which can lead to a significant decline in milk production. Reduced milk production is often used in various studies as an indicator of reduced welfare of animals that are already susceptible to diseases such as mastitis (Gröhn et al., 2004). Rushen et al. (2001) reported that milk yield decreased instantaneously when cows were exposed to a stressful or unfamiliar environment. In this regard, it is often accepted that milk production can be interpreted as a direct indicator of animal welfare and can be used by farmers as a way to assess the condition of cattle in changes in their environment (e.g., increase in ambient temperature or changes in diet). Others have challenged milk production as an acceptable indicator of well-being (von Keyserlingk et al., 2009), especially in cows exposed to heat stress. The reason is the delay in registering a decline in milk production after the animals have already been exposed to high ambient temperatures. Collier et al. (1981) reported a delay of 24 to 48 hours from an increase in ambient temperature to a decline in milk production. Additional evidence provided by Linvill and Pardue (1992) indicates that milk production only begins to decline when the THI consistently exceeds 74 for the previous 4 days. From this, it is clear that if changes in milk production are detected only on days after which the animals have already been under heat stress, this measure is limited and at best indirect to assess the welfare of cattle (von Keyserlingk et al., 2009). Despite the identified barriers to the use of milk production as an indicator of welfare in dairy cows, recent data suggest that changes in milk composition may be far more useful in assessing the condition of animals exposed to immediate heat stress (Hu et al., 2016).

Relationship between heat stress, dry matter intake and milk production

Many scientific publications show a link between the occurrence of heat stress and reduced dry matter intake (DMI), as this is an immediate adaptive response in animals (Kadzere et al., 2002; West, 2003; Rhoads et al., 2009). The reduced productivity

of the animals during heat stress is explained only by parts with reduced DMI. Baumgard et al. (2011) claim that lower consumption of heat-stressed cattle explains only 35–50% of the decline in milk yield. According to Slimen et al. (2016), heat stress causes a reorganization in the use of body resources such as fat, protein and energy. Post-absorption metabolism is altered, and this occurs regardless of the decline in food intake (Slimen et al., 2016). Noordhuizen and Bonnefoy (2015) found a decrease in milk production of 600–900 kg of milk per lactating cow and a decrease in feed intake with 0.85 kg DMI per cow less for each 1° increase in ambient temperature C (West, 2003). According to Kadzere et al. (2002), DMI in cows can report a decrease of up to 40% when the ambient temperature exceeds 30°C, which leads to a deterioration of the energy balance. According to research by Bouraoui et al. (2002), increasing the value of THI from 68 to 78, leads to a decrease in DMI by up to 9.6%. In West's study (2003), food intake began to decline one day after the onset of heat stress. In addition, West (2003) found a decrease in milking 2 days after the animals were under heat stress. The study by Herbut et al. (2018), conducted in free barns, also revealed a 2- to 4-day delay before a decline in milk production was found. Studies show that the decline in milk production depends on both the strength of the heat wave and the length of previous warm periods. The large number of hot days in July and August leads to a rapid response of animals to subsequent changes in thermal conditions in the coming months (Herbut et al., 2018a).

Relationship between heat stress, water intake and milk production

Water intake is extremely important for dairy cattle. For cows producing 41.5 kg of milk per day under thermo-neutral conditions, the water intake is about 135 kg per day (Kadzere et al., 2002). Variations in water intake are closely related to DMI and milk yield, ambient temperature, and relative humidity (Cardot et al., 2008). Water for the animals must be provided in appropriate quantities and temperatures. A 10% decrease in the body's water supply in cows can adversely affect milk production (González Pereyra et al., 2010).

Quality composition of milk

Results from studies by various authors (Bouraoui et al., 2002; Hammami et al., 2013) also report a direct link between heat stress and deterioration in milk quality. Poor temperature and humidity conditions lead to an increase in the number of somatic cells in milk and a decrease in fat and protein (Hammami et al., 2013; Lambertz et al., 2014). As THI increases, so does the number of somatic cells.

Lipids are one of the main components of milk. The dominant fraction of milk fat is TAG (about 98%), present in the form of fat globules (Mansson, 2008). In addition to being an energy source, the composition of TAG is important for human health and the properties of dairy products (Jensen, 2002; Palmquist, 2006). The second most important fraction of milk fat are polar lipids, which are a major structural element of the membrane of fat globules and thus play the role of emulsifier, ensuring the stability of the milk emulsion system (Fong et al., 2007, Sánchez-Juanes et al. 2009). The main classes of lactic polar lipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), sphingolinositol (PI), sphingomylyscholine LP, lactosylceramide (GC) and laczylceramide (2002).

By correlating the meteorological data with the characteristics of fatty acids (FA), it was found that an increase in THI shows a decrease in the content of short-chain and medium-chain fatty acids and an increase in long-chain (Hammami et al., 2015). A similar conclusion was made in other studies of heat-stressed cows (Lacetera et al., 2003). However, these results have not always been convincing, as the effect of heat stress is often confused with different eating patterns over the seasons. Regarding the effect of heat stress on the TAG profile and polar lipid composition, no information is available.

Influence of heat stress on reproductive indicators

The decline in the number of cows bred during the summer season can vary between 20 and 30%, despite the presence of animals that clearly show estrus (De Rensis and Scaramuzzi, 2003). High ambient temperatures have a negative effect on a cow's ability to behave naturally during heat, as it reduces the duration and intensity of estrous expression (Orihuela, 2000). The reason for this is considered to be the reduced intake of dry matter and the subsequent disturbance in the production of hormones (Westwood et al., 2002). An additional reason is the desire of man to turn cows from a "seasonal" to a "year-round" breeding unit. The adverse effects of heat stress on the reproductive cycle are year-round, but significantly more severe during the summer months. Hansen and Aréchiga (1999) report reduced estrus in heat-stressed dairy cows. These authors believe that heat stress causes physical lethargy, which acts as an adaptive mechanism that limits the additional heat production of the animal already generated by activities during estrus. Additional evidence suggests that jumps as an indicator of estrus in beef cattle are significantly less in summer than in winter (White et al., 2002). A shorter duration of estrus is found when European breeds move to the tropics, with differences in climate and nutrition (Orihuela, 2000). Reproductive indicators are often used as an indicator of well-being in heat-stressed cows, as problems with animal breeding (De Rensis and Scaramuzzi, 2003), ovum quality disorders (Roth et al., 2001) and abortion or early embryonic mortality (Silanikove, 2000) are common during these periods. However,

these indicators are retrospective in nature and only give us information that the animal was already in a state of stress. Therefore, these data are of greater value in the management of future nurseries and as a means of determining the need to implement improved strategies to combat heat stress. A more accurate and useful indicator for assessing well-being is measuring the rectal temperature on the day of insemination. Pereira et al. (2013) reported that the chance of fertilization up to 60 days registered a decrease from 21% to 15% at a rectal temperature higher than 39.1°C found during artificial insemination.

Heat stress alters the reactions along the hypothalamic-pituitary-ovarian axis

Because the main hormones that regulate ovarian function are gonadotropin-releasing hormone from the hypothalamus and gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary gland, some authors have investigated the effect of heat secretion on stress. Changes in LH concentration under the influence of heat stress in the peripheral blood are intermittent. Some studies report unchanged concentrations (Gwazdauskas et al., 1985; Gauthier, 1986), while others report an increase (Roman-Ponce et al., 1981) and reduced concentrations (Madan and Johnson et al., 1973; Wise et al., 1988; Gilad et al., 1993; Lee, 1993). Regarding the model of LH secretion in cows subjected to heat stress, there is a decrease in the amplitude of the LH pulse (Gilad et al., 1993) and LH pulse, as well as in their frequency (Wise et al., 1988). The effect of heat stress on the LH preovulatory peak is also controversial: a decrease in the endogenous LH peak from heat stress has been reported in heifers (Madan and Johnson, 1973) but not in cows (Gwazdauskas et al., 1981; Gauthier, 1986; Rosemberg et al., 1981). The reasons for these discrepancies are unclear. These differences are thought to be related to preovulatory estradiol levels, as the amplitude of tonic LH impulses and the GnRH-induced preovulatory plasma peak of LH are lower in cows with low plasma estradiol concentrations but not in cows with high plasma concentrations of estradiol (Gilad et al., 1993). Plasma inhibin concentrations in summer are lower in heat-stressed cows (Wolfenson et al., 1995), which may reflect impaired folliculogenesis, as a significant proportion of plasma inhibin comes from small and medium-sized follicles. Plasma FSH concentrations are higher during the preovulatory period in summer; this is associated with lower circulating concentrations of inhibin (Ingraham et al., 1974).

Influence of heat stress on gametogenesis and embryo

Gametogenesis is sensitive to temperature changes. Normal spermatogenesis requires a temperature that is below normal body temperature. Recent evidence suggests that oocyte development is also sensitive to temperature (Rutledge et al., 1999). The negative effects of heat stress on fertility may be the result of the direct effect of high temperatures on the ovaries and the quality of oocytes, respectively.

The intrauterine environment is also compromised in cows that are subjected to heat stress; decreased blood flow to the uterus and increased temperature (Roman-Ponce et al., 1978; Gwazdauskas et al., 1975). These changes inhibit embryonic development (Rivera and Hansen, 2001), increase early embryonic death, and lead to unsuccessful inseminations. The high ambient temperature indicates a negative effect on the embryos in the pre-attachment stage (Ray et al. 1992), but the degree of this effect decreases gradually with the development of the embryo (Ealy et al., 1993). Heat stress can affect the endometrium of the uterus, leading to premature secretion of prostaglandins (Putney et al., 1989), followed by luteolysis and fetal loss. Most often, embryonic death occurs by the 42nd day.

Influence of heat stress on the development of follicles

Heat stress slows follicle expression and prolongs follicular wave, leading to adverse effects on oocyte quality (Roth et al., 2001; Badinga et al., 1993) and follicular steroidogenesis (Roth et al., 2001; Howell et al., 1994; Palta et al., 1997). Heat stress suppresses the development of dominant follicles, which causes more medium-sized follicles to survive (Wolfenson et al., 1995; Roth et al., 2000; Wilson et al. 1998; Vasconcelos et al., 1998; Badinga et al., 1993). Thus, the duration of preovulatory follicle dominance increases in summer, which in heifers is negatively related to fertility (Mihm et al., 1994). When the expression of an individual dominant follicle is suppressed, it is possible to develop more than one dominant follicle, which is reflected in twins, which can often be observed in summer (Ryan et al., 1991).

Conclusion

As a result of the review, it became clear that the topic of heat stress and its impact on dairy cows has been widely studied in many parts of the world. Despite the many data from various authors, there is still no unanimous opinion on which indices are the most accurate and at which values of the temperature-humidity index measures need to be taken. Particularly interesting is the question of the adaptability of dairy cattle to heat stress and its effects on their physiological, productive and reproductive indicators. Following the review, it is clear that research on the issue is likely to continue in order to find adequate solutions to the issue of heat stress and its impact on dairy cows.

References

1. Ammer S., Lambertz C., Gauly M. (2016). Comparison of different measuring methods for body temperature in lactating cows under different climatic conditions. J. Dairy. Res., 83: 165–172.
2. Angrecka S., Herbut P. (2016). Impact of barn orientation on insolation and temperature of stalls surface. Ann. Anim. Sci., 16: 887–896.
3. Akers, R.M., D.E. Bauman, A.V. Capuco, G.T. Goodman and H.A. Tucker, 1981. Prolactin regulation of milk secretion and biochemical differentiation of mammary epithelial cells in periparturient cows. Endocrinology, 109: 23-30.
4. Allen, T.E. and J. Bligh, 1969. A comparative study of the temporal patterns of cutaneous water vapor loss from some domesticated mammals with epithelial sweat glands. Comp. Biochem. Physiol., 31: 347.
5. Alvarez, M.B. and J.D. Johnson, 1973. Environmental heat exposure on cattle plasma catecholamine and glucocorticoids. J. Dairy Sci., 56: 189-194.
6. Bailey T., Sheet s J., McClary D., Smith S., Bridges A. (2016). Heat Abatement. Elanco.
7. Baumgard L.H., Wheelock J.B., Sanders S.R., Moore C.E., Green H.B., Waldron M.R., Rhoads R.P. (2011). Post absorptive carbohydrate adaptations to heat stress and monensin supplementation in lactating Holstein cows. J. Dairy Sci., 94: 5620–5633.
8. Berman A. (2005). Estimates of heat stress relief needs for Holstein dairy cows. J. Anim. Sci., 83: 1377–1384.
9. Berman A., Folman Y., Kaim M., Mamen M., Herz Z., Wolfenson D., Arieli A., Graber Y. (1985). Upper critical temperatures and forced ventilation effects for high yielding dairy cows in a sub-tropical climate. J. Dairy Sci., 68: 1488–1495.
10. Bouraoui R., Lahmar M., Majdoub A., Djemali M., Belyea R. (2002). The relationship of temperature-humidity index with milk production of dairy cows in a Mediterranean climate. Anim. Res., 51: 479–491.
11. Badinga L, Thatcher WW, Diaz T, Drost M, Wolfenson D. Effect of environmental heat stress on follicular development and steroidogenesis in lactating Holstein cows. Theriogenology 1993;39:797–810.
12. Bauman, D.E. and W.B. Currie, 1980. Partitioning of nutrients during pregnancy and lactation. A review of mechanisms involving homesostasis and homeorhesis. J. Dairy. Sci., 63: 1514.
13. Beede, D.K. and R.J. Collier, 1986. Potential nutritional strategies for intensively managed cattle during thermal stress. J. Anim. Sci., 62: 543-554.
14. Benjamin, M.M., 1981. Fluid and electrolytes. Outline of Veterinary Clinical Pathology. Iowa State Univ. Press, Ames. USA.
15. Bun C., Watanabe Y., Uenoyama Y., Inoue N., Ieda N., Matsuda F., Tsukamura H., Kuwahara M., Maeda K.I., Ohkura S., P h e n g V. (2018). Evaluation of heat stress response in crossbred dairy cows under tropical climate by analysis of heart rate variability. J. Vet. Med. Sci., 80: 181–185.
16. Buttle, H.L., A.T. Cowie, E.A. Jones and A. Turvey, 1979. Mammary growth during pregnancy in hypophysectomized or bromocriptine-treated goats. J. Endocr., 80: 343-351.
17. Cardot V., Le Roux Y., Jurjanz S. (2008). Drinking behavior of lactating dairy cows and prediction of their water intake. J. Dairy Sci., 91: 2257–2264.
18. Chaiyabutr N., Chanpongsang S., Suadsong S. (2008). Effects of evaporative cooling on the regulation of body water and milk production in crossbred Holstein cattle in a tropical environment. Int. J. Biometerol., 52: 575–585.
19. Collier R.J., Dahl G.E., VanBaale M.J. (2006). Major advances associated with environmental effects on dairy cattle. J. Dairy Sci., 89: 1244–1253.
20. Collier R.J., Gebremedhin K., Macko A.R., Roy K.S. (2012). Genes involved in the thermal tolerance of livestock. In: Environmental stress and amelioration in livestock production, Sejian V., Naqvi S.M.K., Ezeji T., Lakritz J., Lal R. (eds). Springer-Verlag (publisher), Berlin Heidelberg, Germany, pp. 379–410.
21. Chebel, R. C., J. E. P. Santos, J. P. Reynolds, R. L. A. Cerri, S. O. Juchem, and M. Overton. 2004. Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. Anim. Reprod. Sci. 84:239–255. https://doi.org/10.1016/j .anireprosci.2003.12.012.
22. Chowers, I., H.T. Hammel, J. Eisenman, R.M. Abrams and S.M. McCann, 1966. Comparison of effect of environmental and preoptic heating and pyrogen on plasma cortisol. Amer. J. Physiol., 210: 606.
23. Collier, R. J., R. M. Eley, A. K. Sharma, R. M. Pereira, and D. E. Buffington. 1981. Shade management in subtropical environment for milk yield and composition in Holstein and Jersey cows. J. Dairy Sci. 64:844–849. https://doi.org/10.3168/jds.s0022-0302(81)82656 -2.
24. Collier, R.J., D.K. Beede, W.W. Thatcher, L.A. Israel and C.J. Wilcox, 1982. Influences of environment and its modification on dairy animal health and production. J. Dairy Sci., 65: 2213-2227.
25. Cunningham, J.G. and B.G. Klein, 2007. Veterinary Physiology. (4 .Ed.) Saunders Elsevier. Missouri. th USA.
26. Dalcin V.C., Fischer V., Daltro D.D., Alfonzo E.P., Stumpf M.T., Kolling G.J., Sil-va M.V., McManus C. (2016). Physiological parameters for thermal stress in dairy cattle. R. Bras. Zootec., 45: 458–465
27. Da Silva R.G., Guilhermino M.M., Morais D.A.E.F. (2010). Thermal radiation absorbed by dairy cows in pasture. Int. J. Biometeorol., 54: 5–11.
28. Davis S., Mader T. (2003). Adjustments for wind speed and solar radiation to the temperaturehumidity index. Nebr. Beef. Cattle Rep., 224: 48–51.
29. De Rensis, F., and R. J. Scaramuzzi. 2003. Heat stress and seasonal effects on reproduction in the dairy cow—A review. Theriogenology 60:1139–1151. https://doi.org/10.1016/s0093-691x(03)00126-2.
30. Dikmen, S., and P. J. Hansen. 2009. Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? J. Dairy Sci. 92:109–116. https://doi .org/10.3168/jds.2008-1370.
31. Eigenberg R.A., Brown - Brandl T.M., Nienaber J.A., Hahn G.L. (2005). Dynamic response indicators of heat stress in shaded and non-shaded feedlot cattle, Part 2: Predictive Relationships. Biosyst. Eng., 91: 111–118
32. Ealy AD, Drost M, Robinson OW, Britt JH. Developmental changes in embryonic resistance to adverse effects of maternal heat stress in cows. J Dairy Sci 1993;76:2899–905.
33. Ellis, W.C., J.H. Matis, K.R. Pond, C.E. Lascano and J.P. Telford, 1984. Dietary influences on flow rate and digestive capacity. In: F.M.C. Gilchrist and R. I. Mackie (Ed.) Herbivore Nutrition in the Subtropics and Tropics. pp.269-293. The Science Press (PTY) Ltd., Craighill, South Africa.
34. El-Nouty, F.D., I.M. Elbanna, T.P. Davis and H.D. Johnson, 1980. Aldosterone and ADH response to heat and dehydration in cattle. J. Appl. Physiol. Respir. Environ. Exercise Physiol., 48: 249.
35. Engelhardt, W. Von and J.R.S. Hales, 1977. Partition of capillary blood flow in rumen, reticulum and omasum of sheep. Amer. J. Physiol., 232: E53.
36. Fong, B. Y., Norris, C. S. & MacGibbon, A. K. H. Protein and lipid composition of bovine milk-fat-globule membrane. International Dairy Journal 17, 275–288 (2007).
37. Godyń D., Herbut E., Walczak J. (2013). Infrared thermography as a method for evaluating the welfare of animals subjected to invasive procedures – a review. Ann. Anim. Sci., 13: 423–434.
38. González Pereyra A.V., Maldonado May V., Catracchia C.G., Herrero M.A., Flores M.C., Mazzini M. (2010). Influence of water temperature and heat stress on drinking water intake in dairy cows. Chil. J. Agric. Res., 70: 328–336.
39. Gauthier D. The influence of season and shade on estrous behaviour, timing of preovulatory LH surge and the pattern of progesterone secretion in FFPN and Creole heifers in a tropical climate. Reprod Nutr Dev 1986;26:767–75.
40. Gilad E, Meidan R, Berman A, Graber Y, Wolfenson D. Effect of heat stress on tonic and GnRH-induced gonadotrophin secretion in relation to concentration of oestradiol in plasma of cyclic cows. J Reprod Fertil 1993;99:315–21.
41. Gröhn, Y. T., D. J. Wilson, R. N. González, J. A. Hertl, H.

Schulte, G. Bennett, and Y. H. Schukken. 2004. Effect of pathogen-specific clinical mastitis on milk yield in dairy cows. J. Dairy Sci. 87:3358– 3374. https://doi.org/10.3168/jds.s0022-0302(04)73472-4.
42. Gwazdauskas FC, Thatcher WW, Kiddy CA, Pape MJ, Wilcox CJ. Hormonal pattern during heat stress following PGF2alpha-tham salt induced luteal regression in heifers. Theriogenology 1981;16:271–85.
43. Gwazdauskas FC, Wilcox CJ, Thatcher WW. Environmental and management factors affecting conception rate in a subtropical climate. J Dairy Sci 1975;58:88–92.
44. Hammami H., Bormann J., M'Hamdi N., Montaldo H.H., Gengler N. (2013). Evaluation of heat stress effects on production traits and somatic cell score of Holsteins in a temperate environment. J. Dairy Sci., 96: 1844–1855.
45. Hempel S., König M., Menz C., Janke D., Amon B., Banhazi T.M., Estellés F., Amon T. (2018). Uncertainty in the measurement of indoor temperature and humidity in naturally ventilated dairy buildings as influenced by measurement technique and data variability. Biosyst. Eng., 166: 58–75.
46. Herbut P., Angrecka S., Godyń D. (2018 a). Effect of the duration of high air temperature on cow's milking performance in moderate climate conditions. Ann. Anim. Sci., 18: 195–207.
47. Herbut P., Angrecka S., Nawalany G. (2013). Influence of wind on air movement in a free stall barn during the summer period. Ann. Anim. Sci., 13: 109–119.
48. Herbut P., Angrecka S., Walczak J. (2018 b). Environmental parameters to assessing of heat stress in dairy cattle – a review. Int. J. Biometerol., 62: 2089–2097
49. Hill D.L., Wall E. (2015). Dairy cattle in a temperate climate: the effects of weather on milk yield and composition depend on management. Animal, 9: 138–149.
50. Hoffmann G., Schmidt M., Ammon C., Rose - Meierhöfer S., Burfeind O., Heu - wieser W., Berg W. (2013). Monitoring the body temperature of cows and calves using video recordings from an infrared thermography camera. Vet. Res. Commun., 37: 91–99.
51. Hammami, H. et al. Genetic analysis of heat stress effects on yield traits, udder health, and fatty acids of Walloon Holstein cows. J Dairy Sci 98, 4956–68 (2015)
52. Hansen, P. J., and C. F. Aréchiga. 1999. Strategies for managing reproduction in the heat-stressed dairy cow. J. Anim. Sci. 77:36. https://doi.org/10.2527/1997.77suppl_236x.
53. Howell JL, Fuquay JW, Smith AE. Corpus luteum growth and function in lactating Holstein cows during spring and summer. J Dairy Sci 1994;77:735–9.
54. Hu, H., Y. Zhang, N. Zheng, J. Cheng, and J. Wang. 2016. The effect of heat stress on gene expression and synthesis of heat-shock and milk proteins in bovine mammary epithelial cells. Anim. Sci. J. 87:84–91. https://doi.org/10.1111/asj.12375.
55. Igono, M.O., H.D. Johnson, B.J. Steevens, W.A. Hainen and M.D. Shanklin, 1988. Effect of season on milk temperature, milk growth hormone, prolactin and somatic cell counts of lactating cattle. Intl. J. Biometeor., 32: 194-200.
56. Ingraham RH, Gillette DD, Wagner WD. Relationship of temperature and humidity to conception rate of Holstein cows in subtropical climate. J Dairy Sci 1974;57:476–81.
57. Johnson J.M., Proppe D.W. (1996). Cardiovascular adjustments to heat stress. In: Handbook of physiology: Environmental physiology, Fregly M.J., Blatteis C.M. (eds). Oxford University Press, New York, USA, pp. 215–243.
58. Jensen, R. G. The composition of bovine milk lipids: January 1995 to December 2000. J Dairy Sci 85, 295–350 (2002).
59. Johnson, H.D., P.S. Katti, L. Hahn and M.D. Shanklin, 1988. Short-term heat acclimation effects on hormonal profile of lactating cows. Univ. of Missouri Rsch. Bull. No. 1061. Columbia. USA.
60. Kadzere C.T., Murphy M.R., Silanikove N., Maltz E. (2002). Heat stress in lactating dairy cows: a review. Livest. Prod. Sci., 77: 59–91.
61. Kovács L., Kézér F.L., Ruff F., Jurkovich V., Szenci O. (2018). Assessment of heat stress in 7-week old dairy calves with non-invasive physiological parameters in different thermal environments. Plos One, 13: e0200622.
62. Lambertz C., Sanker C., Gauly M. (2014). Climatic effects on milk production traits and somatic cell score in lactating Holstein-Friesian cows in different housing systems. J. Dairy. Sci., 97: 319–329.
63. Lees A.M., Lees J.C., Lisle A.T., Sullivan M.L., Gaughan J.B. (2018). Effect of heat stress on rumen temperature of three breeds of cattle. Int. J. Biometeorol., 62: 207–215.
64. Lemerle C., Goddard M.E. (1986). Assessment of heat stress in dairy cattle in Papua New Guinea. Trop. Anim. Health Prod., 18: 232–242.
65. Lacetera, N., Bernabucci, U., Ronchi, B. & Nardone, A. Physiological and productive consequences of heat stress: the case of dairy ruminants. In Interactions between climate and animal production (eds. Lacetera, N. et al.) 45–59 (Wageningen Academic Publishers, 2003).
66. Lee CN. Environmental stress effect on bovine reproduction. Vet Clin North Am 1993;9:263–73.
67. Lindner, H.R., 1964. Comparative aspects of cortisol transport. Lack of firm binding to plasma proteins in domestic ruminants. J. Endocrinol., 28: 301.
68. Linvill, D. E., and F. E. Pardue. 1992. Heat stress and milk production in the South Carolina coastal plains. J. Dairy Sci. 75:2598–2604. https://doi.org/10.3168/jds.s0022-0302(92)78022-9.
69. Lippke, H., 1975. Digestibility and volatile fatty acids in steers and wethers at 21 and 32 C ambient temperatures. J, Dairy Sci., 58: 1860.
70. Lippsett, M.B., T.L. Schwartz and N.A. Thon, 1961. Hormonal control of sodium, potassium, chloride and water metabolism. In: Comar, C.L. and F. bonner (Ed.) Mineral Metabolism, Vol. lB. Academic Press, New York. USA.
71. Madan ML, Johnson HD. Environmental heat effects on bovine luteinizing hormone. J Dairy Sci 1973;56:1420–3.
72. Magdub, A., H.D. Johnson and R.L. Belvea, 1982. Effect of environmental heat and dietary fiber on thyroid physiology of lactating cows. J. Dairy. Sci., 65: 2323.
73. Mansson, H. L. Fatty acids in bovine milk fat. Food Nutr Res 52 (2008)
74. McGuire, M.A., D.K. Beede, M.A. DeLorenzo, C.J. Wilcox, G.B. Huntington, C.K. Reynolds and R.J. Collier, 1989. Effects of thermal stress and level of feed intake on portal plasma flow and net fluxes of metabolites in lactating Holstein cows. J. Anim. Sci., 67: 1050-1060.
75. Mihm M, Baguisi A, Boland MO, Roche JF. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. J Reprod Fertil 1994;102:123–30.
76. Mitra, R., G.I. Christison and H.D. Johnson, 1972. Effect of prolonged thermal exposure on growth hormone (GH) secretion in cattle. J. Anim. Sci., 34: 776-779.
77. Noordhuizen J., Bonnefoy J.M. (2015). Heat stress in dairy cattle: major effects and practical management measures for prevention and control. SOJ Vet. Sci., 1: 1–7. Doi: http://dx.doi.org/10.15226/2381-2907/1/1/00103.
78. NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
79. Orihuela, A. 2000. Some factors affecting the behavioral manifestation of oestrus in cattle: A review. Appl. Anim. Behav. Sci. 70:1–16. https://doi.org/10.1016/s0168-1591(00)00139-8
80. Palmquist, D. L. Milk Fat: Origin of Fatty Acids and Influence of Nutritional Factors Thereon. In Advanced Dairy Chemistry Volume 2 Lipids (eds Fox, P. F. & McSweeney, P. L. H.) 43–92 (Springer US, Boston, MA, 2006).
81. Palta P, Mondal S, Prakas BS, Madan ML. Peripheral inhibin levels in relation to climatic variations and stage of estrous cycle in buffalo (Bubalus bubalis). Theriogenology 1997;47:898–995
82. Pereira, M. H. C., A. D. P. Rodrigues, T. Martins, W. V. C. Oliveira, P. S. A. Silveira, M. C. Wiltbank, and J. L. M. Vasconcelos. 2013. Timed artificial insemination programs during the summer in lactating dairy cows: Comparison of the 5-d Cosynch protocol with an estrogen/progesterone-based protocol. J. Dairy Sci. 96:6904– 6914. https://doi.org/10.3168/jds.2012-6260
83. Purwanto, B. P., Y. Abo, R. Sakamoto, F. Furumoto, and S. Yamamoto. 1990. Diurnal patterns of heat production and

heart rate under thermoneutral conditions in Holstein Friesian cows differing in milk production. J. Agric. Sci. 114:139 https://doi.org/10.1017/ s0021859600072117.
84. Putney DJ, Mullins S, Thatcher WW, Drost M, Gross TS. Embryonic development in superovulated dairy cattle exposed to elevated ambient temperature between the onset of estrus and insemination. Anim Reprod Sci 1989;19:37–51.
85. Ray DE, Halbach TJ, Armstrong DV. Season and lactation number effects on milk production and reproduction in dairy cattle in Arizona. J Dairy Sci 1992;75:2976–83.
86. Renaudeau, D., A. Collin, S. Yahav, V. De Basilio, J. L. Gourdine, and R. J. Collier. 2012. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6:707–728. https://doi.org/10.1017/s1751731111002448.
87. Rhoads, M. L., R. P. Rhoads, M. J. VanBaale, R. J. Collier, S. R. Sanders, W. J. Weber, B. A. Crooker, and L. H. Baumgard. 2009. Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. J. Dairy Sci. 92:1986–1997. https://doi.org/10.3168/ jds.2008-1641.
88. Rivera RM, Hansen PJ. Development of cultured bovine embryos after exposure to high temperatures in the physiological range. Reproduction 2001;121:107–15.
89. Roman-Ponce H, Thatcher WW, Canton D, Barron DH, Wilcox CJ. Thermal stress efects on uterine blood flow in dairy cows. J Anim Sci 1978;46:175–80.
90. Roman-Ponce H, Thatcher WW, Wilcox CJ. Hormonal interrelationship and physiological responses of lactating dairy cows to shade management system in a subtropical environment. Theriogenology 1981;16:139–54.
91. Rosemberg M, Folman Y, Herz Z, Flamenbaum I, Berman A, Kaim M. Effect of climatic condition on peripheral concentrations of LH, progesterone and oestradio-17beta in high milk-yielding cows. J Reprod Fertil 1982;66:139–46.
92. Roth Z, Meidan R, Braw-Tal R, Wolfenson D. Immediate and delayed effect of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. J Reprod Fertil 2000;120:83–90.
93. Roth, Z., A. Arav, A. Bor, Y. Zeron, R. Braw-Tal, and D. Wolfenson. 2001. Improvement of quality of oocytes collected in the autumn by enhanced removal of impaired follicles from previously heat-stressed cows. Reproduction 122:737–744. https://doi.org/10 .1530/rep.0.1220737.
94. Rushen, J., L. Munksgaard, P. G. Marnet, and A. M. De Passillé. 2001. Human contact and the effects of acute stress on cows at milking. Appl. Anim. Behav. Sci. 73:1–14. https://doi.org/10.1016/s0168 -1591(01)00105-8.
95. Rutledge JJ, Monson RL, Northey DL, Leibfried-Rutledge ML. Seasonality of cattle embryo production in a temperate region. Theriogenology 1999;51:330 [abstract].
96. Ryan DP, Boland MP. Frequency of twin births among Holstein X Friesian cows in a warm dry climate. Theriogenology 1991;36:1–10.
97. Schutz K.E, Rogers A.R., Cox N.R., Tucker C.B. (2009). Dairy cows prefer shade that offers greater protection against solar radiation in summer: Shade use, behaviour, and body temperature. Appl. Anim. Behav. Sci., 116: 28–34.
98. Schutz K.E., Cox N.R., Mattews L.R. (2008). How important is shade to dairy cattle? Choice between shade or lying following different levels of lying deprivation. Appl. Anim. Behav. Sci., 114: 307–318.
99. Slimen B.I., Taha N., Abdeljelil G., Manef A. (2016). Heat stress effects on livestock: molecular, cellular and metabolic aspects, a review. J. Anim. Physiol. Anim. Nutr., 100: 401–412.
100. Stevens D.C. (1981). A model of respiratory vapor loss in Holstein dairy cattle. Trans ASAE, 24: 151–153.
101. Sánchez-Juanes, F., Alonso, J. M., Zancada, L. & Hueso, P. Distribution and fatty acid content of phospholipids from bovine milk and bovine milk fat globule membranes. International Dairy Journal 19, 273–278 (2009).
102. Schär, C., P. L. Vidale, D. Lüthi, C. Frei, C. Häberli, M. A. Liniger, and C. Appenzeller. 2004. The role of increasing temperature variability in European summer heatwaves. Nature 427:332–336. https://doi.org/10.1038/nature02300.
103. Schüller, L. K., O. Burfeind, and W. Heuwieser. 2014. Impact of heat stress on conception rate of dairy cows in the moderate climate considering different temperature–humidity index thresholds, periods relative to breeding, and heat load indices. Theriogenology 81:1050–1057. https://doi.org/10.1016/j.theriogenology.2014.01 .029.
104. Silanikove, N., 2000. Effects of heat stress on the welfare of extensively managed domestic ruminants. Livestock Production Science, 67: 1-18.
105. Spiers, D. E., J. N. Spain, J. D. Sampson, and R. P. Rhoads. 2004. Use of physiological parameters to predict milk yield and feed intake in heat-stressed dairy cows. J. Therm. Biol. 29:759–764. https://doi .org/10.1016/j.jtherbio.2004.08.051.
106. Taylor N.A., Tipton M.J., Kenny G.P. (2014). Considerations for the measurement of core, skin and mean body temperatures. J. Therm. Biol., 46: 72–101.
107. Tucker C.B., Rogers A.R., Schutz K.E. (2008). Effect of solar radiation on dairy cattle behaviour, use of shade and body temperature in a pasture-based system. App. Anim. Behav. Sci., 109: 141–154.
108. Unruh E.M., Theurer M.E., White B.J., Larson R.L., Drouillard J.S., Schrag N. (2017). Evaluation of infrared thermography as a diagnostic tool to predict heat stress events in feedlot cattle. Am. J. Vet. Res., 78: 771–777.
109. Vasconcelos JLM, Silcox RW, Lacerda JA, Pursley GR, Wiltbank MC. Pregnancy rate, pregnancy loss, and response to heat stress after AI at 2 different times from ovulation in dairy cows. Biol Reprod 1998;56(Suppl 1):140 [abstract].
110. von Keyserlingk, M. A. G., and M. J. Hötzel. 2015. The ticking clock: Addressing farm animal welfare in emerging countries. J. Agric. Environ. Ethics 28:179–195.
111. von Keyserlingk, M. A. G., J. Rushen, A. M. de Passillé, and D. M. Weary. 2009. Invited review: The welfare of dairy cattle—Key concepts and the role of science. J. Dairy Sci. 92:4101–4111. https:// doi.org/10.3168/jds.2012-6354.
112. Wilson T.E., Crandall C.G. (2011). Effect of thermal stress on cardiac function. Exerc. Sport Sci. Rev., 39: 12–17.
113. Werner J., Mekjavic I.B., Taylor N.A.S. (2008). Concepts in physiological regulation: a thermoregulatory perspective. In: Physiological bases of human performance during work and exercise, Taylor N.A.S., Groeller H. (eds). Churchill Livingstone, London, United Kingdom, pp. 325–340.
114. West, J. W. 2003. Effects of heat-stress on production in dairy cattle. J. Dairy Sci. 86:2131–2144. https://doi.org/10.3168/jds.s0022 -0302(03)73803-x.
115. Westwood, C. T., I. J. Lean, and J. K. Garvin. 2002. Factors influencing fertility of Holstein dairy cows: a multivariate description. J. Dairy Sci. 85:3225–3237. https://doi.org/10.3168/jds.s0022 -0302(02)74411-1.
116. Wetteman, R.P. and H.A. Tucker, 1979. Relationship of ambient temperature to serum prolactin in heifers. Proc. Soc. Exp. Biol. Med., 146: 909-911.
117. White, F. J., R. P. Wettemann, M. L. Looper, T. M. Prado, and G. L. Morgan. 2002. Seasonal effects on estrous behavior and time of ovulation in nonlactating beef cows. J. Anim. Sci. 80:3053–3059. https://doi.org/10.2527/2002.80123053x.
118. Wilde, C.J. and W.L. Hurley, 1996. Animal models for the study of milk secretion. J. Mammary Gland Biol. Neoplasia, 1: 123-134.
119. Wilson SJ, Marion RS, Spain JN, Spiers DE, Keisler DH, Lucy MC. Effect of controlled heat stress on ovarian function in dairy cattle: I. Lactating cows. J Dairy Sci 1998;1:2124–31.
120. Wise ME, Armstrong DV, Huber JT, Hunter R, Wiersma F. Hormonal alterations in the lactating dairy cow in response to thermal stress. J Dairy Sci 1988;71:2480–5.
121. Wolfenson D, Thatcher WW, Badinga L, Savio JD, Meidan R, Lew BJ, et al. The effect of heat stress on follicular development during the estrous cycle dairy cattle. Biol Reprod 1995;52:1106–13.
122. Yousef, M.K. and Johnson, H.D. 1966a. Blood thyroxine degradation rate in cattle as influenced by temperature and feed intake. Life Sci., 5: 1349.

Received 17 May 2022
Accepted 21 September 2022

Effects of Different Cooling Systems on Cows' Behaviour and Comfort during the Hot Period

Oleksandr V. Borshch[1], Sergiy Ruban[2], Vasyl Kostenko[2], Oleksandr O. Borshch[1,2], Oleksandr Cherniavskyi[1], Lesia Korol-Bezpala[1], Maksym Fedorchenko[1], Mykhailo Matvieiev[2]

[1]Department of Technology of Milk and Meat Production, Faculty of Biotechnological, Bila Tserkva National Agrarian University, pl. 8/1 Soborna, 09117, Bila Tserkva, Ukraine

[2]Department of Genetics, Breeding and Reproductive Biotechnology, Faculty of Livestock Raising and Water Bioresources, National University of Life and Environmental Sciences of Ukraine, General Rodimtsev str., 19, Kyiv, 03041, Ukraine

Keywords: *dairy cows, barns, heat stress, fans, irrigation, behavior, comfort.*

Abstract. *The purpose of this study was to compare the effects of high temperatures on the behavior, comfort and thermoregulation energy consumption of cows at free-stall keeping in two barns. The first barn had the system of forced ventilation and air irrigation, and the second one had only fans. Barn parameters were (Length × Width × Height) 94 × 32.1 × 10.5 m. The research was conducted in central Ukraine (Kyiv region) during July 2021. The average daily ambient temperature during the study period was +26.4°C. In each of the barns, the groups of similar of non-pregnant and lactating animals (107 ± 14 days in milking) of Holstein breed were formed. Cows were fed the total mixed ration twice a day in both barns. Hours of air cooling elements operation in the barns were from 10.00 to 18.00. The use of fans in combination with irrigation systems during hot periods had a positive effect on the heat transfer of cows compared with the heat transfer of cows that were in the barn with fans. With this combination, the temperature of the skin and the resting place under the lying cow and energy consumption for heat production were 0.5°C and 0.8°C and 3.1 MJ lower. In addition, this combination of air cooling systems had a positive effect on the duration of lying down and eating food (32 and 16 minutes longer, respectively), and the indicators of standing time, physical activity and drinking were lower by 16, 21 and 6 minutes, respectively. Accordingly, the best values were the cow comfort index (CCI), stall use index (SUI) and cow feeding index (CFI) (4.78, 4.87 and 0.08). In terms of stall standing index (SSI), stall perching index (SPI) and cow drinking index (CDI), slightly higher rates were observed in the barn using fans only (3.72, 1.06 and 0.013).*

Introduction

Dairy cow breeds are the most vulnerable animals to heat stress, and highly productive lactating cows stress at temperatures above +25°C or even +20°C. Heat stress is a global phenomenon and is studied even in countries with moderate temperate climates (Dunn et al., 2014; Angrecka and Herbut, 2016). The world is experiencing global warming, which has manifested itself in the form of rising average annual temperatures, prolonging of the year hot period, increasing of the number and duration of heat waves (Collier et al., 2017; Borshch et al., 2021b). Each degree of global temperature increase leads to a multiple increase in the frequency of heat waves and increased heat stress in dairy cattle (Polsky and von Keyserlingk, 2017).

Temperature stress is the condition of the body when it is unable to dissipate metabolic heat effectively, which leads to an increase of internal body temperature and reduction of living organism's physical activity (Kadzere et al., 2002; Borshch et al., 2019; Ruban et al., 2020; Borshch et al., 2021a). Heat dissipation is carried out by conduction, convection, radiation, and evaporation (Kadzere et al., 2002). Heat stress is observed when the sum of heat produced by the body and received from outside exceeds the total heat loss (Kadzere et al., 2002). The amount of produced and absorbed heat depends on the physiological state, level of productivity, age in calving, lactation stage, color of an animal, as well as genetic factors (Kim et al., 2017; Laporta et al., 2017). Heat stress affects the productive and reproductive qualities of animals, as well as leads to other changes. Changes in productive properties include reduction of milk productivity, reduction of protein, fat, and dry skim milk residue level, slowdown in growth, reduction of feed consumption (West, 2003). Changes in reproductive properties include reduction of fertilization rate, less intense manifestation of sexual desire, deterioration of sperm production (Allen et al., 2015).

Behavioral changes manifest in reducing of lying down duration to 30%, reducing of the duration of cud chewing, increased water consumption, increasing of the motor activity of the animal (Herbut et al., 2020).

Genetic changes are represented by the release of heat shock proteins, in particular *HSP70*, which are synthesized by the genes *HSF-1* and *HSPA6* (Baena et

Correspondence to Oleksandr O. Borshch, Department of Technology of Milk and Meat Production, Faculty of Biotechnological, Bila Tserkva National Agrarian University, pl. 8/1 Soborna, 09117 Bila Tserkva, Ukraine. Email: borshcha@outlook.com

al., 2018). Physiological changes include an increase in body temperature, faster heart and respiration rate, lowering of the pH level in the rumen. Increased soreness is manifested by mastitis, acidosis and scar ketosis, weakening of the immune system, increasing of the number of heat strokes and even increasing mortality (Kadzere et al., 2002).

Deterioration in the welfare of cows depends on discomfort, increased soreness, skin contamination due to frequent lying in the manure alley, lameness (due to prolonged standing and increased activity), malnutrition, increased thirst, frustration and manifestation of aggression (Herbut and Angrecka, 2017). In order to effectively cope with heat stress, it is necessary to take measures related to the use of forced ventilation and cooling of animals (fans and irrigation systems), the use of mattresses for rest with pumping chilled water, the use of feedlot with canopies for rest and feeding, as well as their combinations (Gebremedhin et al., 2016; D'Emilio et al., 2018).

The aim of the study was to compare the effect of high temperatures on the behavior and comfort of cows kept in similar barns with the use of forced ventilation and air irrigation systems and only fans.

Material and methods

The research was conducted at a commercial dairy farm in Kyiv region, Ukraine (49°51-27-N, 30°6-36-E) during July 2021. The average weather indicators for the study period are shown in Table 1. Parameters of placements (Length × Width × Height): 94 × 32.1 × 10.5 m.

The farm uses free-stall keeping of cows with rest in boxes. The animals are kept in three easy-to-assemble barns. Two barns were used for the study. In the first barn, fans (located above the manure and feed aisles) were used during the summer, and in the second one, fans (located above the feed and manure alleys) and irrigation systems (located above the feed alleys) were used (Figure 1).

Technical characteristics and the timetable of cooling systems are given in Table 2.

Table 1. The main weather indicators during the research period (Mean ± SD)

Indicators	Ambient	Barns with:	
		fans	fans+sprinklers
Air temperature, °C	26.4 ± 0.9	23.0 ± 0.4	21.8 ± 0.2
Min–Max	21.7–34.3	20.4–25.6	19.8–23.2
Relative humidity, %	42.5 ± 0.3	58.1 ± 0.5	73.6 ± 1.7
Min–Max	36.2–51.0	53.7–66.1	62.0–78.4
Wind speed, m s^{-1}	6.1 ± 0.2	1.7 ± 0.02	1.7 ± 0.03
Min–Max	2.5–8.6	0.7–2.1	0.8–2.3

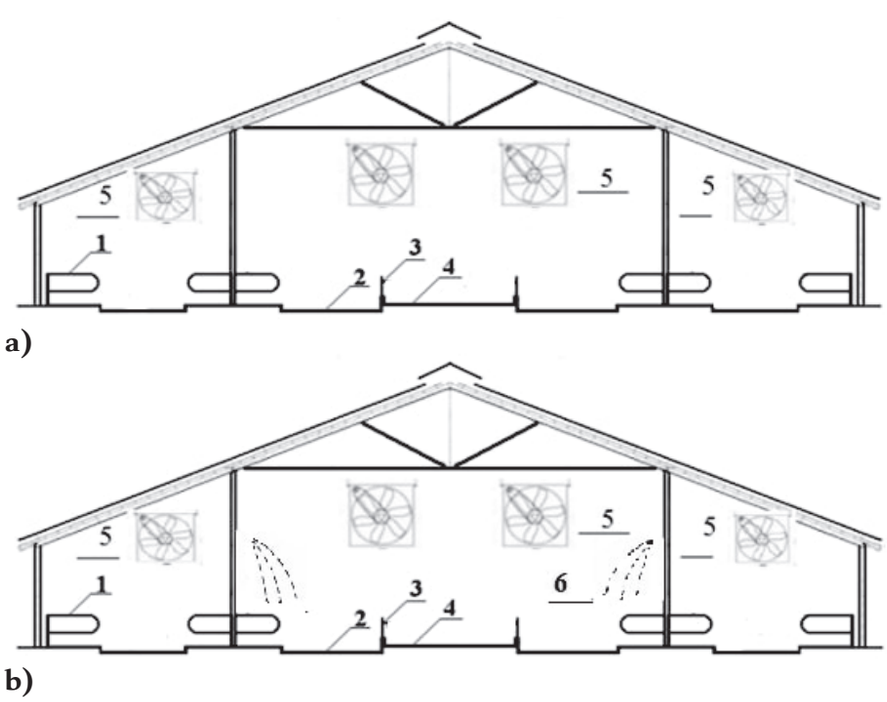

Figure 1. Easy-to-assemble barns with fans (a); with fans and irrigation (b).
1 – boxes for cows rest; 2 – feed and manure alley; 3 – feed passage fences; 4 – feed passage; 5 – fans; 6 – irrigation.

Table 2. Activation timetable and technical specification of the cooling systems

Technical specifications	Barn with:	Barn with:	
	fans	fans	irrigation
Manufacturer	Multifan, Vostermans Ventilation, (Netherlands)	Multifan, Vostermans Ventilation, (Netherlands)	Vdykh-Nova, (Ukraine)
Production capacity	to 48000 m^3/h	to 48000 m^3/h	pressure: 200 kPa
Activation time and operating conditions	Always on with when ambient T > 23°C	Always on with ambient T > 23°C	Operative for 30 s every 10 min with ambient T > 25°C

In each of the barns, groups of similar (n = 36) non-pregnant, lactating animals (107 ± 14 days in milking) of Holstein breed were formed. The average productivity of experimental cows is 25.32–25.63 kg/day. The cows were fed the total mixed ration twice a day in both barns. The cows were milked three times per day at the Parallel installation (DeLaval 2×16, Sweden) in the farm. Milking of the cows took place at 06.00, 12.00 and 18.00 hours.

Weather indicators of the environment during the study period were recorded according to the meteorological station of Bila Tserkva (Kyiv region, Ukraine).

The temperature, relative humidity and wind speed were measured by MISOL WN-5300CA (PRC). The sensors were placed in the zone occupied by cows 0.5 m above the floor. All the measurement results were recorded automatically every 10 min. The cows skin surface temperature was determined in two places: on rumen and in the region of the last inter costal space by using a remote infrared thermometer Thermo Spot Plus (Germany). The temperature at the resting place as well as under the lying cow was determined by the thermometer A36PF-D43 (USA). Costs of energy for heat production were calculated according to the methods of Kadzere et al. (2002).

The cows' behavior during the hot period was determined using internal surveillance cameras. In each barn, 16 Hikvision cameras (Full HD) were installed. Filming in both barns took place around the clock. Placing cameras in the barns allows you to record a recreation area, feeding passage and drinking bowl area and also cows moving. Every 10 minutes, in experimental groups, the number of cows, which during the observation consumed food, were resting by lying, standed, were moving and drinking water and also contact with the stall was recorded. The effect of free-stall housing on stall comfort, welfare, and natural behavior of cows following behavioural indices were calculated: cow comfort index (CCI): number of cows lying in stalls per number of cows in contact with stalls: (Nelson, 1996); stall standing index (SSI): number of cows standing in stalls per number of cows in contact with stalls (Cook et al., 2007); stall perching index (SPI): number of cows standing with 2 front feet in the stall and the rear feet in the alley per number of cows in contact with stalls (Tucker et al, 2005); stall use index (SUI): number of cows lying in stalls per number of cows not actively feeding (Overton et al., 2002); cow feeding index (CFI): defined as the ratio between the number of feeding cows and the total number of cows in the pen (DeVries et al., 2003); cow drinking index (CDI): defined as the ratio between the number of drinking cows and the total number of cows in the pen (Fregonesi et al., 2007).

The obtained data were statistically processed using STATISTICA (Version 11.0, 2012) software. The Student *t* test was used to estimate the statistical significance of the obtained values. The data were considered significant at $P < 0.05$, $P < 0.01$, $P < 0.001$.

Results

It was found that in the barn with the use of fans and irrigation systems the value of the skin temperature during the period of high temperature load was 0.5°C lower compared with the barn in which only fans were used (Table 3). Also, with such a combination of air cooling elements, lower indicators of resting place temperature (by 3.3°C) and resting place under a lying cow (by 0.8°C) were observed. In a room with fans and cooling sprinklers, the energy consumption for heat exchange was 3.1 MJ lower compared with a barn where only fans were used to cool the air.

When keeping cows indoors using fans and sprinklers, the indicators of the main daily behavioral reactions during the period of high temperature load, which characterizes comfort, were slightly better compared with keeping cows in the barn using only fans (Table 3). Thus, the durations of lying down and eating food were 32 and 16 minutes longer, respectively. At the same time, the duration of standing, motor activity and watering was dominated by animals kept in the barn with the use of only fans as elements of air cooling by 16, 21 and 6 minutes, respectively.

To more fully study the impact of using different options for cooling barns during hot periods, we studied the values of six comfort indices for free-stall keeping of cows, which depend on the indicators of daily behavior (Table 5).

The values of the cow comfort index (CCI) and the stall use index (SUI), which depend on the daily duration of lying down, were higher (by 4.78 and 4.87) than those of cows kept in a barn that used fans and sprinklers. The value of the cow feeding index (CFI),

Table 3. Temperature indices of rest places and energy expenditure for heat production during hot period (Mean ± SD)

Indicators	Barns with:	
	fans	fans + sprinklers
Skin temperature, °C	34.9 ± 0.2	34.4 ± 0.1*
Rest place temperature, °C	28.1 ± 0.3	24.8 ± 0.6***
Rest place temperature under lying cow, °C	28.9 ± 0.3	28.1 ± 0.2*
Σ energy for heat production, MJ	64.4 ± 1.6	61.3 ± 1.4

Note: as compared with the barn with only fans *$P < 0.05$; ***$P < 0.001$.

Table 4. Duration of main daily behavior reactions‡ during the hot period (Mean ± SD)

Behavior reactions, min	Barns with:	
	fans	fans + sprinklers
Lying	706 ± 9.2	738 ± 8.8*
Feeding	253 ± 4.6	269 ± 4.3*
Moving	66 ± 1.8	51 ± 1.4***
Standing	189 ± 8.3	168 ± 5.7*
Drinking	49 ± 0.4	43 ± 0.9***

Note: as compared with the barn with only fans; *$P < 0.05$; ***$P < 0.001$; ‡ – excluding milking time.

Table 5. Values of indices that characterize cow comfort during the hot period (Mean ± SD)

Comfort indices	Barns with:	
	fans	fans + sprinklers
CCI	83.64 ± 3.18	88.42 ± 2.76
SSI	8.75 ± 0.42	5.03 ± 0.28***
SPI	7.61 ± 0.21	6.55 ± 0.23**
SUI	72.16 ± 2.03	77.03 ± 1.27*
CFI	0.39 ± 0.02	0.47 ± 0.03*
CDI	0.064 ± 0.0003	0.051 ± 0.0004***

Note: as compared with the barn with only fans; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; CCI – cow comfort index; SSI – stall standing index; SPI – stall perching index; SUI – stall use index; CFI – cow feeding index; CDI – cow drinking index.

which depends on the value of the daily foraging activity of the animals, was also higher than under the keeping of cows in the barn that used fans and sprinklers (by 0.08). The stall standing index (SSI) and stall perching index (SPI), which depend on the daily standing time of cows, were slightly higher in the barn that used fans only (3.72 and 1.06). The cow drinking index (CDI), the value of which depends on the duration of daily watering of animals, was higher (by 0.013) under keeping in a barn that used only fans as cooling elements.

Discussion

The negative effects of high temperatures on dairy cattle during global climate change are a significant problem for the industry (Collier et al., 2017). According to Johnson (2018), to reduce the impact of heat stress is possible through the breeding of heat-resistant breed, the use of microclimate control and modernization of feeding management methods. However, the most effective in the short term is the use of microclimate control tools that can reduce the temperature in barns during hot periods (Gebremedhin et al., 2016; Polsky and von Keyserlingk, 2017).

The results of our studies partially coincide with the studies of Her et al. (1988) and Wolfenson et al. (1988), conducted at commercial farms in Israel where automated irrigation systems (30 s) were used, followed by ventilation (4.5 min) during 30-minute periods. The results showed that this combination of cooling was effective and helped to reduce heat stress in cows, as well as to improve their heat balance and lower body temperature, and met the recommended duration of behavioral responses. Frazzi et al. (2000) reports that, during the hot period, whose cows are

kept in pens with only fans less time lying down (from 15.00 to 20.00 hours) and more time standing (from 15.00 to 17.00 hours) in comparison with cows that kept in pens with fans plus misting. These data partially coincide with our results. Barbari et al. (2010) indicate that under the option of keeping cows when fans and irrigation systems are located above the feed passage, the animals spend less time per day for lying down, which does not coincide with our data. Somewhat different from our data, Matarazzo et al. (2007) reported that the standing time of a group of cows housed in a section using fans and irrigation systems was longer than in the section without air cooling elements.

Tao Ding et al. (2019) in their studies conducted in China reported about lower values of skin temperature in cows during periods of high temperature load using irrigation systems that also coincide to some extent with our data. Gaughan et al. (2004) have also reported similar research results.

Our studies do not coincide with the data obtained by Lovarelli et al. (2020) in northern Italy, which indicate that in barns with fans in the rest area and irrigation systems in the feeding area, the duration of animals' resting lying down was lower than in barns with only fans in the rest area.

It has been found that the use of fans in combination with irrigation systems during hot periods had a positive effect on the heat transfer of cows compared with the keeping of cows in the barn with fans. With this combination, the skin temperature and the resting place under the lying cow were 0.5°C and 0.8°C lower. The duration of cows' lying down and eating fodder with this combination was 32 and 16 minutes longer, respectively. At the same time, the duration of standing, motor activity and watering was dominated in animals kept in the barn with the use of only fans as elements of air cooling by 16, 21 and 6 minutes, respectively. In addition, the combination of fans and irrigation systems contributed to better comfort indices for dairy cows under free-stall keeping.

References

1. Allen J. D., Hall L.W., Collier R.J., Smith J.F. Effect of core body temperature, time of day, and climate conditions on behavioral patterns of lactating dairy cows experiencing mild to moderate heat stress. Journal of Dairy Science. 2015. T. 98(1). P. 118–127. DOI: https://doi.org/10.3168/jds.2013-7704
2. Angrecka S., Herbut P. Impact of barn orientation on insolation and temperature of stalls surface. Annals of Animal Science. 2016. T. 16. P. 887–896. DOI: https://doi.org/10.1515/aoas-2015-0096
3. Baena M., Tizioto P.C., Meirelles S.L.C., Regitano L.C.A. HSF1 and HSPA6 as functional candidate genes associated with heat tolerance in Angus cattle. Revista Brasileira de Zootecnia. 2018. T. 47. Article number: e20160390. DOI: https://doi.org/10.1590/rbz4720160390
4. Barbari M., Leso L., Rossi G., Scaramelli A., Simonini S. Influence of cooling systems on the behaviour of dairy cows housed in cubicle barn. In: CIGR XVII World Congress, Québec City, Canada, June 13–17. 2010. P. 1–10.
5. Borshch A.A., Ruban S., Borshch A.V., Babenko O. Effect of three bedding materials on the microclimate conditions, cows behavior and milk yield. Polish Journal of Natural Sciences. 2019. T. 34. P. 19–31.
6. Borshch O.O., Borshch O.V., Mashkin Y., Malina V., Fedorchenko M. Behavior and energy losses of cows during the period of low temperatures. Scientific Horizons. 2021a. T. 24 (5). P. 46–53. DOI: 10.48077/scihor.24(5).2021.46-53
7. Borshch O.O., Ruban S.Yu., Borshch O.V. The influence of genotypic and phenotypic factors on the comfort and welfare rates of cows during the period of global climate changes. Agraarteadus. 2021b. T. 1. P. 25–34. DOI: https://doi.org/10.15159/jas.21.12.
8. Collier R., Renquist B., Xiao Y. A 100-Year Review: Stress physiology including heat stress. Journal of Dairy Science. 2017. **T**. 100. № 12. **P**. 10367–10380. DOI: https://doi.org/10.3168/jds.2017-13676
9. Cook N.B. The Dual Roles of Cow Comfort in the 'Get Lame – Stay Lame' Hypothesis. Sixth International Dairy Housing Conference Proceeding, 16–18 June. Minneapolis, Minnesota, USA: ASABE. 2007. Article number: 701P0507e. DOI: 10.13031/2013.22819.
10. D'Emilio A., Cascone G., Lanteri P., Porto S.M.C. Effects of different cooling systems on heat stress behaviour of dairy cows. CIGR Journal. 2018. P. 1–14. Retrieved May 31, 2022, https://cigrjournal.org/index.php/Ejounral/article/view/4784/2737.
11. DeVries T. J., von Keyserlingk M.A.G., Weary D.M., Beauchemin K.A. Technical Note: Validation of a System for Monitoring Feeding Behavior of Dairy Cows. Journal of Dairy Science. 2003. T. 86(11). P. 3571–3574. DOI: https://doi.org/10.3168/jds.S0022-0302(03)73962-9
12. Ding T., Sun B., Shi Z., Li B. Optimizing Water Droplet Diameter of Spray Cooling for Dairy Cow in Summer Based on Enthalpy Difference Theory. Energies. 2019. T. 12. Article number: 3637. DOI:10.3390/en12193637
13. Dunn R., Mead N.E., Willett K.M., Parker D.E. Analysis of heat stress in UK dairy cattle and impact on milk yields. Environmental research letters. 2014. T. 9(6). Article number: 064006. DOI: http://dx.doi.org/10.1088/1748-9326/9/6/064006
14. Frazzi E., Calamari L., Calegari F., Stefanini L. Behavior of dairy cows in response to different barn cooling systems. Transactions of the ASAE. 2000. T. 42(2). P. 387–394.
15. Fregonesi J. A., Leaver J.D. Behaviour, performance and health indicators of welfare for dairy cows housed in straw yard or cubicle systems. Livestock Production Science. 2001. T. 68 (2–3). P. 205–216.
16. Gaughan J. B., Davis M.S., Mader T. L. Wetting and the physiological responses of grain-fed cattle in a heated environment. Australian Journal of Agricultural Research. 2004. T. 55. P. 253–260. DOI: https://doi.org/10.1071/AR03110
17. Gebremedhin K.G., Wu B., Perano K. Modeling conductive cooling for thermally stressed dairy cows. Journal of Thermal Biology. 2016. T. 56. P. 91–99. DOI: https://doi.org/10.1016/j.jtherbio.2016.01.004
18. Her E., Wolfenson D., Flamenbaum I., Folman Y., Kaim M., Berman A. Thermal, productive and reproductive response of high yielding cows exposed to short-term cooling in summer. Journal of Dairy Science. 1988. T. 71. P. 1085–1092. DOI: https://doi.org/10.3168/jds.S0022-0302(88)79656-3
19. Herbut P., Angrecka S. Relationship between THI level and dairy cows' be-haviour during summer period. Italian Journal of Animal Science. 2017. T. 17 (1). P. 226–233. DOI: https://doi.org/10.1080/1828051X.2017.1333892
20. Herbut P., Hoffmann G., Angrecka S., Godyń D., Corrêa Vieira F.M., Adamczyk K., Kupczyński R. The effects of heat stress on the behaviour of dairy cows – a review. Annals of Animal Science. 2020. T. 21(2). P. 385–402. DOI: 10.2478/aoas-2020-0116
21. Johnson J. S. Heat stress: Impact on livestock well-being and productivity and mitigation strategies to alleviate the negative effects. Animal Production Science. 2018. Vol. 58 (8). P. 1404–1413. DOI: https://doi.org/10.1071/AN17725

22. Kadzere C.T., Murphy M.R., Silanikove N., Maltz E. Heat stress in lactating dairy cows: a review. Livestock Production Science. 2002. T. 77. P. 59–91. DOI: https://doi.org/10.1016/S0301-6226(01)00330-X
23. Kim J., Hanotte O., Mwai O.A., Dessie T., Bashir S. Diallo, B., Agaba M., Kim K., Kwak W., Sung S., Seo M., Jeong H., Kwon T., Taye M., Song K.D., Lim D., Cho S., Lee H.J., Yoon D., Oh S.J., Kemp S., Lee H.K., Kim H. The genome landscape of indigenous African cattle. Genome Biology. 2017. T. 18. Article number: 34. DOI: 10.1186/s13059-017-1153-y
24. Laporta J., Fabris T.F., Skibiel A.L., Powell J.L., Hayen M.J., Horvath K., Miller-Cushon E.K., Dahl G.E. 2017. In utero exposure to heat stress during late gestation has prolonged effects on the activity patterns and growth of dairy calves. Journal of Dairy Science. 2017. T. 100(4). P. 2976–2984. DOI: 10.3168/jds.2016-11993
25. Lovarelli D., Finzi A., Mattachini G., Riva E. A Survey of Dairy Cattle Behavior in Different Barns in Northern Italy. Animals. 2020. T. 10. Article number: 713. DOI:10.3390/ani10040713
26. Matarazzo S.V., Perissinotto M., Oliveira da Silva I.J., Moura D.J., Fernandes S.A.A. Sixth International Dairy Housing Conference Proceeding, 16–18 June 2007, (Minneapolis, Minnesota). Article number: 701P0507e. DOI:10.13031/2013.22794
27. Nelson A.J. On-farm nutrition diagnostics. Proceedings 29th Annual Conference. American Association of Bovine Practitioners, September 12–14. San Diego, California, USA: Frontier Printers, Inc. 1996. P. 76–85. DOI: https://doi.org/10.21423/aabppro19965940
28. Overton M.W., Sischo W.M., Temple G.H., Moore D.A. Using time-lapse video photography to assess dairy cattle lying behavior in a free-stall barn. Journal of Dairy Science. 2002. T. 85. P. 2407–2413. DOI:10.3168/jds.S0022-0302(02)74323-3
29. Polsky L., von Keyserlingk M. Invited review: Effects of heat stress on dairy cattle welfare. Journal of Dairy Science. 2017. *T*. 100. №. 11. *P*. 8645–8657. DOI: https://doi.org/10.3168/jds.2017-12651
30. Ruban S., Borshch O.O., Borshch O.V., Orischuk O., Balatskiy Y., Fedorchenko M., Kachan A., Zlochevskiy M. The impact of high temperatures on respiration rate, breathing condition and productivity of dairy cows in different production systems. Animal Science Papers and Reports. 2020. T. 38(l). P. 61–72.
31. Tucker C.B., Weary D.M., Fraser D. Influence of neck-rail placement on free-stall preference, use, and cleanliness. Journal of Dairy Science. 2005. T. 88. P. 2730–2737. DOI:10.3168/jds.S0022-0302(05)72952-0
32. West J. Effects of heat-stress on production in dairy cattle. Journal of Dairy Science. 2003. T. 86. P. 2131–2144. DOI: 10.3168/jds.S0022-0302(03)73803-X
33. Wolfenson D., Flamenbaum I., Berman A. Hyperthermia and body energy store effects on estrous behavior, conception rate, and corpus luteum function in dairy cows. Journal of Dairy Science. 1988. T. 71. P. 3497–3504. DOI: https://doi.org/10.3168/jds.S0022-0302(88)79956-7

Received 26 June 2022
Accepted 27 September 2022

Possibilities of Using Ginseng in Diets of Goldfish

Taşkın Değirmencioğlu[1]
[1]*Department of Milk and Fattening, Bursa Karacabey Vocational School, Uludag University, Bursa, Republic of Turkiye,*

Keywords: *goldfish, carassius auratus auratus, ginseng, growth performance, head growth promoting diet.*

Abstract. *This research was carried out to determine the effects of adding 0, 30, 60 and 120 mg/kg ginseng to the diets (T_1, T_2, T_3 and T_4) on growth performance and head development of goldfish. The trial was randomly divided into four groups, 30 goldfish in each group for 90 days.*

As a result of the research, it was determined that the addition of ginseng (60 and 120 mg/kg) to the diets (T_3 and T_4) significantly increased the specific growth rate, total live weight gains, head and body height of the fish compared with the fish fed with the control diet (T_1) ($P < 0.05$) ($P < 0.01$). However, there was no positive effect on live weight gain, head height, and body height in fish fed the T_2 diet. All ginseng feeds positively affected head widths, feed consumption, and feed efficiency of fish. On the other hand, in this study, the body lengths of the fish were not significantly affected by ginseng application. Especially because of the risk caused by the use of ginseng, it seems that 60 mg/kg ginseng can be used as an appropriate dose in goldfish.

Introduction

Goldfish care has been a popular hobby for centuries, with steady expansion in its trade, growing interest, and over 125 countries. Over 2500 species are involved in the global ornamental fish industry, of which over 60% are of freshwater origin (Dey, 2016). Global imports of ornamental fish also increased from US$247.9 million in 2000 to US$ 275.2 million in 2016 (Anonymous , 2016a). The goldfish is the top freshwater seller in the Netherlands. Goldfish ranks second in sales of fish in the US (Anonymous, 2016 b). Goldfish (*Carassius auratus auratus*) belongs to the family *Cyprinidae* and is one of the most loved aquarium fish because of its color, body shape, and simple breeding that can be seen in ponds and aquariums in the world (Degirmencioglu, 2021). The head of some fish has a uniform texture, just like a blackberry. However, head texture is rough like cauliflower and is known as the tiger head (Smart, 2001). Goldfish are omnivorous fish. Trout feeds with excessive oil and protein content cause goldfish digestive disorders and developmental disorders. That is why cold-water fish have a balanced diet (Degirmencioglu, 2021). Crude protein and crude fat of a goldfish diet are respectively 30–35% and 5–7%. For reproductively active, breeding fish or juveniles, a diet higher in protein and fat needs to be selected (Anonymous, 2021). Goldfish puppies have increased protein requirements due to rapid tissue development. With an ideal feeding and soil pond, fry reaches their selling length in 3–4 months. It takes 2 years for the head to develop (Degirmencioglu, 2021). Therefore, goldfish feeds also need natural aromatic plants, growth-promoting as well as rich nutrient content. Among these aromatic plants, ginseng can be given as an example. Some studies have reported that adding ginseng to fish rations can reduce the death rate by stimulating the immune system, and positively affect the live weight, weight gain and feed conversion ratio of the animal by promoting growth; (Ashraf and Goda, 2008; Tawwab. 2012; Li et al., 2022; Mehrim et al., 2022).

Studies on the use of ginseng as a growth promoter in ornamental fish are rare. Therefore, reliable doses of ginseng in fish feed need to be investigated. The aim of the study was to determine the optimal ginseng dose in diets of goldfish.

Materials and methods

Study site

Twins aquarium, a commercial pet store located in the Bursa-sehreküstü district, was chosen as the experimental area. The experiment was conducted between September and November 2021 and lasted 90 days.

Animals, treatments, and experimental design

As the animal material of the research, a total of 120 of oranda (goldfish) (the age of about 2 months) were used. The trial was randomly divided into four groups, 30 goldfish in each group for 90 days. In this study, 600 mg of Panax Ginseng (G)containing ginseng root extract as a ginseng source were used. During the 90-day trial period, the goldfish in the 1st, 2nd, 3rd and 4th groups, were fed with diets supplemented with 0, 30, 60 and 120 mg/kg ginseng, respectively.

The concentrate feed mixture CFM consisted of 10% fish meal, 10% wheat gluten, 21% krill meal,

Correspondence to: Taşkın Değirmencioğlu, Department of Milk and Fattening, Bursa Karacabey Vocational School, Uludag University, Bursa, Republic of Turkiye, http://orcid.org/0000-0002-1512-1302 E mail taskin@uludag.edu.tr

15% soybean meal, 20% oat, 10% wheat germ, 3% spirulina, 9% wheat bran, 0.5% molasses, 0.5% vitamins and minerals mix, 0.5% probiotic, 0.4% Beta-xylanase + Phytase and 0.1% garlic powder (Table 1).

During the experiment, the fish in the 1st, 2nd, 3rd and 4th groups were fed with the respective diets (T_1: 35.99 CP % and 4495.28 Kcal kg-$^{-1}$DM; T_2: 36.50 CP % and 4513.39 Kcal kg^{-1}DM: T_3: 35.48 CP % and 4447.13 Kcal kg^{-1}DM; and T_4: 36.28 CP % and 4537.54 Kcal kg^{-1}DM) (Table 1). While preparing concentrate feed mixes, it was aimed that the ratios of nutrients were close to each other. Before the experiment, the fish were photographed and individually numbered.

The fish were randomly divided into 4 groups, with 30 fish in each aquarium (90 × 40 × 50). In the determination of the nutrient contents of the ration used in the research, the nutrient limits of the cold water fish feed ration produced by a feed company of Far East origin were taken as the basis (CP min 32%, EE min 4%, CELL max 4% and crude ash max 12% moisture max 10%) (Anonymous, 2021b). The feed was brought to a dough consistency with a mixture of water and molasses, then the dough was passed through a meat grinder, and then left to dry in an oven at 65°C. The length and the diameter of the pellet were adjusted to 1–1.5 inches so that the fish could easily eat the prepared food mixture (shown in Figure 1). The body weights, feed consumption, and body forms of the fish were determined every 15 days throughout the experiment (shown in Figure 2).

Scales capable of measuring 0.001 g were used to determine fish weights. During the experiment, the live weights and live weight gains of the fish were determined by control weighing every two weeks. Daily feed consumption of fish was determined at the level of 2% of live weight. The feed was given by soaking in a small bowl so that bubbles do not form

Table 1. The composition of feed mixtures used in the research

Feed			**Diet**			
	100	Nutrient[3] composition	T_1 0.00 mg kg^{-1}	T_2 30.00 mg kg^{-1}	T_3 60.mg kg^{-1}	T_4 120 mg kg^{-1}
Fish meal	10	DM	96.88	96.44	96.78	97.09
Wheat gluten	10	OM	89.8	89.39	89.68	90
Krill meal	21	CP	35.99	36.50	35.48	36.28
Soya meal	15	EE	6.97	7.36	6.44	7.63
Wheat bran	9	CEL	2.86	2.71	3.03	3.00
Oat	20	CA	7.08	7.05	7.10	7.09
Wheat germ	10	NFE	43.98	42.82	44.73	43.09
Spirulina	3	ME[4] Kcal kg^{-1} DM	4495.28	4513.39	4447.13	4537.54
Molasses	0.5					
Vitamin + Mineral[1]	0.5					
Probiotic[2]	0.5					
Beta-xylanase + Phytase[3]	0.4					
Garlic powder	0.1					
TOTAL	100.					

[1]Trace minerals and vitamins (per kg): Dicalcium phospote, Sodium chloride, Magnesium oxide, Calcium carbonate Analyze: Calcium 17.90%, Phosphorus 10.80%, Natrium 5.5%; Retinolpalmitaat (Vitamin A) 2000.000 IE; Cholecol-ciferol (Vitamin D3) 200.000 IE; α Tocopherolacetat (Vitamine E) 8.000 mg; Asccorbinebinezuur (Vitamin C) 20.000 mg; Thiamine (Vitamine B1) 2.000 mg; Riboflovine (Vitamine B2) 4500 mg; Phyridoxine (Vitamine B5) 1500 mg; Nicotinumide (Vitamine pP) 5000 mg; calcium-D-pentothenote 1500 mg; Foliumzuur 400 mg; Menadion (Vitamine K3) 250 mg; Vitamin B12 30.000 mcg; Biotin (Vitamin H) 25.000 mcg; Magnesiumoxide 22.000 mg; Zinkoxide 50.000 mg; Nikkel (II)-Sülfat 10 mg; Natriumfluoride 50 mg; Borax 100 mg; Kaliumiodide 110 mg; Natriumbromid 100 mg; Mangan (II)-sulfaat 500 mg; Aluminisulfaat 500 mg; Litniumcarbonaat 500 mg; Kaliumsulfaat 5000 mgLizer (II)-Sulfaat 1500 mg Koper(II)-Sulfaat 400 mg.

[2](Saccharomyces cerevisiae) + lactobacillus acidophilus)

[3] **4a1617** Endo-1.4-beta-xylanase (EC 3.2.1.8) was obtained from Trichoderma citrinoviride Bisset (IM SD 135) 1.100.000 EPU/kg, **4a12** 6-Phytase (EC 3.1.3.26) was obtained from Trichoderma reset (CBS 122001) 83.400 PPU/Kg.

[3]DM: dry matter; OM: organic matter; CP: crude protein; EE: ether extract; CELL: cellulose; CA: crude ash; NFE: nitrogen free extract;

[4]ME: metabolizable energy.

Figure 1. Goldfish food (Degirmencioglu, 2021)

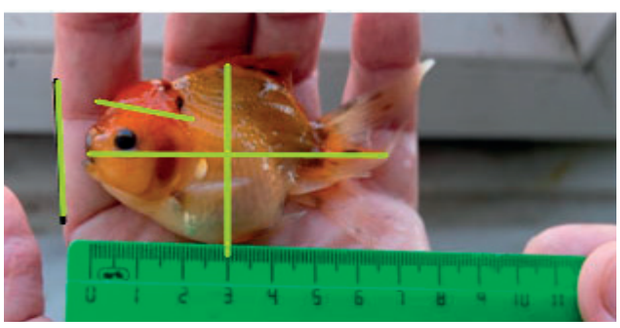

Figure 2. Calico oranda (Degirmencioglu, 2021)

in the air sacs while the fish are eating. Experimental groups were fed three times a day at 8:00 am, at 13:00 pm, and 6:00 pm. The feed and residual feed amounts to each aquarium were weighed and recorded daily. The individual feed consumption was not determined because a group feeding protocol was used in the study. The feed consumption was obtained by dividing the total fish number in each aquarium.

Growth performance values

Specific growth rate (SGR) = [(Final live weight (FLW) – İnitial live weight (ILW) / 90 day] × 100, Weight gain (WG) (g) = (FLW) (g) – (ILW)(g);

Feed conversion Ratio (FCR) = Feed intake (g) / Weight gain (g) (Mahghani et al., 2014)

Quality water and high protein feed positively affect the head and color development of fish. In essence, feed residues and fish excrement increase harmful gases such as ammonia, nitrate, and hydrogen sulfur in the water over time. 30% water withdrawal was drawn from the aquariums with a bottom siphon 4 times a week to eliminate such formations. Tap water was added to aquariums after resting for three days. Oxygen was supplied to the aquarium with a hose and air stone assembly connected to the air motor. Water temperature, pH, and micro siemens measurements in aquariums were made daily with the Hanna device. See Figures 3, 4, and 5.

Chemical composition

The metabolizable energy (ME) value of the diet was calculated based on chemical analyses according to the National Research Council (NRC 1981) and Halver (1973). The following main ME formula was:

Total ME (Kcal kg^{-1}) = 5.65 × (CP%) + + 9.45 × (EE%) + 4.10 × (NFE) × 10.

The nutrient contents of the diet were analyzed according to the AOC method (1990).

Statistical analysis

Data for growth performance and head development in the aquarium were tested by analysis of variance using the SPSS version 15.0 Statistical Package (2006) and the means were analyzed with the general linear model procedure using the following model described by Cochran and Cox (1957):

Yijk = μ + Ti + Dj + Eijk

where:

Yijk – observation, μ – population mean, Ti – Dietaries (I = T1, T2, T3 or T4),

Dj – animals (k = 1, 2, 3,....119 or 120),

Eijk – residual error.

Means were separated by Duncan's multiple range test.

Results and Discussion

In nature, due to plant respiration and photosynthesis, pH generally drops at night and rises during the day. Juvenile fish are extremely sensitive to pH fluctuations. Amazonian fish such as discus and neon lay eggs in slightly acidic and soft fish. In contrast, goldfish prefer neutral waters (pH: 7.0–7.5). The metabolism of fish is affected by water temperature (Degirmencioglu, 2021). The warmer the water, the hungrier the fish will be. With water temperatures below 70F (21°C), feed goldfish at least once a day. Above this, goldfish need to be fed twice a day. Give the fish as much food as they will eat in 3–5 minutes.

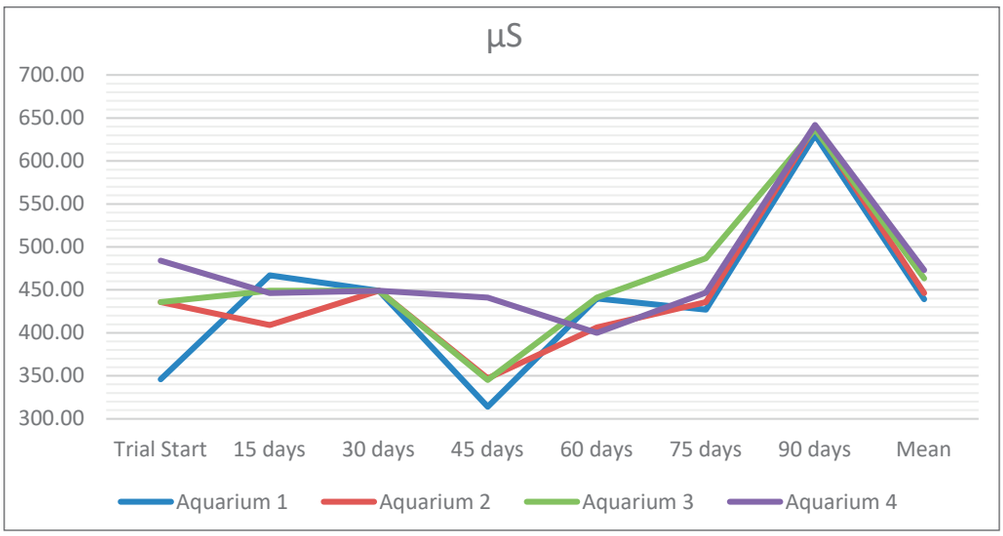

Figure 3. Micro siemens measurements in experimental aquariums

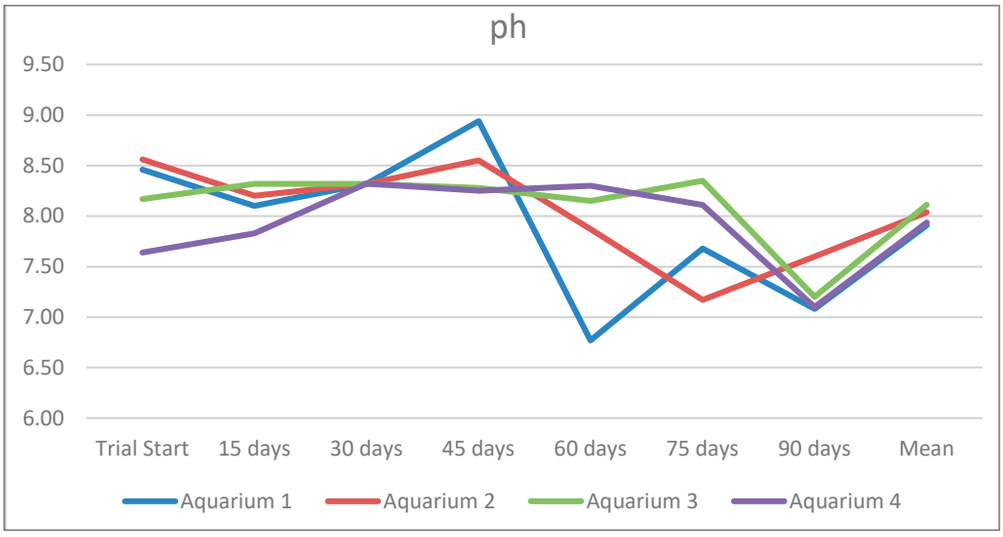

Figure 4. pH measurements in experimental aquariums

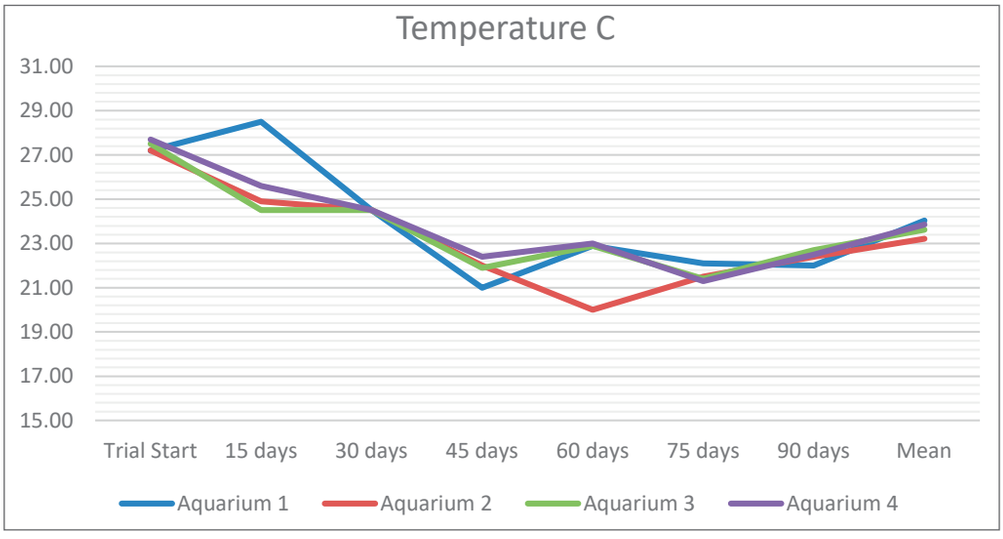

Figure 5. Temperature °C measurements in experimental aquariums

When the temperature of the water drops below 4°C, the fish stop consuming the feed (Anonymous, 2021). The MS (micro siemens), pH and temperature values in the fish aquarium recorded during the experimental period varied within 314–663, 7.08–8.94, and 20–28.5, respectively. According to the previous literature, values for pH and temperature were within the normal limits (7.0–7.5 and 4–27, respectively) (Anonym, 2021) and can be considered as not dangerous for fish.

The effects of ginseng on feed consumption

The results of the live weights of the fish at various growth periods and the total live weight increases, specific growth rate, and feed intake during the trial period are reported in Table 2.

As seen in Table 2, in the present experiment, the live weights for goldfish fed the T_1, T_2, T_3 and T_4 diets were 3.49 ± 0.20, 3.23 ± 0.20, 3.60 ± 0.19 and 3.50 ± 019 g/day, respectively, at the beginning of the study ($P > 0.05$). At the end of the 90-day trial, their live weights increased and ranged from 13.00 ± 0.85 to 17.94 ± 1.51 g. The highest live weight was determined in the 4th group and the lowest live weight was determined in the 1st group. The live weights of the other groups were similar to each other. The total live weight gains for fish fed the T_1, T_2, T_3, and T_4 diets were 9.51 ± 0.66, 12.03 ± 0.94, 12.74 ± 1.10, and 14.44 ± 1.39 g/day, respectively, during the trial (Table 2). In the present study, the live weight gains in fish fed treatments T_3 (G 60 mg/kg) and T_4 (G 120 mg/kg) were greater ($P < 0.05$) than those in fish

Table 2. Results on performance growth of ornamental fish

Groups	1st Group		2nd Group		3rd Group		4th Group	
Parameters	n	$\overline{X} \pm S_{\overline{X}}$	n	$\overline{X} \pm S_{\overline{X}}$	n	$\overline{X} \pm S_{\overline{X}}$	n	$\overline{X} \pm S_{\overline{X}}$
İnitial live weight (g/fish)	30	3.49 ± 0.20	30	3.23 ± 0.20	30	3.60 ± 0.19	30	3.50 ± 019
15. day	30	4.15 ± 026^{a}	30	4.17 ± 0.24^{a}	30	4.55 ± 0.25^{a}	30	5.01 ± 0.20^{b}
30. day	30	4.38 ± 0.26^{a}	30	4.88 ± 0.31^{a}	30	5.11 ± 0.29^{a}	30	5.44 ± 0.27^{b}
45. day	30	5.39 ± 0.36	30	5.44 ± 0.41	30	5.82 ± 0.35	30	5.99 ± 0.38
60. day	30	5.84 ± 0.40^{a}	30	6.21 ± 0.49^{ab}	30	7.03 ± 0.44^{ab}	30	7.43 ± 0.62^{b}
75. day	30	7.44 ± 0.43^{a}	30	8.24 ± 0.62^{ab}	30	8.51 ± 0.57^{ab}	30	9.21 ± 0.79^{b}
90. day Final live weight	30	13.00 ± 0.85^{a}	30	15.26 ± 1.12^{ab}	30	16.34 ± 1.25^{ab}	30	17.94 ± 1.51^{b}
Total live weight gain	30	9.51 ± 0.66^{a}	30	12.03 ± 0.94^{ab}	30	12.74 ± 1.10^{b}	30	14.44 ± 1.39^{b}
Specific growth rate (%/d)		10.55 ± 0.73^{a}		13.36 ± 1.04^{ba}	30	14.15 ± 1.23^{b}	30	16.04 ± 1.55^{b}
Daily live weight gain (g/fish)								
0–15.day	30	0.043 ± 0.047^{c}	30	0.062 ± 0.044^{c}	30	0.063 ± 0.008^{c}	30	0.100 ± 0.008^{d}
16–30 day	30	0.015 ± 0.001^{c}	30	0.047 ± 0.005^{d}	30	0.036 ± 0.004^{cd}	30	0.028 ± 0.005^{c}
31–45. day	30	0.067 ± 0.007^{a}	30	0.037 ± 0.006^{b}	30	0.047 ± 0.006^{ab}	30	0.036 ± 0.008^{b}
46–60. day	30	0.030 ± 0.04^{c}	30	0.051 ± 0.06^{c}	30	0.080 ± 0.008^{cd}	30	0.096 ± 0.017^{d}
61–75. day	30	0.106 ± 0.004^{a}	30	0.135 ± 0.009^{a}	30	0.098 ± 0.009^{ab}	30	0.118 ± 0.012^{a}
76–90. day	30	0.370 ± 0.030^{c}	30	0.467 ± 0.034^{cd}	30	0.522 ± 0.046^{c}	30	0.582 ± 0.048^{c}
Mean	30	0.105 ± 0.005^{a}	30	0.133 ± 0.006^{a}	30	0.141 ± 0.005^{a}	30	0.160 ± 0.005^{b}
Feed intake (g/fish)								
0–15. day		0.055		0.061		0.069		0.072
16–30 day		0.067		0.095		0.096		0.110
31–45. day		0.087		0.085		0.102		0.139
46–60. day		0.106		0.123		0.125		0.115
61–75. day		0.124		0.134		0.096		0.106
76–90. day		0.136		0.136		0.136		0.128
Mean		0.095		0.105		0.104		0.111
FCR		0.904		0.789		0.737		0.693

FCR; Feed conversion ratio,
a–b, c–d–e: (P < 0.05), (P > 0.01), Different letters in the same line are significantly different.

fed treatments T_1 (0.0 mg/kg) and T_2 (G 30 mg/kg), respectively, during the trial.

In a study on the subject, Ashraf and Goda (2008) reported that the live weight in Nile tilapia fish ranged widely from 254.8 to 252.3 g/fish/ginseng 200–250 mg/kg. These values are different to those for 12.74–14.44 g/fish/60–120 mg/kg. However, goldfish showed better performance in low dosage than that in Tilapia (ginseng 60 mg versus 200 mg). As seen in Table 2, The specific growth rates (SGR) (%/d) were higher in goldfish fed T_3 and T_4 diets than in goldfish fed T_1 and T_2 diets (25.44%, and 34.22%; $P > 0.05$). Significant differences were observed between T_3, T_4 diets and T_1, T_2 diets ($P < 0.05$).

Experimental results in fish showed that the highest daily live weight gain value during the trial was obtained from the T_4 diet with 0.160 ± 0.005 g day^{-1}; this was followed by the T_3 diet, T_2 diet, and T_1 diet with 0.141 ± 0.005, 0.133 ± 0.006, and 0.105 ± 0.005, respectively. The differences between the means of diet T_4 and the other diets were found statistically significant ($P < 0.05$). The findings of this study are different from those of Ashraf and Goda (2008) who found that the highest live weight gain in Nile tilapias ranged from ginseng 200 to 250 mg/kg DM, and those of Mehrim et al. (2022) who noted that the highest live weight gain in African catfish (*Clarias gariepinus*) was with a dose of 200 mg ginseng/kg diet.

The variations observed were due to differences in fish species and diet used in the trial, the characteristics of the soil in which ginseng was grown, the drying process, leaf-branch ratio, and climate, etc.

The average daily feed consumption of fish showed a continuous increase during the trial period. It ranged within 0.095–0.111 g. The highest feed consumption was determined in the 4th group and the lowest feed consumption was determined in the 1st group. It was observed that the average daily feed consumption of fish increased due to the increase in the ginseng level added to the ration. It was determined that the average FCR of the experimental fish varied within 0.904–0.693, respectively, during the trial (Table 2). The highest FCR was determined in the 4th group and the lowest FCR was determined in the 1st group. Ginseng administration increased FCR (0.904, 0.789, 0.737 and 0.693 for T_1, T_2, T_3 and T_4, respectively). The dietary supplementation with ginseng extract did not affect growth performance and feed utilization. Since group feeding was applied in the experiment, statistical analyses of the results obtained regarding the feed consumption and FCR of fish in different groups could not be performed. The obtained results were similar to the results reported by Ashraf and Goda (2008) and Mehrim et al., (2022) who reported that the FCR increased in fish fed with different levels of ginseng. However, Bulfon et al. (2017) reported that there was no significant difference in growth performance and feed utilization in rainbow trout (*Oncorhynchus mykiss*) fed with a dietary containing 0.0%, 0.01%, 0.02%, 0.03% of ginseng ethanolic extract. The observed response variance may be related to several factors, such as ginseng type, feeding strategy, animal differences, the trial length tested, the amount of ginseng added, the source of the ginseng product and seasonal effects.

The effects of developmental body

When the effects of using ginseng at different levels in concentrate feed on the developmental body of fish were examined, it was determined that the average body lengths of the experimental animals varied between 3.28 ± 0.099 and 3.35 ± 0.075, respectively, at the beginning of the trial (Table 3).

At the end of the 90-day trial, their body lengths increased and ranged from 6.1 0 ±0.132 to 6.35 ± 0.166 cm. Differences between diets in terms of body lengths were statistically insignificant. In the current study, an increase in head and body heights was observed during the development periods of fish (Table 3). The highest body height was determined in the 4th group fed with the T_4 diet, and the lowest body height value was determined in the 1st group fed with the T_1 diet, as in Table 2. The differences between the means of 3rd and 4th groups and 1st and 2nd groups were found statistically significant. As a result of the research, it was determined that adding ginseng at the level of 60 or 120 mg/kg to the ratio significantly increases the body height lengths in fish ($P < 0.01$; 2.69 ± 0.060, 2.76 ± 0.061, 2.93 ± 0.070 and 3.03 ± 0.072 for T_1, T_2, T_3, and T_4, respectively). The differences between the means of 3rd and 4th groups and 1st and 2nd groups were found statistically significant. As shown in Table 3, the head heights of the fish in the groups at the beginning of the experiment were determined to vary between 1.04 ± 0.033 and 1.05 ± 0.029 cm ($P > 0.05$). At the end of the 90-day trial, the head height of goldfish were positively affected with increasing levels of ginseng. It was determined that the head height increased by 0.35 cm in the fish fed T_4 diet compared with the fish fed T_1 diet. Similarly, while the head height was 0.28 cm in the fish fed the T_3 diet, this increase was only 0.13 cm in the fish fed the T_2 diet. In the present study, the head height of goldfish in treatments T_3 and T_4 were greater ($P < 0.01$) than those in T_1 and T_2, respectively. Similarly, at the end of the 90-day trial, the head widths were higher in fish fed the diets with ginseng (T_2, T_3, and T_4) than in fish fed the control diet (11.32%, 14.02% and 12.96%, $P < 0.01$). The differences between the means of diet T_1 and diets with ginseng (T_2, T_3, and T_4) were significant ($P < 0.01$).

The present trial results showed that adding ginseng of 60 and 120 mg/kg to the diet could positively affect the total live weight, specific growth rate, feed consumption, and FCR of fish. It can be said that this condition is a result of the positive effect

Table 3. Results on developmental body of ornamental fish

Groups	1st, Group		2nd Group		3rd Group		4th Gr0up	
Parameters	n	$\overline{X} \pm S_{\overline{X}}$	n	$\overline{X} \pm S_{\overline{X}}$	n	$\overline{X} \pm S_{\overline{X}}$	n	$\overline{X} \pm S_{\overline{X}}$
İnitial body lengths (cm)	30	3.28 ± 0.099	30	3.33 ± 0.077	30	3.35 ± 0.075	30	3.28 ± 0.067
15. day	30	3.61 ± 0.107	30	3.57 ± 0.068	30	3.64 ± 0.077	30	3.71 ± 0.063
30. day	30	3.87 ± 0.091	30	3.82 ± 0.078	30	389 ± 0.086	30	3.87 ± 0.076
45. day	30	4.01 ± 0.078^{a}	30	4.05 ± 0.089^{ab}	30	3.99 ± 0.089^{a}	30	4.19 ± 0.081^{b}
60. day	30	4.27 ± 0.077^{a}	30	4.30 ± 0.107^{ab}	30	4.50 ± 0.106^{b}	30	4.47 ± 0.091^{b}
75. day	30	5.54 ± 0.131	30	5.51 ± 0.125	30	5.76 ± 0.153	30	5.68 ± 0.152
90. day	30	6.10 ± 0.132	30	6.10 ± 0.138	30	6.35 ± 0.166	30	6.29 ± 0.152
İnitial body height of goldfish (cm)		1.82 ± 0.041		1.84 ± 0.061		1.85 ± 0.046		1.79 ± 0.079
15. day	30	2.04 ± 0.047	30	2.07 ± 0.053	30	2.08 ± 0.057	30	2.01 ± 0.045
30. day	30	2.17 ± 0.043^{a}	30	2.21 ± 0.056^{ab}	30	2.30 ± 0.061^{b}	30	2.30 ± 0.056^{b}
45. day	30	2.34 ± 0.051^{a}	30	2.36 ± 0.063^{a}	30	2.46 ± 0.061^{b}	30	2.47 ± 0.055^{b}
60. day	30	2.49 ± 0.051^{a}	30	2.47 ± 0.070^{a}	30	2.63 ± 0.066^{b}	30	2.68 ± 0.063^{b}
75. day	30	2.61 ± 0.055^{a}	30	2.64 ± 0.071^{a}	30	2.81 ± 0.067^{b}	30	2.88 ± 0.071^{b}
90. day	30	2.69 ± 0.060^{c}	30	2.76 ± 0.061^{c}	30	2.93 ± 0.070^{d}	30	3.03 ± 0.072^{d}
İnitial head height of goldfish (cm)	30	1.04 ± 0.033	30	1.05 ± 0.029	30	1.04 ± 0.029	30	1.04 ± 0.018
15. day	30	1.27 ± 0.037	30	1.27 ± 0.033	30	1.28 ± 0.042	30	1.30 ± 0.024
30. day	30	1.38 ± 0.038^{c}	30	1.40 ± 0.028^{cd}	30	1.43 ± 0.034^{d}	30	1.59 ± 0.032^{e}
45. day	30	1.55 ± 0.034^{c}	30	1.53 ± 0.042^{c}	30	1.56 ± 0.040^{c}	30	1.77 ± 0.041^{d}
60. day	30	1.67 ± 0.050^{c}	30	1.67 ± 0.048^{c}	30	1.79 ± 0.046^{c}	30	2.00 ± 0.047^{d}
75. day	30	1.86 ± 0.057^{c}	30	1.86 ± 0.059^{c}	30	2.02 ± 0.059^{d}	30	2.10 ± 0.038^{d}
90. day	30	2.59 ± 0.067^{c}	30	2.72 ± 0.066^{cd}	30	2.87 ± 0.075^{d}	30	2.94 ± 0.077^{d}
İnitial head width (cm)	30	0.82 ± 0.028	30	0.81 ± 0.024	30	0.80 ± 0.024	30	0.082 ± .019
15. day	30	0.99 ± 0.021	30	1.00 ± 0.023	30	1.02 ± 0.024	30	1.04 ± 0.028
30. day	30	1.06 ± 0.029^{a}	30	1.14 ± 0.026^{ab}	30	1.16 ± 0.030^{b}	30	1.15 ± 0.031^{b}
45. day	30	1.17 ± 0.031c	30	1.24 ± 0.033^{cd}	30	1.33 ± 0.037^{d}	30	1.30 ± 0.036^{d}
60. day	30	1.24 ± 0.034^{c}	30	1.42 ± 0.041^{d}	30	1.44 ± 0.035^{d}	30	1.45 ± 0.040^{d}
75. day	30	1.30 ± 0.030^{c}	30	1.51 ± 0.035^{d}	30	1.54 ± 0.038^{d}	30	1.55 ± 0.041^{d}
90. day	30	1.41 ± 0.033^{c}	30	1.59 ± 0.035^{d}	30	1.64 ± 0.036^{d}	30	1.62 ± 0.040^{d}

a-b, c-d-e: ($P < 0.05$), ($P < 0.01$) Different letters in a same line are significantly different.

of saponin on fish due to the increase in the level of ginseng included in the ratio.

Francis et al. (2002a) reported that the main components found in ginseng roots were a series of triterpene saponins called ginsenosides. Johnson et al. (1986) found that some saponins positively affected nutrient absorption by increasing the size of intestinal mucosal cells (Attele et al., 1999; Briskin, 2002).

Francis et al. (2001a, 2001b, 2002b, and 2002c) reported that increasing saponin in the diet promotes growth and feed consumption of fish such as *Cyprinus carpio* and Nile tilapia. Similarly, Tawwab (2012) stated that Nile tilapia (*Oreochromis niloticus*) fed with mixed feed with 1.0–5.0 g ginseng /kg diet obtained a higher growth than tilapias fed with mixed feed with control 0.0 /kg diet. Another study (Mehrim et al. 2022) reported that African catfish (*Clarias gariepinus*) fed 200 mg/kg ginseng /kg diet significantly ($P < 0.05$) increased growth performance and feed efficiency, compared with other treatments. Another study by Li et al. (2022) found that Tilapia *(Oreochromis niloticus)* fed with a diet supplemented with 0.4–0.8‰ of ginseng water extract improved the growth performance and increased the specific growth rate (SGR). Ginseng contains a sufficient amount of ginsenosides (*triterpene saponins*). These substances affect the nutrient absorption positively by changing the villi permeability of the intestine. In addition, it is believed that ginseng prevents the colonization of pathogenic microorganisms in the digestive tract,

increasing the population of beneficial microorganisms and promoting growth due to its positive contribution to microbial enzyme activity (Hu et al., 2003).

Another subject on the developmental body of goldfish may help to explain the positive effect in nutrition intake (Table 3). Findings obtained from the research regarding the growth performance of fish are consistent with the research findings conducted on a similar topic (Francis et al., 2001a, 2001b, 2002a, 2002b; Francis et al., 2002c; Ashraf and Goda, 2008; Tawwab, 2012; Mehrim et al., 2022; Li et al., 2022).

Conclusions

In the light of the scientific results obtained in this research, the following main conclusions can be drawn. It has been observed that the application of ginseng in goldfish increases FCR, body height, and head development compared with body length. Essentially among hobbyists, egg-shaped fish with the ability to develop heads and bodies are preferred. The obtained results indicated that the optimal dosage of ginseng to a diet of goldfish was 60 mg/kg on a DM basis. Therefore, ginseng had the potential to be used as an alternative aromatic plant source for goldfish without a negative impact on the digestive system and growth.

Ethics approval and consent to participate

No blood sample was taken because the fish were small. There was no loss of death from the trial animals.

Acknowledgment

Grant and thank the Matlı A.Ş Feed Labrotory for providing support in facilities and also Dr Mucahit Palaz, Erdinç Değirmencioğlu.

References

1. AOAC (1990) Official methods of analysis. 15th ed. Assoc. Off. Anal Chem. Arlington, VA, USA. (73)1: 189 – 191, https://doi.org/10.1093/jaoac/73.1.189.
2. Anonymous (2016 a) Catfish, [Online]. Available: http://www.factfish.com/statistic/ornamental%20fish%2C%20live%2C%20export%20value
3. Anonymous (2016b) The global ornamental aquarium industry: Facts and figures – Part 2. [Online]. Available: https://www.bassleer.com/ornamentalfishexporters/wp-content/uploads/sites/3/2016/11/GlobalAquariumIndustryPart2OFI-Journal-78_029g14-16.pdf
4. Anonymous (2021) https://www.thesprucepets.com/aquarium-water-ph-1378801.
5. Attele AS, Wu JA & Yuan CS (1999) Ginseng pharmacology: multiple constituents and multiple actions. Biochemical Pharmacology, 58:1685–1693, https://doi.org/10.1016/s0006-2952(99)00212-9.
6. Ashraf MA& Goda S (2008) Effect of Dietary Ginseng Herb (Ginsana_ G115) Supplementation on Growth, Feed Utilization, and Hematological Indices of Nile Tilapia, *Oreochromis niloticus* (L.), Fingerlings. journal of the World Aquaculture Society,39:2 205-214, https://doi.org/10.1111/j.1749-7345.2008.00153.x.
7. Briskin DP (2002) Production of Phytomedicinal Chemicals by Plants. Book Chapter published 18 Sep 2001 in Handbook of Plant and Crop Physiology on pages 507 to 522. https://doi.org/10.1201/9780203908426-26.
8. Bulfon C, Bongiorno T, Messina M, Volpatti D, Tibaldi E & Tulli F (2017) Effects of Panax ginseng extract in practical diets for rainbow trout (*Oncorhynchus mykiss*) on growth performance, immune response and resistance to Yersinia ruckeri. Aquaculture Research, (48)5:2369-2379 https://doi.org/10.1111/are.13072
9. Cochran WG & Cox GM (1957) In Experimental Designs. 22nd ed. Pp. xiv, 611. 82s. (John Wiley and Sons, New York; Chapman and Hall, London). https://doi.org/10.2307/3610494.
10. Dey VK (2016) The Global Trade in Ornamental Fish." Infofish International vol. 4, pp. 52-55. [Online]. Available: https://www.bassleer.com/ornamentalfishexporters/wp-content/uploads/sites/3/2016/12/GLOBAL-TRADE-IN-ORNAMENTAL-FISH.pdf.
11. Degirmencioglu T (2021) Pet Animals' s Nutrition. Lecturer Note. 125 Page. Unpublished.
12. Francis G (2021)Effects of low dietary levels of saponins on two common culture fish—common carp (*Cyprinus carpio* L.) and Nile tilapia (*Oreochromis niloticus* L.). Ph.D. thesis, University of Hohenheim, Stuttgart, Germany.
13. Francis G, Makkar HPS & Becker K (2001 a) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture, 199:197–227.
14. Francis G, Makkar HPS & Becker K (2001 b) Effects of Quillaja saponins on growth, metabolism, egg production, and muscle cholesterol in individually reared Nile tilapia (*Oreochromis niloticus*). Comparative Biochemistry and Physiology 1 Part C: Toxicology & Pharmacology, 129:105–114. https://doi.org/10.1016/s1532-0456(01)00189-2.
15. Francis G, Kerem Z, Makkar HPS & Becker K (2002a) The biological action of saponins in animal systems: a review. British Journal of Nutrition, 88 (6):587–605. https://doi.org/10.1079/bjn2002725.
16. Francis G, Makkar HPS & Becker K (2002b)Dietary supplementation with a Quillaja saponin mixture improves growth performance and metabolic efficiency in common carp (*Cyprinus carpio L*). Aquaculture, 203:311–320. https://doi.org/10.1016/s0044-8486(01)00628-7.
17. Francis G, Makkar HPS & Becker K (2002c) Effects of cyclic and regular feeding of Quillaja saponin supplemented diet on growth and metabolism of common carp (*Cyprinus carpio L).* Fish Physiology and Biochemistry, 24:343–350.
18. Halver JE (1973) Fish nutrition. Academic Press XII+713. London https://doi.org/10.1126/science.180.4092.1270-a.
19. Hu S, Concha C, Lin F & Waller KP (2003) Adjuvant effect of ginseng extract on the immune responses to immunization against Staphylococcus aureus in dairy cattle. Veterinary Immunology and Immunopathology, 91:29–37. https://doi.org/10.1016/s0165-2427(02)00264-7.
20. Johnson T, Gee JM, Price K, Curl C & Fenwick GR (1986). Influence of saponins on gut permeability and active nutrient transport in vitro. J. Nutri. 116: 2270-2277.
21. Li M. Qiang J, Zhu X, Bao J, Tao Y & Zhu H (2022) Effect of Siberian Ginseng Water Extract as a Dietary Additive on Growth Performance, Blood Biochemical Indexes, Lipid Metabolism, and Expression of PPARs Pathway-Related Genes in Genetically Improved Farmed Tilapia (*Oreochromis niloticus*). Fishes, 7(4): 149. https://doi.org/10.3390/fishes7040149.
22. Mahghani F, Gharaei A, Ghaffari M & Akrami R (2014) Dietary synbiotic improves the growth performance, survival, and innate immune response of Gibel carp (*Carassius auratus gibelio*) juveniles, International Journal of Aquatic Biology, 2: 99-104. https://doi.org/10.22034/ijab.v2i2.37.
23. Mehrim AI, Refaey MM, Hassan MAE, Zaki MA & Zenhom OA (2022) Ginseng® as a reproductive enhancer agent for African catfish, *Clarias gariepinus* (Burchell, 1822). Fish Physiol Biochem, 48(1):15-32. https://doi.org/10.1007/s10695-021-00969-y
24. NRC (1981) (National Research Council) Nutrient Requirements of Coldwater fishes National Academy Press Washington, D.C. https://doi.org/0.17226/19731.
25. Siegel RK (1979) Ginseng abuse syndrome, problems

with the panacea. Jama, 241(15):1614–1615. https://doi.org/10.1001/jama.1979.03290410046024.
26. Smart J (2001) Gold Fishes Varieties and Genetics Hand-Book for Breeders Printed and bound in Great Britain by MPG Books Ltd, Bodmin, Cornwall ISBN 0-85238-265-0. 139-157. https://doi.org/10.1002/9780470999790.ch6.
27. SPSS (2006). Statistical package for social sciences, book Chapter published 24 Mar 2006 in Handbook of Univariate and Multivariate Data Analysis and Interpretation with SPSS on pages 11 to 24. https://doi.org/10.1201/9781420011111.ch2.
28. Tawwab MA (2012) The Use of American Ginseng (Panax quinquefolium) in Practical Diets for Nile Tilapia (*Oreochromis niloticus*): Growth Performance and Challenge with *Aeromonas hydrophila*. 24(4):366-376. https://doi.org/10.1080/10454438.2012.733593
29. Van Soest PJ, Robertson JB & Lewis BA.(1991) Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition Journal of Dairy Science, 74 (10): 3583 -3597.https://doi.org/10.3168/jds.s0022-0302(91)78551-2.

Received 8 October 2022
Accepted 27 September 2022

Case Report: Angioinvasive Pulmonary Aspergillosis in an Adult Captive Green Java Peafowl (*Pavo muticus*)

A. D. Anjum[1] and Asad Aslam Khan[1]
[1]*Department of Pathobiology, Riphah College of Veterinary Sciences (RCVetS), Riphah International University, Lahore, Pakistan*

Keywords: *Mycotic pneumonia, Green Java, Peafowl, Pulmonary, Aspergillosis*

Abstract. *An adult Green Java peafowl, from a private aviary, was presented for treatment at the Riphah Pet Hospital, Lahore, Pakistan. Physical examination of the bird revealed lethargy, gasping and head jerking but no other clinical signs. The bird died before any intervention. At necropsy, multiple off-white hard nodules were observed in the lungs and two off-white flat growths in abdominal air sacs. Systemic spread of the fungus to the liver causing hepatitis was also noticed. Kidneys were pale, yellow and atrophied but fungus hyphae or conidia could not be detected in kidneys.*

Microscopically, the lung nodules were typical granulomas consisting of Aspergillus spp. hyphae and necrosis in the centre surrounded by inflammatory cells, mainly heterophils and macrophages and rare multinucleated giant cells. From the literature search, it appears to be the first report of angioinvasive mycotic pneumonia in Green Java peafowl.

Statement of novelty

Pulmonary aspergillosis is not uncommon in poultry and avian wildlife. This is the first report describing the condition in Green Java Peafowl kept in captivity.

History and clinical signs

An adult Green Java peafowl of age 2.5 years was presented for examination with severe illness. History included anorexia, depression, weight loss and severe respiratory distress. The bird received, poorly described, extensive medication by the owner using antibiotics, etc., but the sickness remained progressive. The live body weight of the bird was 2.84 kg.

Physical examination of the bird revealed depression and severe open-mouth difficult breathing. The bird died soon after arrival at the hospital before any intervention and no ante mortem tests could be performed.

Post-mortem lesions

Necropsy examination revealed pectoral muscle atrophy and fibrosis at the site of intramuscular injection(s).

Grossly, the lungs were congested haemorrhagic and consolidated with multifocal off-white nodules on the surface, as well as deep in the parenchyma, varying from 0.4 cm to 1.0 cm in diameter (Fig. 1A). Microscopically, the interalveolar septa were thickened with fibrin, heterophils, lymphocytes and macrophages. There was congestion, haemorrhages and extensive consolidation, and many areas contained abundant hemosiderin pigment (Fig. 2A) in the lungs. The nodules were typical fungal granulomas (Fig. 2B) comprised of septated branching hyphae of Aspergillus spp. (Fig. 3B) and conidia heads, which were stained with Haematoxylin and Eosin stain (Fig. 3A) with mainly heterophil infiltration followed by infiltration of macrophages and rare multinucleated giant cells. Fungal hyphae caused extensive necrosis and also invaded blood vessels (Fig. 4).

Two fungal growths were also present in the left abdominal air sac (9 mm diameter x 1mm thick and 4 mm diameter x 0.5 mm thick), off-white in colour, surrounded by general cloudiness of air sacs (Fig. 1B). The bigger colony was attached to the gizzard serosal surface but it had no physical contact with the lungs. Wet smears, stained with Wright's stain, revealed fungal hyphae and conidia in the thickened inflamed air sacs.

Grossly, the oral cavity showed stomatitis. Lesions consisted of yellowish-green, fur-like tongue and palate. Scrapings, stained with Wright's stain, showed conidial heads and fungal hyphae.

The liver was enlarged and inflamed with numerous tiny aspergillus colonies. Fungal hyphae were seen in impression smears from the cut surface of the liver stained with Wright's stain. Kidneys were pale and atrophied.

Discussion

Soil is the natural reservoir of Aspergillus from which aerosols of conidia are released that are inhaled and deposited deep in the respiratory tract. Aspergillosis, a respiratory disease, is common in young chickens (Dykstra et al., 2013), captive (and wild) birds (Khosravi et al., 2008; Samson et al., 2014; Hauck et al., 2020; Arne et al., 2021), aquatic birds (Melo et al., 2020). The published literature is limited to case reports in various birds, but information on

Corresponding to Asad Aslam Khan, Department of Pathobiology, Riphah College of Veterinary Sciences, Lahore, Pakistan. Email: asad.aslam@riphah.edu.pk

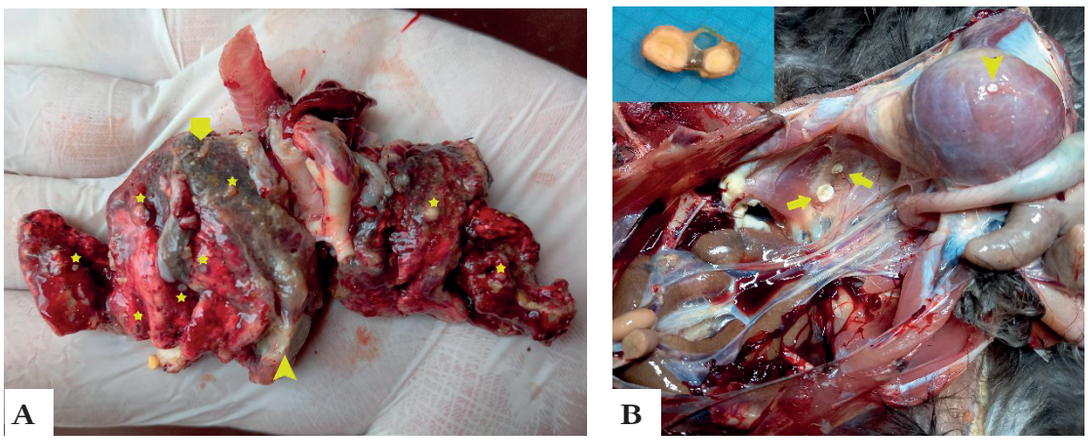

Fig. 1. The lungs of a Green Java Peafowl showing (A) congestion, consolidation and multifocal off-white nodules (*). The pleural covering of the lungs is thickened due to oedema and fibrinous inflammation (arrow). A large fungal colony involves pleura and lung parenchyma (arrowhead). (B) Grossly, two fungal growths were observed in the left abdominal air sac (arrows). The inset shows a closer view of the two colonies. The bigger colony was attached to the gizzard serosa (arrowhead).

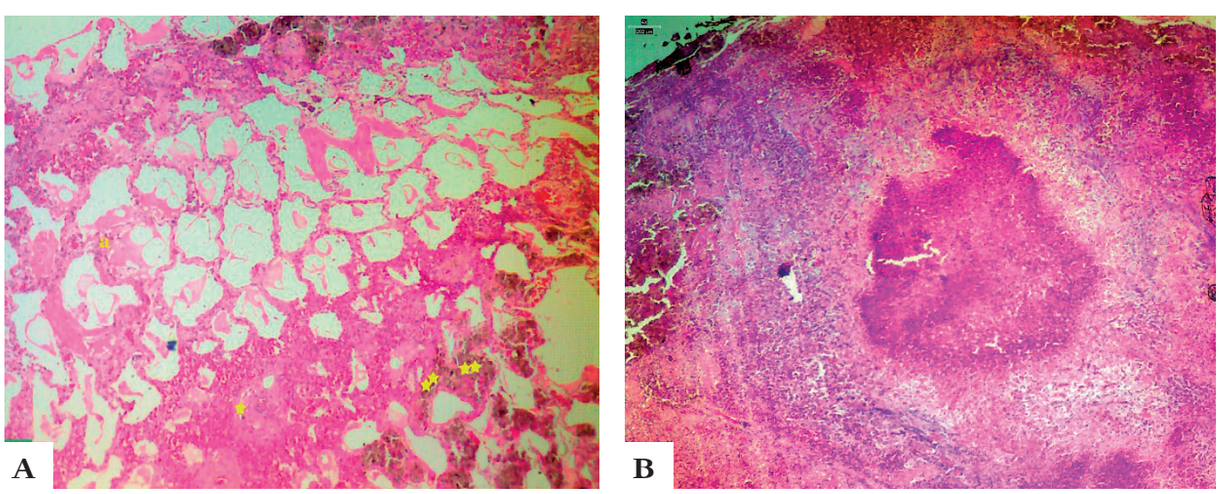

Fig. 2. A photomicrograph of the lung: (A) thickened inter-alveolar septa (a) with fibrin, heterophils, lymphocytes and macrophages; necrosis sounded by consolidation (*) and hemosiderin pigment (**); (B) a typical Aspergillus granuloma in the lungs (H&E stain).

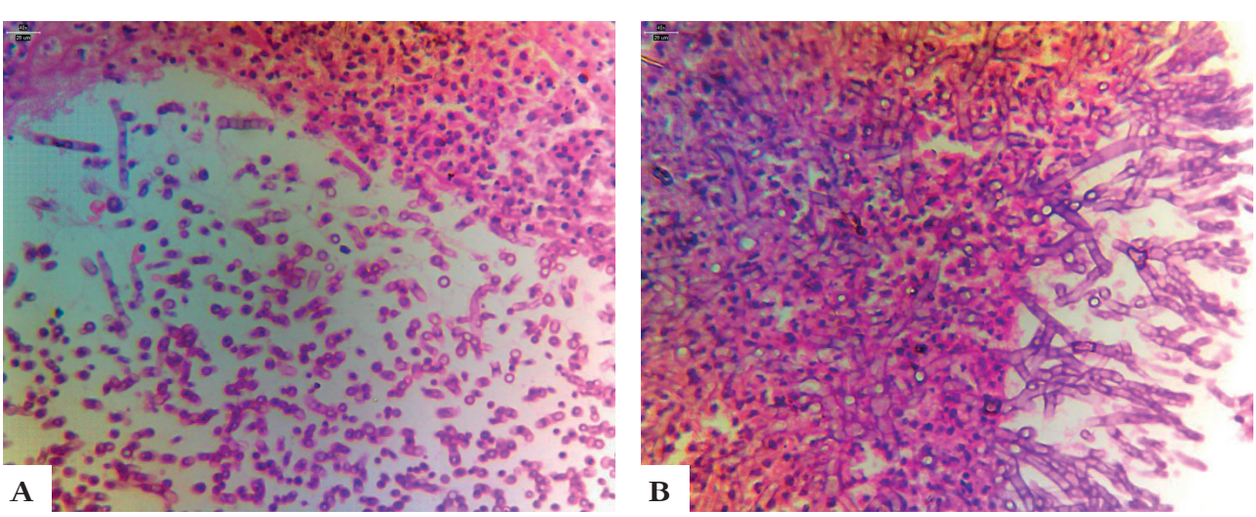

Fig. 3. (A, B) A photomicrograph of the lung of the Green Java peafowl showing extensive septated hyphae and conidia typical of Aspergillus spp., stained with Haematoxylin and Eosin stain.

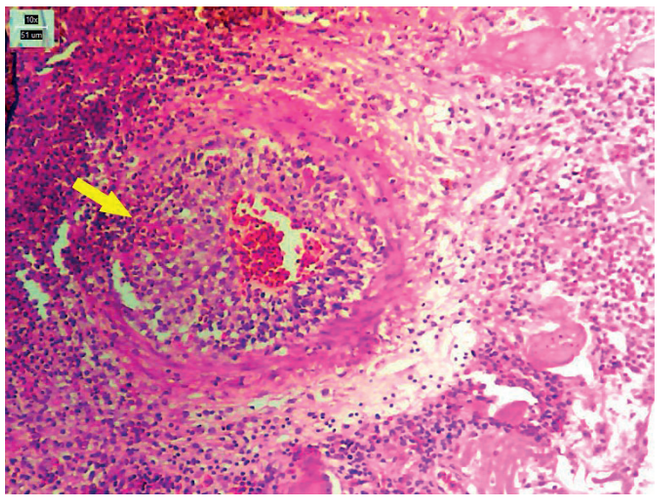

Fig. 4. The lung section of the Green Java peafowl shows an invasion of hyphae into an arteriole (arrow), and fungus growth has narrowed the arteriolar lumen to less than a half.

A photomicrograph of the lung with thickened interalveolar septa with fibrin, heterophils, lymphocytes and macrophages. HE × 320
A photomicrograph of the lung showing congestion and oedema. HE × 200

pulmonary mycosis in Green Java peafowl is scarce or not available.

Aspergillus flavus is a more prevalent species in Pakistan (Hedayati et al., 2007). However, in the Green Java peafowl, *Aspergillus fumigatus* was confirmed. Noteworthy, *A. fumigatus* conidia being smaller in size (2 to 3.5 µm) than *A. flavus* conidia (3 to 6 µm) allows *A. fumigatus* conidia to reach the pulmonary alveoli much easier than those of *A. flavus* (Hedayati et al., 2007).

Past case reports in various birds have emphasized a granulomatous type of lesion in the lungs. In the present case, an extensive and widespread inflammatory reaction was also seen in the lungs (Fig. 1). According to Ahamad et al. (2018), mycelial development causes tissue necrosis and incites a strong host reaction. Hence, a massive inflammatory response inducing severe necrosis and inflammation in the air sacs could be linked to extensive hyphae sprouting.

In humans, pulmonary nodules are a less common manifestation of aspergillosis in immunocompetent patients (Muldoon et al., 2016) but they have been associated with drastic immunosuppressive therapies (Hedayati et al., 2007). Also, Frank et al. (2005) induced severe mycotic air sacculitis and pneumonia with *A. fumigatus* by prolonged prednisolone therapy in parrots. There was no traceable obvious immunosuppressive factor in the present case. However, healthy birds exposed to large numbers of aerosol conidia may be infected without immunosuppression. Furthermore, *A. fumigatus* itself secretes molecules which are potentially immunosuppressive (Latgé, 1999).

In general, mononuclear cells predominate in chronic granulomatous lesions. However, in this case of aspergillus granuloma, heterophils predominated in the necrotic tissue. As reviewed by Latgé (1999), neutrophils remain responsible primarily for hyphal killing, and conidia are killed by macrophages. Therefore, extensive development of mycelium (see Fig. 3) justifies heterophil predominance in aspergillus lesions.

Aspergillosis can cause local lesions in the respiratory tract or systemic infections involving internal organs such as the liver, kidneys, or brain (Dykstra et al., 2013). The inflammation of the liver in this case appears to be systemic dissemination of the Aspergillus from the lungs (note angio invasion in Fig. 4).

There was no gross or histological evidence of fungal invasion in the kidneys. Pale, atrophied kidneys suggest chronic damage by mycotoxins; however, feed samples could not be obtained for spores or mycotoxins analysis due to the non-availability of feed consumed in the past. Kidney damage could also be related to injudicious and excessive medication or synthesis of mycotoxins by Aspergillus while growing in tissues.

The green Java was purchased around 6 months ago. Keeping in view the history and longevity of lung lesions in the case and hygienic management at the aviary, *a priori*, the bird was a latent carrier at the time of its arrival to this location. Five of the mates at this aviary are apparently healthy. Therefore, it appears that the *A. fumigatus* was not bird-to-bird transmitted.

Declaration of interest

The authors report no conflicts of interest. The research was conducted in the absence of any commercial or financial relationships. The authors are responsible for the content and writing of the paper.

Acknowledgements

We are thankful to Mr Khizar Hayat (Centre of Animal Diagnosis, Lahore) and Mr Nabeel Shafqat (RCVetS) for helping in the post-mortem of the peafowl and laboratory processing of samples.

References

1. Ahamad DB, Ranganathan V, Punniyamurthy N, Sivaseelan S, & Puvarajan B (2018) Pathomorfology of aspergillosis in a Japanese quail. Indian Veterinary Journal, 5 (4): 36-42.
2. Arné P, Risco-Castillo V, Jouvion G, Le Barzic C & Guillot J (2021) Aspergillosis in Wild Birds - Review Journal of Fungi, 7, 241.
3. Dykstra MJ, Charlton BR, Chin RP & Barnes HJ (2013) Fungal infections. In: Diseases of Poultry, 13th edition. DE Swayne. Wiley-Blackwell., p-1077.
4. Frank A, Verstappen LM & Dorrestein GM (2005) Aspergillosis in Amazon Parrots after Corticosteroid Therapy for Smoke-inhalation Injury. Journal of Avian Medicine and Surgery, 19 (2):138-141.
5. Hauck R, Cray C & França M (2020) Spotlight on avian pathology: aspergillosis. Avian Pathology, 49 (2): 115-118.
6. Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P & Denning DW (2007) Aspergillus flavus: human pathogen, allergen and mycotoxin producer. Microbiology, 153: pp. 1677-1692.
7. Khosravi AR, Shokri H, Ziglari T, Naeini AR, Mousavi Z & Hashemi H (2008) Outbreak of severe disseminated aspergillosis in a flock of ostrich (Struthio camelus). Mycoses, 51(6): 557-559.
8. Latgé JP (1999) Aspergillus fumigatus and Aspergillosis. Clinical Microbiology Reviews 12 (2): 310–350.
9. Melo AM, Silva-Filho RPD, Poester VR, von Groll A, Fernandes CG, Stevens DA, Sabino R & Xavier MO (2020) Aspergillosis in free-ranging aquatic birds. Medical Mycology Case Reports, 28: 36–38.
10. Muldoon EG, Sharman A, Page I, Bishop P & Denning DW (2016) Aspergillus nodules; another presentation of Chronic Pulmonary Aspergillosis. BMC Pulmonary Medicine, 16 (1):123.
11. Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH (2014) Phylogeny, identification and nomenclature of the genus Aspergillus. Studies in Mycology, 78: 141–173.

Received 19 July 2022
Accepted 17 October 2022

Microbiological Studies on the Prevalence of *Staphylococcus* Spp., Involved in the Etiology of Mastitis in Cattle and their Susceptibility to Antimicrobial Agents

Mariana Nikolova[1], Valentina Urumova[1]
[1]*Department of Veterinary Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria*

Keywords: *mastitis, cattle, staphylococci, resistance.*

Abstract. *For the period between June 2020 and -March 2022, a total of 8 dairy cattle farms were surveyed in terms of the prevalence of clinical and subclinical mastitis. Four of them were located in Northern Bulgaria (Targovishte, Shumen and Dobrich districts) and another four in Southern Bulgaria (Stara Zagora, Plovdiv and Haskovo districts). In these target farms, a rapid mastitis screening test was initially performed to detect the presence of subclinical mastitis or samples with high somatic cell counts. A total of 312 milk samples were obtained from milk quarters who reacted with 3+ or 4+ to which 34 samples of inflammatory exudate from cows with clinical mastitis were added.*

During the microbiological investigation of the 346 samples, 272 of them (79.1%) were bacteriologically positive. Streptococcus spp., were isolated from 151 samples (55.5%). The second most common species was Staphylococcus spp., detected in 110 (40.4%) of the tested samples. In total, the Gram-positive cocci finding exceeded 95% of the microbial species. The remaining 11 (4.0%) isolates belonged to another 6 taxa. These included four strains identified by prior phenotypic identification as Trueperella pyogenes, three Escherichia coli isolates, and one strain of each Pasteurella multocida, Nocardia asteroides, Klebsiella pneumoniae, and Acinetobacter spp.

Staphylococci were also studied for their sensitivity to 11 chemotherapeutic agents. The highest percentage of resistance (50%) was determined to lincomycin, followed by that to tetracycline (37.3%), and beta-lactams ampicillin (24.5%), and oxacillin (13.6%). The resistance rates to cefoxitin and cephalotin were 5.5% and 0.9% respectively. Also, lower values of resistant strains were observed for the combination of trimethoprim/sulfonamides (7.3%), ciprofloxacin (1.8%), and rifampicin (0.9%). Resistance to gentamicin and amoxicillin/ clavulanic acid was not established. Minimum inhibitory concentrations were determined for the studied chemotherapeutics, with the highest MIC_{90} values of 128 μg/mL and 2 μg/mL for tetracycline and lincomycin, and the lowest MIC_{90} values of 0.001 μg/mL for rifampicin and ciprofloxacin, respectively. A MIC_{90} of 0.125 μg/mL was obtained for oxacillin, 1.5 μg/mL for trimethoprim/sulfonamides and 1.0 μg/mL for ampicillin, cephalotin and cefoxitin.

Introduction

Staphylococci are among the commonest agents of bovine mastitis, causing substantial economic losses and threatening public health. Apart the economic status of an affected farm, mastitis also influences the quality of milk, and respectively the health of offspring. On the other hand, the spread of multi-resistant microbial strains involved in mastitis etiology is also an important health issue related to animal welfare and risks for human health through the food chain.

Subclinical mastitis is one of the most frequent form of bovine mastitis, prevalent in many geographic regions, increasing milk somatic cell counts and altering milk physical and chemical properties (Abebe *et al*., 2016; Jagielski *et al*., 2014; Léon-Galvan *et al*., 2015; Salvador et al., 2014).

Corresponding to Valentina Urumova, Trakia University Faculty of Veterinary Medicine Department of Veterinary Microbiology, Infectious and Parasitic Diseases Stara Zagora, Bulgaria.
E-mail: valentina_62@abv.bg

The spread of resistance to chemotherapeutics used for treatment of mastitis is a serious challenge for control of these infections, and also a specific process influencing the therapeutic approach to affected populations (Sakwinska *et al*., 2011; Silveira- Filho *et al*., 2014; Vakkamäki *et al*., 2017). The monitoring of antimicrobial resistance is beneficial for objective decision-making on the therapy and prevention of mastitis, and at the same time demonstrates the possible trends of resistance development, which is important for the correct use of antimicrobial drugs in veterinary practice (Mader *et al*., 2021). The monitoring of resistance to beta-lactam chemotherapeutics is of particular interest, as they are frequently used for treatment of bovine mastitis, and some pathogenic strains, e.g. methicillin-resistant staphylococci have a public health impact as well (Schnitt and Tenhagen, 2020). The production of beta-lactamases is the commonest mechanism of resistance against beta-lactams among both Gram-negative and Gram-positive bacteria (Livermore and Brown, 2001).

The aim of the present investigation was to perform a two-year survey on the prevalence of the most prevalent etiological agents of bovine mastitis and to analyze their sensitivity to most commonly used chemotherapeutic drugs.

Material and methods

From June 2020 to March 2022, a total of 8 dairy cattle farms were surveyed for prevalence of clinical and subclinical mastitis. Of them, four were located in North Bulgaria (2 in Targovishte district, 1 in Shumen and 1 in Dobrich districts); the other four were in South Bulgaria (Stara Zagora, Plovdiv and Haskovo districts). Initially, the cows at the farms were screened with the rapid Kerba mastitis test to detect subclinical mastitis or samples with high somatic cell counts. A total of 312 milk samples were collected from milk quarters having reacted with 3+ or 4+. To them, 34 samples of inflammatory exudate from cows with clinical mastitis were added.

All milk samples were cultured aerobically on Columbia blood agar (5% sheep red blood cells) and McConkey agar at 37°C for 24– 48 hours. The samples were determined as contaminated after the detection of growth of more than two morphotypes and absence of the specific growth of the main etiological agents. Strains from the positive samples were the first type depending on the expression of haemolytic activity: α-, β-, or double haemolytic zone, Gram staining, production of catalase and oxidase, free coagulase production and mannitol utilisation.

DNA extraction from suspect colonies of both coagulase-negative and coagulase-positive staphylococci was done with DNeasy Blood Tissue kit (Qiagen, Germany) and the identification of staphylococci was performed with commercial Microbial DNA qPCR assay kits- Qiagen, Germany. The emphasis in this study was on the genetic determination of *Staphylococcus epidermidis* and *Staphylococcus aureus*. The genetic assays employed TaqMan probes for detection of amplification regions determining the species affiliation of staphylococci, labelled with FAM reporter dye and ROX Reference Dye (passive reference dye). Amplification reactions were run in STRATAGENE Mx3000P qPCR (Agilent Technologies, USA). The reaction protocol was as followed:

Table 1. PCR protocol

Components	n=1
PCR water	6.5 µL
Master mix	12.5 µL
qPCR assay	1 µL
Mastermix volume	20 µL
DNA tamplate	5 µL
Total volume	25 µL

The temperature region of amplification reaction comprised: the initial step of denaturation at 95°C for 10 minutes, two steps of 40 cycles each consisting of denaturation (95°C for 15 sec), and annealing/ elongation (60°C for 2 min).

For phenotype analysis of resistance of isolated staphylococci to antimicrobial drugs, the disk diffusion method and the method for determination of minimum inhibitory concentrations (MIC) (E-test, Hi Comb™) were used. The disks and test strips were produced by Himedia Biosciences, India. MICs of lincomycin were determined by the dilution in Muller-Hinton agar method. Lincomycin was provided by the Veterinary Preparations Enterprise in Zavet, Razgrad district, Bulgaria. The sensitivity of staphylococci was tested to eleven chemotherapeutical drugs from several classes. The used antibiotic disks included beta-lactams: ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), oxacillin (1 µg), cephalothin (30 µg) and cefoxitin (30 µg); aminoglycosides-aminocyclitols: gentamicin (10 µg), tetracyclines: tetracycline (30 µg), fluoroquinolones: ciprofloxacin (5 µg), as well as lincomycin (15 µg), rifampicin (5 µg) and the combination trimethoprim/ sulfonamide (23.75/1.25 µg). The reference strain *Staphylococcus aureus* ATTC 25923 was used as control in the phenotype analysis of resistance. The strains were interpreted as resistant or sensitive based on critical thresholds recommended by EUCAST (2022).

Statistical analysis of the data was performed with GraphPad Instat 3 software.

Results

The data from performed surveys are presented in Table 2. Of all 6568 tested milk quarters of dairy cows, 312 (4.7%) were found positive or suspect by the rapid mastitis test. The samples from all positive quarters were sent for microbiological examination together with another 34 samples from clinical mastitis.

The highest percentage of subclinical mastitis was found out in the farm in Mengishevo (29.8%), followed by the Karapelit farm (24.0%), the least prevalence was identified at the Kamburovo farm (7.4%), where the *Staphylococcus aureus* isolates were more spread (11 of the total 16 strains from bovine subclinical mastitis). The cows at the Karapelit farm exhibited a higher prevalence of coagulase-negative staphylococci (29 of 61 strains from bovine subclinical mastitis) followed by the Popovitsa farm (18 out of 28 strains from bovine subclinical mastitis). Staphylococci were the least prevalent at the Harmanli farm (2 of the total 4 strains from bovine subclinical mastitis).

A positive microbial finding was detected in 272 of all the tested 346 samples (79.1%). *Streptococcus* spp. was presented in 151 samples (55.5%). Second came the members of genus *Staphylococcus*. They were found out in 110 of the tested samples (40.5%). Of them, 78 (71.0%) were determined as coagulase-

negative and 32 (29.1%) as coagulase-positive. In general, Gram-positive cocci exceeded 95% of all identified microbial species. Eleven isolates (4%) belonged to other 6 taxa. They included 4 strains phenotypically determined as *Trueperella pyogenes*, 3 *E.coli* strains, and a single strain of species *Pasteurella multocida*, *Nocardia asteroides*, *Klebsiella pneumoniae* and *Acinetobacter* spp.

Staphylococci were tested for their sensitivity to 11 chemotherapeutic drugs. Table 4 presents the results about the spread of antimicrobial resistance among staphylococci. The highest resistance rate (50%) was shown against lincomycin, followed by that against tetracycline (37.3%) and beta-lactams ampicillin (24.5%) and oxacillin (13.6%). The occurrence of resistance to cefoxitin and cephalothin was lower, 5.45% and 0.95%. respectively. Also, a lower prevalence was detected with respect to strains resistant to trimethoprim/sulfonamides: 7.3%, ciprofloxacin: 1.8% and rifampicin: 0.9%. Studied staphylococci showed no resistance to gentamicin and amoxicillin/clavulanic acid.

Table 5 presents the determined MIC_{90} values to 9 of the tested chemotherapeutics. The highest MIC_{90}, 128 μg/mL and 2 μg/mL, were obtained for tetracycline and lincomycin, respectively. The lowest MIC of 0.001 μg/mL were found out for rifampicin and ciprofloxacin. With regard to oxacillin, MIC_{90} was 0.125 μg/mL; for trimethoprim/sulfamethoxazole it was 1.5 μg/mL; and for ampicillin, cephalothin and cefoxitin it was 1 μg/mL.

Resistance to beta-lactams, lincomycin and tetracycline was present in 15.4% of multi-resistant coagulase-negative staphylococci. The multi-resistance patterns in 21.9% of coagulase-positive staphylococci referred to beta-lactams and rifampicin.

The genetic analysis of strains showed that 10.2% of coagulase-negative staphylococci belonged to *Staphylococcus epidermidis* species, whereas 31.2% of coagulase-positive staphylococci were identified as *Staphylococcus aureus*. Figure 1 presents amplification plots and Cт values obtained from genetic identification of staphylococci. Positive results have C_T values < 34.

Discussion

In this survey, the proportion of staphylococcal isolates from bovine mastitis was 40.5%, coming second after isolates from *Streptococcus* spp. The major part of staphylococcal isolates from milk samples was coagulase-negative (71%), whereas the share of *Staphylococcus aureus* was 31.2% from the group of coagulase-positive strains. Seventy-seven coagulase-negative staphylococcal strains (98.7%) and 25 coagulase-positive strains (78.1%) were associated with subclinical mastitis.

A number of authors have affirmed the increasing role of coagulase-negative staphylococci in the etiology of mastitis in Europe (Tenhagen *et al.*, 2009;

Table 2. Surveyed farms, cases with subclinical mastitis, samples submitted to microbiological examination and results

Farm	Total number of cows	Number of cows with clinical mastitis	Number of milk quarters tested with KMT	Of them ++++	Total number of samples for microbiological examination	Number of samples with positive microbiological finding	Positive for *Streptococcus* spp.	Positive for *Staphylococcus* spp spp.	Other species
Trem	280	2	1 112	28	28	22	12	3	7
Kamburovo	114	10	416	23	33	24	8	16	0
Mengishevo	350	10	1360	93	103	67	61	5	1
Karapelit	500	3	1988	75	78	64	30	34	0
Stara Zagora	60	1	236	30	31	26	15	11	0
Popovitsa	96 out of 2500	1	380	34	35	29	10	19	0
Harmanli	61	6	-	-	6	4	2	2	0
Borets	270	1	1076	29	30	36	13	20	3
Total	1731	34	6568	312	346	272	151	110	11

Table 3. Species distribution of microbial isolates from clinical and subclinical bovine mastitis by farms

Farm	Examined samples	Clinical / sub-clinical mastitis	Total number of isolates	Including from clinical / subclinical mastitis	*Streptococcus* spp.		*Staphylococcus* spp.		Other species
					number	species	number	species	
Trem	**30**	2 / 28	**22**	2 / 20	**12**	*S.agalactiae-6* *S.dysgalactiae-4* *S.uberis* - 2	**3**	*S.aureu-2* *CNS-1*	*T.pyogenes–3* *Pasteurella spp.–1* *E.coli-3*
Kamburovo	**33**	10 / 23	**24**	8 / 16	**8**	*S.agalactiae-4* *S.uberis-4*	**16**	*S.aureus-11* *CNS-5*	
Mengishevo	**103**	10 / 93	**67**	10 / 57	**61**	*S.agalactiae-42,* *S.dysgalactiae-7* *S.uberis-12*	**5**	*CNS-5*	*Klebsiella spp.-1*
Karapelit	**78**	3 / 75	**64**	3 / 61	**30**	*S.agalactiae-7* *S.dysgalactiae-8* *S.uberis - 15*	**34**	*S.aureus-5* *CNS-29*	
Stara Zagora	**31**	1 / 30	**26**	1 / 25	**15**	*S.dysgalactiae-9* *S.uberis - 6*	**11**	*S.aureus-3* *CNS-8*	
Popovitsa	**35**	1 / 34	**29**	1 / 28	**10**	*S.dysgalactiae-5* *S.uberis-5*	**19**	*S.aureus-1* *CNS-18*	
Harmanli	**6**	6	**4**	4	**2**	*S.dysgalactiae-2*	**2**	*S.aureus-2*	
Borets	**30**	1 / 29	**36**	1 / 35	**13**	*S.agalactiae-2* *S.dysgalactiae-3* *S.uberis-8*	**20**	*S.aureus-8* *CNS-12*	*T.pyogenes–1* *Acinetobacter spp–1* *Nocardia spp.-1*
Total	**346**	34 / 312	**272**	30 / 242	**151**	*S.agalactiae* – **61** (1 *S.dysgalactiae* – **38** *S.uberis* – **52**	**110**	*S.aureus* – **32** CNS – **78**	**11** *T.pyogenes–3* *Pasteurella spp.–1* *E.coli–3* *Acinetobacter spp.–1* *Nocardia spp.–1*

Table 4. Percentage of staphylococci resistant to 11 chemotherapeutic drugs with confidence limits

Chemotherapeutic drugs	Coagulase-negative staphylococci (n=78)	*Staphylococcus aureus* (n=32)	Total number of staphylococci (n-110)	Confidence limits (CL)
Ampicillin	13 (16.7%)	14 (43.7%)	27 (24.5%)	17.8÷32.8
Amoxicillin/clavulanic acid	-	-	-	
Oxacillin	12 (15.4%)	3 (9.4%)	15 (13.6%)	7.8÷20.6
Cephalothin	1 (1.3%)	-	1 (0.9)	0÷3.5
Cefoxitin	3 (3.8 %)	3 (9.4%)	6 (5.4%)	1.9÷10.3
Lincomycin	50 (64.1%)	5 (15.6%)	55 (50%)	40.7÷59.3
Gentamicin	-	-	-	
Tetracycline	32 (41.0%)	9 (28.1%)	41 (37.3%)	28.5÷46.5
Rifampicin	1 (1.3%)	-	1 (0.9%)	0÷3.5
Ciprofloxacin	2 (2.6%)	-	2 (1.8%)	0.1÷5.1
Trimethoprim/sulphamethoxazole	8 (10.2%)		8 (7.3%)	3.1÷12.8

Table 5. MICs of 9 chemotherapeutic drugs for staphylococcal isolates from subclinical and clinical bovine mastitis (n = 110)

	MIC μg/mL																	
Chemotherapuetic drugs	MIC_{90}	0.001	0.125	0.5	0.75	1	1.5	2	3	4	6	8	16	32	64	96	128	192
Ampicillin	1.0		85	2*	5	8	2	4	1	2					1			
Oxacillin	0.125		98	6*		2	1	3*										
Cephalothin	1.0			32	30	20		27			1*							
Cefoxitin	1.0					54		50		2	*	1	1	2				
Lincomycin	2.0			5	45	5		55*										
Tetracycline	128								*		69			1	9	1	4	26
Rifampicin	0.001	109	*								1							
Ciprofloxacin	0.001	108					*							2				
Trimethoprim/ sulphamethoxazole	1.5					30	40	32		3		1*		4				

Legend: MIC thresholds are marked with asterisks

Persson *et al*., 2011; Piessens *et al*., 2011), as well as in other regions in the world (Mekonnen *et al*., 2017; Mpatswenumugabo *et al*., 2017). In Belgium, Piessens *et al*. (2011) have discussed the thesis that the spread of coagulase-negative staphylococci in mastitis may be associated with the fact that they are human skin commensal organisms that may spread on animals due to the lack of proper hygienic measures. Zadoks *et al*. (2011) and Ndahetuye *et al*. (2019) have emphasized on the fact that the involvement of *Staphylococcus aureus* in the etiology of subclinical mastitis was rather related to chronic and persisting infections.

The genotyping of coagulase-negative staphylococci demonstrated that the commonest species were *S. chromogenes*, *S. epidermidis*, *S. haemolyticus*, *S. simulans*, and *S. xylosus* (Capurro *et al*., 2009; Perry *et al*., 2010; Persson *et al*., 2011; Duse *et al*., 2021). In Germany, Luthje *et al*. (2006) have reported a different species distribution of coagulase-negative staphylococci causing bovine subclinical mastitis, with the highest prevalence of *Staphylococcus chromogenes* (33.2%), followed by *Staphylococcus simulans* (23.2%) and *Staphylococcus epidermidis* (11.7%). In Rwanda, Ndahetuye *et al*. (2019) have found out that among coagulase-negative staphylococcal agents of subclinical mastitis, *Staphylococcus epidermidis* (38.2%) was more commonly isolated than *Staphylococcus sciuri* (19.5%) and *Staphylococcus chromogenes* (9.8%). On the other hand, Ruegg (2020) has commented that streptococci were the leading agents of bovine clinical mastitis,

Table 6. Resistance patterns of multiresistant staphylococci isolated from bovine mastitis (n-110)

Resistance patterns	Number of resistant strains	Resistant staphylococci (%) and confidence limits (CL)
	Coagulase-negative staphylococci (n=78)	
Ox, Amp, L,T	6	7.7% (2.8÷14.5)
Ox, Amp, Fox, L	2	2.6% (0.3÷7.2)
Ox, Amp, L	2	2.6% (0÷0.3)
Ox, Amp, Ceph, L	1	1.3% (0÷4.9)
Ox, Amp, AMC, Ceph, Fox, L, T	1	1.3% (0÷4.9)
	Coagulase-positive staphylococci (n=32)	
L, RIF	4	12.5% (3.5÷26.1)
Ox, Amp, Ceph, RIF	3	9.4% (1.9÷21.7)

Legend: Ox – oxacillin, Amp-ampicillin, AMC – amoxicillin/clavulanic acid, Ceph – cephalothin, Fox – cefoxitin, L – lincomycin, T – tetracycline, RIF – rifampicin.

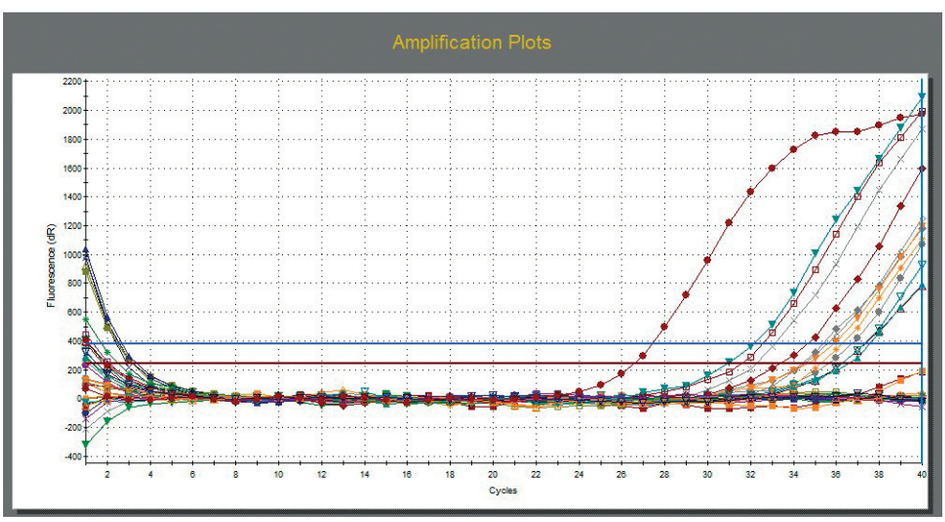

Fig. 1. Amplification plots of *Staphylococcus aureus*

followed by coliform bacteria and *Staphylococcus aureus*. Neelam *et al.* (2022) have also determined *Staphylococcus aureus* as the leading etiological agent of bovine mastitis in India: in 79.7% of cases as seen from genetic analysis of staphylococci isolated from milk samples, whereas only 10.9% were described as belonging to coagulase-negative staphylococci. Also in India, Mahanti *et al.* (2020) have proved the presence of *Staphylococcus aureus* in 21% of milk samples from healthy cows and cows with clinical and subclinical mastitis, tested by phenotype and genetic methods.

From a comparative point of view, the number of genetically determined coagulase-negative *Staphylococcus epidermidis* were encountered at a lower rate (10.2%), comparable with that reported by Luthje *et al.* in Germany (11.8%). Regarding the prevalence of coagulase-positive staphylococci, genetic studies classified 31.2 % of the tested strains as *Staphylococcus aureus*, with a rate higher than those from data of Mahanti *et al.*, and far lower than those reported by Neelam *et al.* in India.

In Poland, Gagielski *et al.* (2014) have discussed the hypothesis that penicillin-resistant staphylococci were largely involved in bovine mastitis etiology (41%). They also underlined that fact that all *Staphylococcus aureus* isolates were sensitive to cefoxitin, gentamicin, doxycycline, ciprofloxacin and trimethoprim/sulfamethoxazole and expressed an intermediate sensitivity to clindamycin. In their view, the monitoring of antimicrobial resistance of *Staphylococcus aureus* strains in dairy cattle farms is an important part of mastitis control and objective therapeutic control.

In Germany, Luthje *et al.* (2006) observed a broader prevalence of ampicillin-resistant coagulase-negative staphylococci (18.1%), as well as of those resistant to pirlimycin from the lincosamides group (6.4%). In Brazil, Olivera *et al.* (2012) have reported a higher prevalence rate to tetracycline (19%) among *Staphylococcus aureus* isolates from bovine subclinical and clinical mastitis. Again in Brazil, Zuniga *et al.*

(2020) have discussed the high rate of coagulase-negative staphylococci, resistant to amoxicillin and ampicillin (59.7%) causing subclinical mastitis in cattle. With regard to gentamicin and oxacillin, the authors recorded a high sensitivity, 95% and 86%. The MIC_{90} values for aminopenicillins exceeded 8 μg/mL.

Our results demonstrated a lower spread of staphylococci resistant to aminopenicillins (24.5%), compared with the data of Zuniga *et al*. from Brazil, with a higher percentage of resistant coagulase-positive strains (43.7%). On the other side, the obtained MIC_{90} values for aminopenicillins were below 1 μg/mL. In India, Mahanti *et al.* (2020) have reported a higher prevalence of *Staphylococcus aureus* resistant to beta-lactam chemotherapeutics ampicillin (71.4%), cefoxitin (42.9%) and amoxicillin/clavulanic acid (38.1%). The authors commented that multi-resistance patterns of methicillin-resistant strains included beta-lactams, tetracyclines and aminoglycosides. In Sweden, possibly due to the antibiotic restriction in livestock husbandry, Duse *et al*. (2021) have reported a high sensitivity of clinical mastitis *Staphylococcus aureus* isolates to chemotherapeutics, except for penicillin-resistant coagulase-negative staphylococci (30.4%).

Also, higher rates of resistance to lincomycin and tetracycline have been reported (50%, 37.3%) compared with data from Germany published by Luthje *et al*. and from Brazil reported by Zuniga *et al*. In India, Neelam *et al*. have also established higher percentages of *Staphylococcus aureus* milk isolates, resistant to lincomycin (49.09%) and oxytetracycline (98.18%). These rates were discussed in the light of the more common use of antimicrobial drugs for bovine mastitis therapy in the country. Our results for microbial resistance to lincomycin and tetracycline showed higher rates in coagulase-negative staphylococci: 64.1% and 41.0%, respectively.

Our data referring to the prevalence of staphylococci sensitive to oxacillin and gentamicin (86.4%, 100%) were similar to the results of Zuniga *et al*. from Brazil.

Conclusion

The information from the present study provides evidence about the higher prevalence of coagulase-negative staphylococci in the etiology of bovine mastitis in Bulgaria and increased rates of resistance among staphylococcal isolates to lincomycin and tetracycline. Multi-resistant patterns including ampicillin, oxacillin, lincomycin and tetracycline were demonstrated in 7.7% a higher prevalence in resistant coagulase-negative staphylococci, whereas 12.5% of *Staphylococcus aureus* strains had multi-resistance patterns including lincomycin and rifampicin.

The monitoring of bacterial antimicrobial resistance, including in agents causing mastitis in animals is recommended by the World Health Organization and the World Organisation for Animal Health to provide valuable information about the trends in therapeutic approaches, namely antibiotic drugs use.

In Bulgaria, beta-lactams, aminopenicillins extended spectrum semi-synthetic penicillins e.g. cloxacillin, and cephalosporins, most commonly cephalothin, are among the drugs most frequently recommended for treatment of bovine mastitis. Intramammary infusions containing combinations of lincomycin and gentamicin, tetracycline, neomycin and bacitracin are also used. In this two-year survey, the demonstrated higher prevalence of lincomycin-resistant and tetracycline-resistant staphylococcal isolates from milk samples is probably a specific feature that should be considered in designing future national-scale studies in this field.

Acknowledgements

This work was supported by a grant from Ministry of Education, Republic of Bulgaria no.12/2020.

References

1. Abebe R, Hatiya H, Abera M, Megersa B & Asmare K (2016) Bovine mastitis: Prevalence, risk factors and isolation of *Staphylococcus aureus* in dairy herds at Hawassa milk shed, South Ethiopia. BMC Veterinary Research, 12: 270
2. Capurro A, Artursson K, Persson Waller K, Bengtsson B, Ericsoon Unnerstad H & Aspan A (2009) Comparison of a commercialized phenotyping system, antimicrobial susceptibility testing, and *tuf* gene sequence-based genotyping for species –level identification of coagulase- negative staphylococci isolated from cases of bovine mastitis. Veterinary Microbiology, 134: 327-333
3. Duse A, Persson-Waller K & Pedersen K (2021) Microbial aetiology, antibiotic susceptibility and pathogen-specific risk factors for udder pathogens from clinical mastitis in dairy cows. Animals, 11, 2113, doi.org/10.3390/ani11072113
4. EUCAST (2021) European Committee on Antimicrobial Susceptibility Testing, Clinical breakpoints-breakpoints and guidance, https://www.eucast.org/clinical breakpoints (23 march 2022 date last accessed)
5. Gagielski T, Puacz E, Lisowski A, Siedlecki P, Dudziak W, Miedzobrodzki J& Krukowski H (2014) Short communication: antimicrobial susceptibility profiling and genotyping of *Staphylococcus aureus* isolated from bovine mastitis in Poland. Journal of Dairy Science, 97: 6122-6128
6. Léon-Galvan MF, Barboza-Corona JE, Lechuga-Arana AA, Valencia-Posadas M, Aguayo DD, Cedillo-Palaez C, Martinez –Ortega EA & Gutierrez-Chavez AJ (2015) Detection and sensitivity to antibiotics and bacteriocins of pathogens isolated from bovine mastitis in family dairy herds of Central Mexico. BioMed Research, 615153
7. Livermore D & Brown D (2001) Detection of beta-lactamase-mediated resistance. Journal of Antimicrobial Chemotherapy, 48, Suppl 1: 59-64
8. Luthje P& Schwarz S (2006) Antimicrobial resistance of coagulase-negative staphylococci from bovine subclinical mastitis with particular reference to macrolide- lincosamide resistance phenotypes and genotypes. Journal of Antimicrobial Chemotherapy, 57: 966-969
9. 9. Mader R, Damborg P, Amat JP, Bengtsson B, Bourély C, Broens EM, Busani L, Crespo P, Filippitzi ME& Fitzgerald W (2021) Building the European Antimicrobial Resistance Surveillance network in veterinary medicine (EARS-Vet). Eurosurveillance: 26
10. Mahanti A, Siddhartha Narayan Joardar, Samiran Bandyopad-

hyay, Jaydeep Banerjee, Sarbaswarup Ghosh, Kunal Batabyal, Tapas Kumar Sad, Tapan Kumar Dutta & Indranil Samanta (2020) Characterization of methicillin- resistant and enterotoxins producing *Staphylococcus aureus* in bovine milk in India. Journal of Agriculture and Food Research, 2, 100017, doi. org/101016/j.jafr.2019. 100017
11. Mekonnen SA, Koop G, Melkie ST, Getahun CD, Hogeveen H & Lam TJGM (2017) Prevalence of subclinical mastitis and associated risk factors at cow and herd level in dairy farms in North-West Ethiopia. Preventive Veterinary Medicine, 145: 23-5877
12. Mpatswenumugabo JP, Bebora LS, Gitao GC, Mobegi VA, Iraguha B, Kamana O, Shumbusho B & Mestorino Nora (2017) Prevalence of subclinical mastitis and distribution of pathogens in dairy farms of Rubavu and Nyabihu districts, Rwanda. Journal of Veterinary Medicine, 8456713, doi: 10.1155/2017/8456713
13. Neelam VK, Jain Mahavir Singh, Vinay G. Joshi, Rajesh Chhabra, Kuldeep Singh & Rana YS (2022) Virulence and antimicrobial resistance gene profiles of *Staphylococcus aureus* associated with clinical mastitis in cattle. PLOS One, doi. org/10.1371/journa.pone.0264762
14. Ndahetuye JB, Persson Y, Nyman Ann-Kristin Tukei M, Ongol MP& Båge R (2019) Etiology and prevalence of subclinical mastitis in dairy herds in peri-urban areas of Kigali in Rwanda. Tropical Animal Health and Production, 51: 2037-2044
15. Oliveira L, Langoni H, Hulland C& Ruegg PL (2012) Minimum inhibitory concentration of *Staphylococcus aureus* recovered from clinical and subclinical cases of bovine mastitis. Journal of Dairy Science, 95:1913-1920
16. Persson Y, Nyman A & Grönlund- Andersson U (2011) Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. Acta Veterinaria Scandinavica, 53: 3
17. Perry J, Midlleton JR, Dufour S, Scholl D, Calloway C, Anderson S& Dohoo I (2010) Association of coagulase-negative staphylococcal species and milk somatic cell count of cows from the Canadian national cohort of dairy farms. MMC Annual Meeting Procedures, Albuquerque, New Mexico: 204-205
18. Piessens V, Van Coillie E, Verbist B, Supré K, Braem G, Van Nuffel A, De Vuyst L, Heyndrickx M & De Vliegher S (2011) Distribution of coagulase-negative *Staphylococcus* spp. from milk and environment of dairy cows differs between herds. Journal of Dairy Science, 94 (6): 2933-2944
19. Ruegg PL (2018) Making of antibiotic treatment decisions for clinical mastitis. Veterinary Clinical Nort American Food and Animal Practice, 34: 413-425
20. Sakwinska OD, Morisset J, Madec JY, Waldvogel A, Moreillon P& Haenni M (2011) Link between genotype and antimicrobial resistance in bovine-mastitis *Staphylococcus aureus* strains, determined by comparing Swiss and French isolates from the Rhóne Valley. Applied Environmental Microbiology, 77: 3428-3432
21. Salvador RT, Soliven RL, Balagan EJY, Abes NS, Gutierrez CA & Mingala CN (2014) Evaluation of portable somatic cell counter in the diagnosis of bubaline subclinical mastitis. Thai Journal of Agricultural Science, 74 (4): 205-209
22. Schnitt A & Tenhagen Bernd-Alois (2020) Risk factors for the occurrence of methicillin-resistant *Staphylococcus aureus* in dairy herds: An update. Foodborne Pathogens and Disease, 17 (10), doi: 10.1089/fdp.2019.2638
23. Silveira-Filho VM, Luz IS, Campos APF, Silva WM, Barros MPS, Medeiros E, Freitas MFL, Mota RA, Sena MJ& Leal-Balbino TC (2014) Antibiotic resistance and molecular analysis of *Staphylococcus aureus* isolated from cow's milk and dairy products in Northeast Brazil. Journal of Food Protection, 77: 583-591
24. Tenhagen BA, Hansen I, Reinecke A & Heuwieser W (2009) Prevalence of pathogens in milk smples of dairy cows with clinical mastitis and in heifers at first parturition. Journal of Dairy Research, 76: 179-187
25. Vakkamäki J, Taponen S, Heikkilä AM & Pyörälä S (2017) Bacteriological etiology and treatment of mastitis in Finnish dairy herds. Acta Veterinaria Scandinavica, 59: 33
26. Zadoks RN, Middleton JR, McDougall S, Katholm K& Schukken YH (2011) Molecular epidemiology of mastitis pathogens of dairy cattle and comparative relevance to humans. Journal Mammary Gland Biology Neoplasia, 16: 357-372
27. Zuniga E, Benites NR, Santana da Hora A, Mello PL, Laes MA, Ribeiro de Olivera LA, Brandäo PE, Oliveira de Souza Silva S, Taniwaki SA & Melville PA(2020) Expression of genes encoding resistance in *Staphylococcus* spp., isolated from bovine subclinical mastitis in Brazil. Journal of Infection in Developing Countries, 14 (7): 772-780

Received 25 October 2022
Accepted 9 December 2022

Study of *MYBPC3* Gene A31P Mutation (c.91 G>C) Causing Hypertrophic Cardiomyopathy in Maine Coon and Other Breed Cats

Kristina Morkūnienė[1], Justina Dambrauskaitė[1], Renata Bižienė[1], Ramutė Mišeikienė[1], Nijolė Pečiulaitienė[1], Laimutis Kučinskas[1]

[1]Lithuanian University of Health Sciences, Institute of Biology Systems and Genetic Research, LT- 47181 Tilžės st. 18, Kaunas, Lithuania

Keywords: *A31P mutation, feline, hypertrophic cardiomyopathy, Maine Coon, MYBPC3 gene.*

Abstract. *Hypertrophic cardiomyopathy (HCM) is one of the most common heart diseases among cats. The aim of the study was to find out the prevalence of the missense A31P mutation of the MYBPC3 gene in the feline population and its dependence on breed and sex. During the study, 130 individuals were tested by PCR-RFLP method. The 242 bp PCR product was digested with AvaI restriction enzyme and the fragments were separated by electrophoresis in 2.5% agarose gel. Besides that, retrospective analysis was performed based on data from the veterinary clinic, collected in a three years period. The mutation (G/C genotype) was detected only in the Maine Coon breed. Data analysis showed that frequency of heterozygous genotype in the tested Main Coon population was 0.23, which is equal to 13.85% of all the studied feline population and 23.1% of the tested experimental (Maine Coon) group. Allele frequencies were calculated in the experimental group (Maine Coon), the control group (feline of other breeds) and for all tested individuals. In all groups, the most frequently repeated allele was G, and in the control group, it was the only detectable allele. Frequency of the C (mutated) allele was calculated for all individuals (0.07) and for experimental (Maine coon) (0.115) groups. Statistically significant Main Coon breed dependence on the mutation was assessed ($P < 0.05$). Information analyzed retrospectively showed that HCM was more often diagnosed for males (87%) than females.*

Introduction

Hypertrophic cardiomyopathy (HCM) is characterized by an abnormal increase in myocardial mass that affects cardiac structure and function. HCM is the most common inherited cardiovascular disease in humans (0.2%) and the most common cardiovascular disease in cats (14.7%) (Gil-Ortuño et al., 2020). Domestic cats of any age from 3 months upward, of either sex and of any breed, can be affected. A higher prevalence in male and domestic shorthair cats has been reported (Kitz et al., 2019).

Feline hypertrophic cardiomyopathy (HCM) is defined by an unexplained thickening of the left ventricular wall without dilation of the chambers and in absence of any other cardiac or noncardiac disease that itself is capable of causing hypertrophy of the heart (Kittleson et al., 2021).

Feline HCM has long been called an idiopathic disease. Viral infections have been implicated as the cause of cardiomyopathy in several mammalian species (Machado et al., 2010). Recently, however, there has been a growing trend towards a different – genetic – diagnosis of HCM. Genetic HCM is thought to occur in American Shorthair, Maine Coon, Persian, Norwegian Forest, Ragdoll and Sphinx cats. Based on research in human medicine in the diagnosis of HCM, the genetic origin of feline HCM was first identified in 2005 by a sequence analysis of the gene encoding sarcomer proteins (Wess et al., 2010; Connolly et al., 2005). More than 1500 variants in *MYBPC3, MYH7* and other sarcomeric genes are associated with human HCM, while in cats, only two causative variants in *MYBPC3* are currently known (Schipper et al., 2019). For the first time, the A31P mutation in the *MYBPC3* gene was found for Maine Coons. It is supposed that about 34 percent world Maine Coon population has the missense A31P mutation (Mary et al., 2010). Frequent prevalence of the disease in Maine Coon population for which this mutation has not been identified indicates that it is probably not the only mutation causing HCM in this breed.

The A31P mutation (c.91 G > C). A change in the nucleotide sequence of the DNA was detected at codon 31, nucleotide 91 of the third exon of the *MYBPC3* gene on chromosome 11, in which the conserved guanine (G) was replaced by cytosine (C). Such a change in the nucleotide sequence at the codon resulted in a change in the GCC triplet, which encodes alanine, to the CCC triplet, which encodes proline. There are two ways to term this mutation: the first is A31P, which contains the codon number and exchanged amino acids, and the second is c.91G>C, which indicates the nucleotide number of the DNA

Corresponding to Kristina Morkūnienė Lithuanian University of Health Sciences, Institute of Biology Systems and Genetic Research, Kaunas, Lithuania.
E-mail: kristina.morkuniene@lsmuni.lt

and substituted nucleotides (Meurs et al., 2005).

The prevalence of the A31P mutation was observed among Maine Coon. In one study, 34% of the studied Maine coons had this mutation and 10% of them were homozygous. This study also suggests that the mutation is observed only in the Maine Coon breed cats (Fries et al., 2008). However, in 2010, an A31P mutation was also detected in one cat of British Shorthair (Mary et al., 2010). The prevalence of the A31P mutation in the *MYBPC3* gene has not been analyzed so far among Maine coons and other breeds of cats bred in Lithuania.

As in humans, the HCM causing mutation in cats may have different penetrantity. Mutation manifestation phenotypically was found to be in 6–8% cats with a heterozygous genotype and 58–80% with a homozygous genotype. Therefore, even one cat of the same origin may have minimal heart changes and others have a severely developing disease. Such differences between genotypes and phenotypes indicate that there may be other genetic and environmental factors that influence the development of a particular phenotype (Kittleson et al., 2015).

So far, the genetic alterations that cause HCM have been identified in the gene *MYBPC3*, which encodes the cardiac isoform of myosin-binding protein C (cMyBP-C). This isoform consists of 2% of myofibril proteins in the heart and is important in the regeneration of muscle cells. Decrease in cMyBP-C and myomezin protein levels were observed in cats affected by the mutation. cMyBP-C is a protein that regulates the heart muscle, which affects the power and the rate of heart contractions. It also contributes to cardiac systolic and diastolic function and the ability of the heart to increase contractions under the influence of an inotropic stimulus (Sadayappan et al., 2012).

Due to the frequent occurrence of HCM in cats, a clear mechanism for the onset of the disease is very relevant in veterinary medicine. Therefore, the aim of the research was to evaluate the prevalence of the A31P mutation in the *MYBPC3* gene in feline population, its dependence on different factors such as breed and sex. Detection of the mutation allows preventive measures to be taken to ensure the well-being and health of cats at a risk of HCM.

Material and methods

MYBPC3 gene polymorphism study

A total of 130 individuals were studied. Biological samples were taken from various breeds of cats (*Table 1*). Samples for research were collected from different regions of Lithuania. They were divided into two parts: control and experimental groups. The control group consisted of cats of various breeds (Scottish Fold, British Shorthair, Siamese, Devon Rex, Persian, Bengal and crossbreed), and the experimental group was comprised only of Maine Coon cats. DNA was isolated from buccal epithelial cells (Aidar et al., 2007). The *MYBPC3* gene polymorphism study was performed by the PCR-RFLP method. This test was used to identify a mutated allele that forms when a missense mutation occurs, guanine changes to cytosine at codon 31 of the third exon (c.91G>C). According to feline *MYBPC3* gene sequence (GeneBank accession No. NC_018732.3), using

Table 1. Distribution of studied individuals by breed and gender

No.	Breed	Number of tested individuals	Females	Males
1.	Maine Coon	78	57	21
2.	Scottish Fold	20	11	9
3.	Persian	3	1	2
4.	Devon Rex	1	0	1
5.	British Shorthair	10	1	9
6.	Crossbreed	13	3	10
7.	Bengal	3	2	1
8.	Siamese	2	0	2

Table 2. Primers, PCR reaction conditions, size of PCR product and restriction enzyme

Patin7Genetic defect	Primers	PCR profile			PCR product size, bp	Restriction enzyme
Hypertrophic cardiomyopathy (HCM)	F: 5'-agccttcagcaagaagcca-3' R: 5'-caaacttgaccttggaggagc-3'	95°C	3 min		242 bp	*AvaI*
		94°C	30 sec	35 cycles		
		56°C	30 sec			
		72°C	40 ses			
		72°C	7 min			

"Primer3" software, oligonucleotide primers were designed. The PCR mode used in the study is shown in *Table 2*. The primers amplify 242 bp fragment. The obtained PCR product was digested with a restriction enzyme. The appropriate restriction enzyme (*AvaI, Thermo Scientific*) was selected by the "CLC Sequence Viewer 8" program. 10 µL of the PCR product was digested with 10 µL of the restriction mixture. Samples were incubated in a thermostat overnight at 37°C. The digested PCR products, stained with 10 µL of ethidium bromide, (CS-300V Cleaver; Scientific Ltd), were fractionated by electrophoresis at 2.5% agarose gel (1 × TAE) for 45 min. Then they were analyzed under UV light (wavelength 300 nm) with a "MiniBisPro" video documentation device (Herolab). *MYBPC3* gene c.91G>C DNA fragments sizes after digestion with the restriction enzyme were: homozygous genotype for the normal allele (G/G) – 242 bp, heterozygous genotype (G/C) – 242, 179, 63 bp, homozygous genotype for the mutated allele (C/C) – 179 and 63 bp. (Fig. 1).

Data for retrospective analysis was collected at the veterinary clinic. Information was collected from patients who referred to the clinic for heart problems. A medical history of the animals was collected during the visit, including the conditions of animal keeping, preventive measures, changes in behavior, general well-being of the animal. Patients underwent general and specific tests.

Fig. 1. Analysis of the genotypes in the agarose gel. A– G/G genotype; B – G/C genotype; C – 50 bp DNA ruler (Thermo Fisher Scientific).

Statistical Analysis

IBM SPSS Statistics software was used to interpret the results. The frequencies of the obtained results were calculated using the Descriptive statistics FREQUENCIES function, averages with the Compute variable MEAN function. The Descriptive statistics Crosstabs CHI-SQUARE function was used to calculate the significance between the different variables. Results were considered significant, when $P < 0.05$.

Results

Genotype frequencies were calculated separately for the experimental (Maine Coon, n = 78) and for the control group (various breeds, n = 52). The calculated data show that in both cases the genotype (G/G) leading to the absence of the mutation is more common. However, comparing the frequencies of the experimental group and the control group genotypes, the frequency of the heterozygous genotype was higher in the experimental group (Table 3).

Breed dependence on the mutation was assessed. A heterozygous genotype was detected only in the experimental (Main coon) group. A statistically significant result was obtained ($P = 0.0002$, $P < 0.05$) (Fig. 2).

Allele frequencies were calculated in the experimental (Maine Coon), the control groups, and for all tested individuals. In all groups, the most frequently repeated allele was G, and in the control group, it was the only detectable allele. Frequency of the C allele was determined in the experimental (0.115) and all individuals (0.07) group (Table 4).

Another relevant aspect of outcome evaluation is the distribution of mutation by gender. Seventy-five females were examined, which accounted for 57.7% of all subjects, and 55 males, which accounted for 42.3% of all studied subjects. Heterozygous genotype (G/C) was identified for 18 cats: 14 females (78%) and 4 (22%) males. However, in the analysis of the data only for cats of the Maine Coon breed, the percentage of females with the heterozygous genotype was 24.56%, and the number of males was 19%, respectively, based on the number of individuals of each gender studied.

After collecting data at the veterinary clinic, it was determined that 15 cats were diagnosed with HCM in the three years period. It was observed that the disease was diagnosed more often in male individuals: 13 males (frequency 0.867). Less frequently, the disease was diagnosed for females (frequency 0.133).

The frequency of the age group at which HCM was diagnosed was calculated. The most common disease was diagnosed in the youngest cats' group (frequency 0.467) (Table 5), and less often in the older cats' group. The mean age of diagnosis with HCM was 4.8 years (Fig. 3).

Table 3. Genotypes frequencies table

Frequencies of experimental group genotypes		Frequencies of control group genotypes	
G/G	0.769	**G/G**	1
G/C	0.231	**G/C**	0
C/C	0	**C/C**	0

Table 4. The frequencies table of alleles

Allele	Experimental group	Control group	All tested individuals
G	0.885	1	0.93
C	0.115	0	0.07

Table 5. Age groups and frequency table

Age group, years	Number of individuals with HCM	Frequency
1–3	7	0.467
3–6	5	0.333
6–9	0	0
9–12	2	0.133
> 12	1	0.067

Discussion

Samples were collected from both males (55) and females (75) belonging to Maine Coon and other breed cats (Scottish Fold, British Shorthair, Siamese, Devon Rex, Persian, Bengal and crossbreed). The mutation (G/C genotype) was detected only in the Maine Coon breed. The prevalence of the A31P mutation was not determined in the other studied breed cats. That makes 13.85% of all studied feline population and 23.1% of the tested experimental (Maine Coon) group. Breed dependence on the mutation was assessed ($P = 0.0002$, $P < 0.05$). The same results are also confirmed by other researches. HCM is one of the most common diagnoses in cats, and thus far only two genetic variants have been reported to be associated with this disease (Ontiveros et al., 2019). According to O'Donnell et al., the lack of the MYBPC3 p.Ala31Pro and other mutation variants in feline HCM population suggests that the clinical utility of genetic testing is greatest in the specific cat breeds in which these causative variants of HCM have been identified (O'Donnell et al., 2021).

Compared with the results obtained by other authors, a similarity is visible. Mary et al. identified a breed dependence of the mutation. Of the 2744 Maine Coon cats tested, 41.5% had the A31P mutation detected. However, in this study, a mutation was also detected in one cat of the British Shorthair breed (Mary et al., 2010). Godiksen et al. indicate that the prevalence of HCM causing mutation in the Maine Coon population is between 9.5% and 26.3% (Godiksen et al., 2011).

When evaluating the distribution of the mutated allele according to the gender of the studied cats,

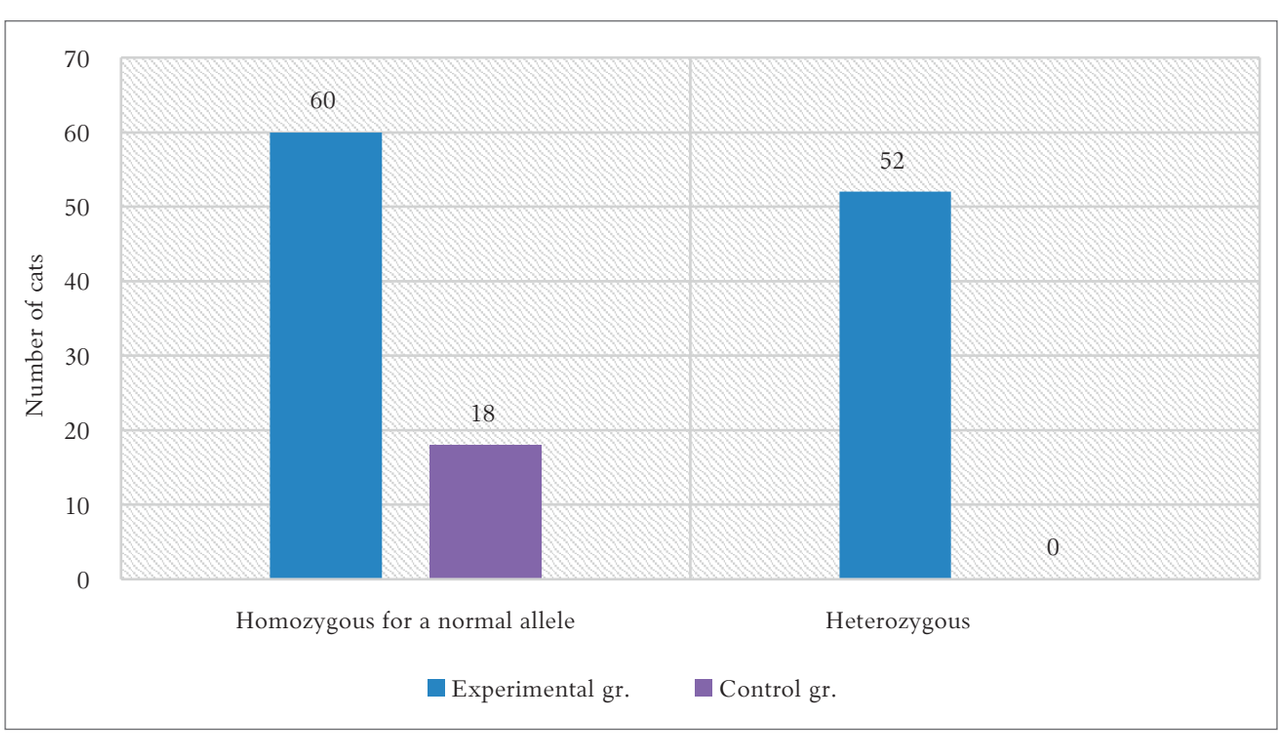

Fig. 2. Distribution of genotypes in experimental and control groups.

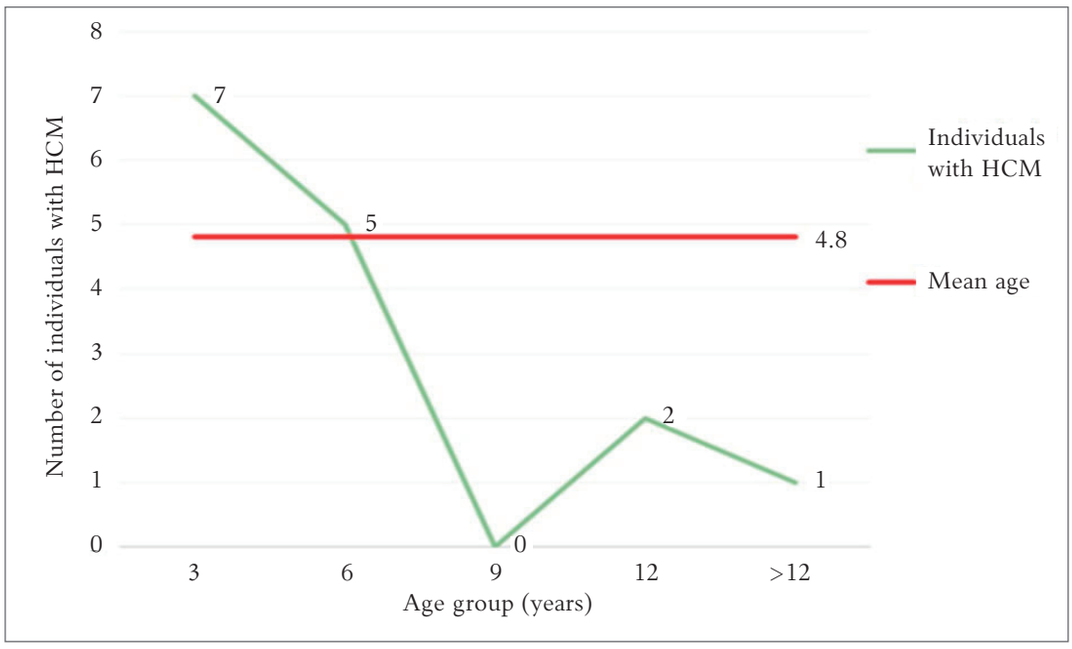

Fig. 3. The number of individuals diagnosed with HCM by age group and mean age.

only the experimental group was analyzed, since the mutated allele was detected only in it. Females with a heterozygous genotype accounted for 24.56%, and males for 19%, respectively, of all tested representatives of each gender.

After information analysis collected at the veterinary clinic, it was observed that the disease was diagnosed more frequently in males (frequency 0.867). This accounted for 87% of all samples. Less frequently, the disease was diagnosed in females (frequency 0.133).

According to Payne et al., the prevalence of the disease is higher in males (62.6%) than in females, suggesting that sex may be one of the predisposing factors, leading to the manifestation of the mutation (Payne et al., 2015). Analysis of Longeri et al. research results also showed the incidence of the disease by gender: HCM was found in 78.9% of males and only 21.1% of females (Longeri et al., 2013). According to Riesen et al., HCM is common in both sexes equally, but males develop symptoms earlier and often have a more severe form of the disease (Riesen et al., 2007).

The age dependence of HCM was assessed by analyzing data from veterinary clinic X. During a three-year period, the disease was the most often diagnosed in the youngest cats' group, aged 1–3 years (46.7%), and slightly less in the middle age cats' group, aged 3–6 years (33.3%). The mean age at which HCM was diagnosed was 4.8 years.

According to Payne et al., the prevalence of the disease was found in 4.3% of young cats (6–12 months of age) and 29.4% of older cats (⩾ 9 years). However, it is thought that age may vary depending on the breed. Maine Coon, Sphinx, British Shorthair and Rag cats have a diagnosis of the disease ranging from 5 months to 4.2 years. This is thought to have led to a higher incidence of younger cats in the study. According to Payne et al., the mean age of diagnosis of HCM in the feline population is 6 years (Payne et al., 2015).

Conclusions

The A31P mutation (c.91 G>C) was detected only in the Maine Coon breed. Based on the data of the study, it can be stated that the nucleotide substitution of the MYBPC3 gene that determines HCM is specific and prevalent only in cats of the Maine Coon breed. However, no significant relationship between the gender and the genotype of the mutated allele was found. On purpose to select cats for prophylactic testing to detect the disease at an early stage or to reduce the incidence of the mutant allele causing the disease, especially in the Maine Coon population, it is most appropriate to perform molecular genetic testing.

Additional information

All tests were performed in accordance with the requirements of national and European Union legal acts: Law of the Republic of Lithuania on the Care, Keeping and Use of Animals No. VIII –500/1997; and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

1. Aidar M., Line S.R. A Simple and Cost-Effective Protocol for DNA Isolation from Buccal Epithelial Cells. Brazilian Dental Journal. 2007. T. 18(2). P. 148-152. doi: 10.1590/s0103-64402007000200012
2. Connolly D.J., Guitian J., Boswood A., Neiger R. Serum troponin I levels in hyperthyroid cats before and after treatment with radioactive iodine. Journal of Feline Medicine and Surgery. 2005. T. 7 (5). P. 289-300. doi: 10.1016/j.jfms.2005.01.002
3. Fries R., Heaney A.M., Meurs K.M. Prevalence of the myosin-binding protein C mutation in Maine Coon cats. Journal of Veterinary Internal Medicine. 2008. T. 22(4). P. 893-896. doi: 10.1111/j.1939-1676.2008.0113.x
4. Gil-Ortuño C., Sebastián-Marcos P., Sabater-Molina M., Nicolas-Rocamora E., Gimeno-Blanes J.R., Fernández del Palacio M.J. Genetics of feline hypertrophic cardiomyopathy. Clin Gene. 2020.T. 98. P 203– 214. doi: 10.1111/cge.13743
5. Godiksen M.T., Granstrøm S., Koch J., Christiansen M. Hypertrophic cardiomyopathy in young Maine Coon cats caused by the p.A31P cMyBP-C mutation--the clinical significance of having the mutation. Acta Veterinaria Scandinavica. 2011. T. 53(1). P. 7. doi: 10.1186/1751-0147-53-7
6. Kittleson M.D., Meurs K.M., Harris S.P. The genetic basis of hypertrophic cardiomyopathy in cats and humans. Journal of Veterinary Cardiology Suppl 1. 2015. T. Suppl 1. P. 53-73. doi: 10.1016/j.jvc.2015.03.001
7. Kittleson M.D., Côté E. The Feline Cardiomyopathies: 2. Hypertrophic cardiomyopathy. J Feline Med Surg. 2021. T. 23(11). P. 1028-1051. doi: 10.1177/1098612X211020162
8. Kitz S., Fonfara S., Hahn S., Hetzel U., Kipar A. Feline Hypertrophic Cardiomyopathy: The Consequence of Cardiomyocyte-Initiated and Macrophage-Driven Remodeling Processes? Vet Pathol. 2019. T. 56(4). P. 565-575. doi: 10.1177/0300985819837717
9. Longeri M., Ferrari P., Knafelz P., Mezzelani A., Marabotti A., Milanesi L. Myosin-binding protein c DNA variants in domestic cats (A31P, A74T, R820W) and their association with hypertrophic cardiomyopathy. Journal of Veterinary Internal Medicine. 2013. T. 27(2). P. 275-285. doi: 10.1111/jvim.12031
10. Machado Rolim V., Assis Casagrande R., Terezinha Barth Wouters A., Driemeier D., Petinatti Pavarini S. Myocarditis caused by Feline Immunodeficiency Virus in Five Cats with Hypertrophic Cardiomyopathy. J Comp Pathol. 2016. T. 154(1). P. 3-8. doi: 10.1016/j.jcpa.2015.10.180
11. Mary J., Chetboul V., Sampedrano C., Abitbol M., Gouni V., Trehiou-Sechi E. et al. Prevalence of the MYBPC3-A31P mutation in a large European feline population and association with hypertrophic cardiomyopathy in the Maine Coon breed. Journal of Veterinary Cardiology. 2010. T. 12(3). P. 155-161. doi: 10.1016/j.jvc.2010.06.004.
12. Meurs K.M., Sanchez X., David R.M., Bowles N.E., Towbin J.A., Reiser P.J. et al. A cardiac myosin binding protein C mutation in the Maine Coon cat with familial hypertrophic cardiomyopathy Human Molecular Genetics. 2005. T. 14(23). P. 3587-3593. doi: 10.1093/hmg/ddi386
13. O'Donnell K., Adin D., Atkins C.E., DeFrancesco T., Keene B.W., Tou S., Meurs K.M. Absence of known feline MYH7 and MYBPC3 variants in a diverse cohort of cats with hypertrophic cardiomyopathy. Anim Genet. 2021. T. 52. P. 542-544. doi: 10.1111/age.13074
14. Ontiveros E.S., Ueda Y., Harris S.P., Stern J.A. Precision medicine validation: identifying the MYBPC3 A31P variant with whole-genome sequencing in two Maine Coon cats with hypertrophic cardiomyopathy. J Feline Med Surg. 2019. T. 21(12). P. 1086-1093. doi:10.1177/1098612X18816460
15. Payne J.R., Brodbelt D.C., Luis Fuentes V. Cardiomyopathy prevalence in 780 apparently healthy cats in rehoming centres (the CatScan study). Journal of Veterinary Cardiology. 2015. T. Dec;17 Suppl 1. P. 244-57. doi: 10.1016/j.jvc.2015.03.008. PMID: 26776583.
16. Riesen S.C., Kovacevic A., Lombard C.W., Amberger C. Prevalence of heart disease in symptomatic cats: an overview from 1998 to 2005. Schweiz Arch Tierheilkd. 2007. T. 149(2) P. 65-71. doi: 10.1024/0036-7281.149.2.65.
17. Sadayappan S., Tombe P.P. Cardiac myosin binding protein-C: redefining its structure and function. Biophysical Reviews 2012. T. 4(2). P. 93-106. doi: 10.1007/s12551-012-0067-x
18. Schipper T., Van Poucke M., Sonck L., Smets P., Ducatelle R., Broeck B., Peelman L. A feline orthologue of the human MYH7 c.5647G>A (p.(Glu1883Lys)) variant causes hypertrophic cardiomyopathy in a Domestic Shorthair cat. Eur J Hum Genet. 2019. T. 27. P. 1724–1730. doi: 10.1038/s41431-019-0431-4
19. Wess G., Schinner C., Weber K. Association of A31P and A74T polymorphisms in the myosin binding protein C3 gene and hypertrophic cardiomyopathy in Maine Coon and other breed cats. Journal of Veterinary Internal Medicine. 2010. T. 24(3). P. 527-532. doi: 10.1111/j.1939-1676.2010.0514.x

Received 14 October 2022
Accepted 22 December 2022

Effect of Additional Dose of Pgf2α Use in Ovsynch During Second Gnrh and at Insemination on Ovulation and Pregnancy Rates in Dairy Cows

Youssef Assadi[1], Mojtaba Goli[2], Saheb Foroutanifar[3]

[1]Faculty of Veterinary Medicine, Razi University, Kermanshah, Sheykh Toussi Bo., Shohada Sq., Iran
[2]Department of clinical science, Faculty of Veterinary Medicine, Razi University, Kermanshah, Sheykh Toussi Bo., Shohada Sq.,Kermanshah, Iran;
[3]Department of Animal science, Faculty of Agriculture and Natural Resources, Razi University, Kermanshah, Razi University; Imam Khomeini highway; Iran

Keywords: *prostaglandin, ovulation rate, pregnancy rate, Ovsynch, lactating dairy cow.*

Abstract. *The objective was to investigate the effect of administering an additional dose of a PGF2α analog concurrent with the second GnRH (GnRH2) and at artificial insemination in the Ovsynch protocol on ovulation (OR) and pregnancy rates (PR) in Holstein lactating cows. Multiparous clinically healthy cows (parity: 3–5 lactation and 50 ± 5 days in milk (DIM)) were randomly allocated to three groups: Ovsynch (n = 23): GnRH (Alarelin acetate, 25 μg)-7 days- PGF2α (d-cloprostenol, 500 μg)-2 days-GnRH-16 to 18 h-FTAI plus 5 mL normal saline; OvsynchPG9 (n = 17): as Ovsynch plus administering another dose of PGF2α concurrent with GnRH2; and OvsynchPG10 (n = 28): as Ovsynch plus administering another dose of PGF2α at insemination. The ovaries of all selected animals were scanned by transrectal ultrasonography on days 9, 10, and 11 after the initiation (day 0) of the Ovsynch protocol to record the incidence of ovulation. Pregnancy diagnosis was performed by transrectal ultrasonography 50 ± 2 days after FTAI.*

The results showed that OR was greater in OvsynchPG9 (82.3%) than that in OvsynchPG10 (81.8%) or Ovsynch (81.2%), although the difference was not significant (P = 0.8205). In addition, PR/FTAI was greater in OvsynchPG9 (41.2%) than in Ovsynch (30.4%) and the difference was not significant (P = 0.8288). In conclusion, it was found that administering an additional dose of a PGF2α did not improve OR or PR in Ovsynch protocol in Holstein lactating cows.

Introduction

It has been evidenced that poor fertility in dairy cattle is still a major concern for the dairy industry all over the world and that both oestrus detection rate and conception rate (CR) influence pregnancy rate (PR) of dairy herds (Ambrose et al., 2015). Incorrect oestrus detection is correlated to the profit loss because of long intervals of calving, loss of milk yield, and costs related to veterinary service (Roelofs et al., 2010). During the past decades, new technologies, particularly those related to animal reproduction, have become very important in improving agricultural production worldwide (Bó et al., 2002). The use of fixed-time artificial insemination (FTAI) programs such as the Ovsynch protocol can obviate the necessity for oestrus detection and increase submission rates for artificial insemination (AI) resulting in improvement of overall PRs of herds (Ambrose et al., 2015). Ovulation can be synchronized with the Ovsynch program within an 8-h period from 24–32 h after giving the second GnRH treatment, so that successful AI can be performed at a fixed time without the need for oestrus detection (Keskin et al., 2010). Based on the experimental evidence (Liu et al., 2018), during the last years, interest has been increased in expanding new synchronization protocols with FTAI to enhance reproductive efficiency in the dairy industry. However, it has been indicated that ovulation synchrony rates after the use of the Ovsynch program are not absolute and range between 80% and 90%; therefore, enhancement of ovulation synchrony following the Ovsynch program may enhance the percentage of pregnant cows (Peters & Pursley, 2002).

It has been revealed that prostaglandin is a biologically very powerful material with several applications for controlling reproduction (Pfeifer et al., 2014). It has been reported that improvement of sperm transport to the uterine tube occurred after adding substances such as prostaglandin F2α (PGF2α) to the semen or administering them to the female animals. Therefore, administration of PGF2α at the same time with AI could enhance pregnancy probability due to induction of oxytocin secretion which stimulates contractions of the uterus and supports transport of semen (Sauls et al., 2018). The most common uses of PGF2α in cattle are because of its luteolytic effects, that is, synchronization of oestrus, regression of luteal tissue, inducing abortion and initiating parturition (Gabriel et al., 2011). It

Corresponding to Mojtaba Goli, Department of clinical science, Faculty of Veterinary Medicine, Razi University, Kermanshah, Sheykh Toussi Bo., Shohada Sq., Kermanshah, Iran.
Email: mojtabagoli@razi.ac.ir

has been already reported that administering PGF2α intramuscularly stimulates ovulation in prepubertal heifers. More lately, intramuscular administration of PGF2α has been used successfully to induce ovulation in FTAI programs in beef and dairy cattle (Pfeifer et al., 2018). In addition, experimental works in cows have demonstrated that intrafollicular prostaglandin during the periovulatory period is necessary for the process of ovulation (Leonardi et al., 2012). Prostaglandin F2α analog augments hypophysial responsiveness to GnRH, thereby increasing LH secretion in a process resulting in ovulation (Pfeifer et al., 2014). Randel et al. (1996) reported that administering alfaprostol, a PGF2α analog to anestrous cows resulted in an increased frequency of LH secretion 6 h after treatment. Additionally, prostaglandin F2α may act on the hypothalamic–hypophysial axis and stimulate LH release and promote ovarian cyclical activity (Weems et al., 2006). Moreover, prostaglandin F2α seems to have a local role in the ovary (Pfeifer et al., 2018). It has been shown that prostaglandin that is secreted by the preovulatory follicles is closely linked to the ovulatory process. Moreover, as ovulation approaches, secretion of PGF2α increases substantially in the follicle; therefore, administering a pharmacologic dose of PGF2α may quicken ovulation (Pfeifer et al., 2014). In addition, intravenous, intramuscular or intrauterine administration of PGF2α to domestic livestock at AI in order to affect conception has been a subject of frequent investigations since the early 1990s (Sauls et al., 2018). Therefore, the objective of this study was to compare the effect of administering an additional dose of a PGF2α analog, d-cloprostenol sodium, at the second GnRH and at insemination on ovulation and pregnancy rates in Holstein lactating dairy cows synchronized with the Ovsynch protocol.

Materials and methods

Ethical approval

All the procedures of the present study were approved (Approval ID: IR.RAZI.REC.1400.059/07-28-2021) by the Research Ethics Committee of Razi University, Kermanshah, Iran.

Animals

The study was conducted on a commercial dairy farm consisting of about 150 Holstein dairy cows located near Kermanshah (with a hot-summer Mediterranean climate (Csa); 34.4576° N, 46.6705° E, altitude: 1,350 m above sea level, average annual precipitation: 478.7 mm, average low and high temperature: –1.7°C and 37.8°C in December and July, and average low and high relative humidity: 23% and 75% in January and July, respectively), the capital city of Kermanshah province, west of Iran in summer, 2021. Cows were housed in barns with open-air and sheltered areas, had free access to fresh water, were fed a mixed diet that had been adjusted to provide their physiological requirements and milked three times daily with an average 8-hour interval and average milk yield of approximately 27 kg/cow/day during the study period. The diet consisted mainly of wheat straw, corn silage, and hay as roughage, and rice bran, soybean meal and, dried grounded corn seeds as concentrates. The reproductive tract and mammary glands of the cows were examined by inspection and manual palpation for the evidence of any clinical abnormalities 30 ± 2 days after calving and again 55 ± 5 days *postpartum* before initiation of the experiment. Those cows that were clinically normal were enrolled in the experiment. Body condition scores (BCS) were determined for all cows at the beginning of the study using a 5-point (1 = thin to 5 = fat) scoring system and those cows with a BCS between 3.25 to 3.50 were selected for the study.

Experimental design

The animals (55 ± 5 days in milk; multiparous) regardless of being observed in oestrus before were randomly allocated to one of the study groups according to their even or odd ear tag numbers and parity, so that an approximately identical distribution of parity occurred in the groups, as follows: Ovsynch (n = 23): cows received 25 μg of a GnRH analog (5 mL of Alarelin acetate, Vetaroline®, 5 μg luliberin A/mL, Abureyhan pharmaceutical Co., Tehran, Iran) on Day 0, 500 μg of d-cloprostenol sodium analog (2 mL of D-Cloprostenol sodium, D-Clo PG®, 250 μg/mL, Royan darou Co., Veterinary Division, Semnan, Iran) on Day 7, and another 25 μg of the GnRH analog plus 5 mL of normal saline solution on Day 9. The cows were inseminated 18 h after the second dose of GnRH and were administered another dose of normal saline at FTAI; OvsynchPG9 (n = 17): as Ovsynch, except that an additional dose of PGF2α was administered concurrent with the second GnRH instead of normal saline; OvsynchPG10 (n = 28): as Ovsynch, except that an additional dose of PGF2α was administered at FTAI. All treatments were given intramuscularly. Those cows that exhibited oestrus signs before the FTAI as well as those that were culled or were treated because of problems such as mastitis, lameness, etc., were excluded from the study. The data of those cows that were not observed in heat until approximately 24 h before FTAI and were inseminated approximately 18 hours after administering the second dose of GnRH were used for the analysis. All cows were inseminated by the same technician with proven commercial frozen-thawed semen from a single bull.

Ovarian ultrasonography

The ovaries of all selected animals were examined by transrectal ultrasound scanning with a 7.5 MHz transducer (ULTRASONIC SCANNER, MODEL: HS-1500V, SN: 70610484, HONDA ELECTRONIC CO. LTD 20 OYAMAZUKA OIWA-CHO, TOYOHASHI, AICHI, JAPAN) on days 9, 10, and 11 after the beginning of the Ovsynch protocol (day 0) in order to record the occurrence of ovulation. The disappearance (from one session of scanning to the next) of a follicle with 8 mm in diameter or greater

that had been identified in the previous scanning session was defined as ovulation (Pfeifer et al., 2018). Ovulation rate (OR) was defined as the number of the cows in a group that ovulated until day 11, divided by the total number of the animals, in which ovaries were scanned by ultrasound in the corresponding group × 100.

Pregnancy diagnosis

Pregnancy diagnosis was performed in all animals by transrectal ultrasound examination of the uterine horns 50 ± 2 days after FTAI. Those cows that returned to oestrus before pregnancy diagnosis were recorded as non-pregnant. Pregnancy rate per insemination (PR/FTAI) was defined as the number of cows in a group that were confirmed pregnant on days 50 ± 2 after FTAI out of the total number of cows in the corresponding group x 100.

Statistical analysis

Data were analyzed using SAS® software (Statistical Analysis System, Release 9.4. Cary, NC, USA: SAS Inst. Inc.). The analyses were performed in two steps including ORs and PRs to FTAI (PRs/FTAI) by Logistic Regression method using Proc Genmod for determining the probability of significant differences among the groups. χ^2 square statistics was used to determine the degree of difference between the groups. The level of significance was set at $P < 0.05$.

The effects of the average milk production during the study period, BCS and parity on OR and PR were statistically analyzed by Logistic Regression method using Proc Genmod.

Results

The average milk yield, BCS and parity showed no significant interaction.

Ovulation rate

The incidence of ovulation and the number of animals that ovulated between days 9 and 11 after the beginning of the Ovsynch protocol are presented in Table 1. Although the incidence of ovulation in OvsynchPG9 group (82.3%) was numerically greater than those in Ovsynch and OvsynchPG10 groups (81.2% and 81.8%, respectively), the differences were not significant ($P = 0.8205$).

Pregnancy rate

PRs to FTAI in the study groups are presented in Table 1. Although PR/FTAI in OvsynchPG9 group (41.2%) was higher than those in Ovsynch (30.4%) and OvsynchPG10 (35.7%) groups, and the differences were not significant ($P = 0.8288$).

Discussion

According to the results of the present study, there was no significant effect of parity, BCS, and milk yield of the cows on the PR. In a previous study, Momcilovic et al. (1998) reported no effect of BCS and number of lactation on pregnancy rate in dairy cows synchronized with GnRH and/or PGF2α for oestrus and ovulation, which is in agreement with the finding of the present study. In addition, in agreement with the finding of the present study, the results of a recent research (Chenault et al., 2014) have demonstrated that dam parity had no significant effect on pregnancy to FTAI in dairy cows that underwent synchronization of ovulation and FTAI. Lajili et al. (1991) also reported no significant effect of parity and milk yield on the conception rate. Moreover, the findings of the present study revealed that parity and milk yield had no significant effect on OR. In agreement with this finding, a more recent research (Liu et al., 2018) has reported that parity had no effect on OR. By contrast, Gümen and Seguin (2003) reported that parity influenced ovulation after administration of GnRH, because it was demonstrated that ovulation was much less likely to occur in first parity cows.

According to the results of the present study, administering an additional dose of PGF2α concurrent with the second GnRH and at insemination in Ovsynch does not improve OR significantly in lactating dairy cows synchronized with the Ovsynch protocol compared with those that did not receive the additional dose of PGF2α. In agreement with these findings, Sauls et al. (2018) reported that following administration of PGF2α at FTAI, an average ovulation risk was higher than 90% in lactating dairy cows, but did not differ between treatments. Pfeifer et al. (2018) reported no difference in the proportion of cows ovulating between cows that received an extra dose of d-cloprostenol and those that did not receive it. Contrary to these findings, López-Gatius et al. (2004) reported that a single injection of PGF2α

Table 1. Ovulation and pregnancy rates at insemination in Holstein lactating dairy cows synchronized with the Ovsynch protocol with or without an additional dose of PGF2α.

Variables	Ovsynch1	OvsynchPG92	OvsynchPG103	P-value
OR (%)*	13/16 (81.2)[a]	14/17 (82.3)[a]	9/11 (81.8)[a]	0.8205
PR/FTAI (%)**	7/23 (30.4)[a]	7/17 (41.2)[a]	10/28 (35.7)[a]	0.8288

* OR: ovulation rate; ** PR/FTAI: pregnancy rate to fixed-time artificial insemination.
[1] Ovsynch protocol (GnRH-7 days-PGF2α-2 days-GnRH-18 hours-FTAI);
[2] OvsynchPG9: GnRH-7 days-PGF2α-2 days-GnRH+PGF2α-18 hours-FTAI;
[3] OvsynchPG10: GnRH-7 days-PGF2α-2 days-GnRH-18 hours-FTAI+ PGF2α;
[a] Means with different superscripts in the same row are significantly different ($P < 0.05$).

intravenously at the time of AI increased OR in dairy cows.

Fricke et al. (1998) reported that the incidence of ovulation after injection of the second GnRH was 84% when Ovsynch was initiated at various stages of the estrous cycle. In another study (Vasconcelos et al., 1999), it was reported that the incidence of ovulation induced by the second GnRH was 81% to 94% and that the total mean synchronization rate was 87%. Yilmazbaś-Mecitoglu et al. (2014) found an 84.5% OR in dairy cows after the second GnRH of the traditional Ovsynch protocol. In a study by Liu et al. (2018), 93.3% of ovulations were successfully induced by GnRH mainly within 36 h of injection in dairy cows synchronized with a modified Ovsynch protocol, in which two low doses of PGF2α were given on days 7 and 8 of the protocol. In the present study, the Ovsynch protocol was initiated at random stages of the oestrus cycle and ultrasonic scanning of the ovaries was performed three times with a 24 h interval beginning at the time of administration of the second dose of GnRH. The results showed 81.2% ovulation within 48 h after administration of the second dose of GnRH in the Ovsynch group, which did not differ significantly with those in the OvsynchPG9 or OvsynchPG10 groups (82.3% and 81.8%, respectively), in which the animals received an additional dose of PGF2α concurrent with the second dose of GnRH or at FTAI, respectively.

According to Pfeifer et al. (2014), for achieving excellent fertility to FTAI, synchronous ovulations must happen (within a few hours) in most of the treated cows, with the least number of failures. It has been shown that exogenous PGF2α can be successfully used as an ovulatory stimulus for FTAI in parous and nulliparous cows (Pfeifer et al., 2014). Ovulation may be successfully induced and synchronized by PGF2α similar to treatments with estradiol benzoate or estradiol cypionate (Sauls et al., 2018). Prostaglandins can increase the response to GnRH (Pfeifer et al., 2014). However, the ovulation process is influenced by many factors such as the environment and diet (Sauls et al., 2018).

The results of the present study showed no significant difference in PR to FTAI between the treatment groups, although the low number of animals in each group was a limitation of the study. Conflicting results have been reported regarding the effect of administering PGF2α or its analog at the time of AI on PRs. Some studies (López-Gatius et al., 2004; Neglia et al., 2008) have reported improved PRs after administration of PGF2α at the time of insemination, but some others (Archbald et al., 1992; Kauffold et al., 2009; Gabriel et al., 2011; Mohammadi et al., 2019) have reported the opposite. Neglia et al. (2008) reported positive results on PRs when 500 µg cloprostenol were administered intramuscularly to Italian Mediterranean buffaloes. Ambrose et al. (2015) found that 10 mg, but not 5 mg, of PGF2α given intramuscularly concurrent with FTAI resulted in a significant increase in conception rate in lactating dairy cattle. López-Gatius et al. (2004) reported that administration of a single dose of PGF2α intravenously at AI improved PR in dairy cows. Meanwhile, in agreement with the results of the present study, Sauls et al. (2018) reported no increase in PR when a single dose of cloprostenol (500 µg) was injected intramuscularly at AI. In addition, in another study (Archbald et al., 1992), it was reported that administering standard luteolytic doses of PGF2α at AI had no positive effect on PR. Similarly, Gabriel et al. (2011) reported that PRs were unaffected by intramuscular injection of 25 mg dinoprost at AI in dairy cows and heifers. In a very recent study (Mohammadi et al., 2019), it has been demonstrated that treatment with d-cloprostenol or buserelin acetate at the time of AI did not have any effect on pregnancy per AI in dairy cows under a condition without heat stress. Kauffold et al. (2009) reported that intramuscular injection of 500 µg cloprostenol immediately after AI had no effects on ovulation and PR in primiparous and multiparous cows and, therefore, cannot be recommended as a means of improving fertility. Pfeifer et al. (2018) reported that an additional injection of PGF2α to FTAI beef cows postpartum did not enhance pregnancy per AI. Sauls et al. (2018) suggested that according to the inconsistent results of treatment with PGF2α, the positive effects seem to be herd specific and depend on unknown factors for success.

Conclusion

In conclusion, the results of the present study showed that administering an additional dose of a PGF2α analog, d-cloprostenol sodium, concurrent with the second GnRH and at insemination of Ovsynch, did not improve ovulation rates on Days 9, 10, and 11 after the initiation of the Ovsynch program or pregnancy rates on Days 50 ± 2 after insemination significantly in Holstein lactating dairy cows. However, further studies with a larger number of cows are needed.

Acknowledgements

The authors would like to acknowledge the valuable assistance of the farm manager and staffs for providing the animals and records and for helping to do the experiment.

References

1. Ambrose, D.J., Gobikrushanth, M., Zuidhof, S., Kastelic, J.P. Low-dose natural prostaglandin F2α (dinoprost) at timed insemination improves conception rate in dairy cattle. Theriogenology. 2015. V. 83(4). P. 529-534.
2. Archbald, L.F., Tran, T., Massey, R., Klapstein, E. Conception rates in dairy cows after timed-insemination and simultaneous treatment with gonadotrophin releasing hormone and/or prostaglandin F2 alpha. Theriogenology. 1992. V. 37(3). T. 723-731.

3. Bó, G.A., Baruselli, P.S., Moreno, D., Cutaia, L., Caccia, M., Tríbulo, R., Tríbulo, H., Mapletoft, R.J. The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology. 2002. V. 57(1). P. 53-72.
4. Chenault, J.R., Meeuwse, D.M., LaGrow, C., Tena, J.K., Wood-Follis, S.L., Hallberg, J.W. Evaluation of gonadotropin-releasing hormone hydrogen chloride at 3 doses with prostaglandin F2α for fixed-time artificial insemination in dairy cows. J Dairy Sci. 2014. V. 97(5). P. 2816-2821.
5. Fricke, P.M., Guenther, J.N., Wiltbank, M.C. Efficacy of decreasing the dose of GnRH used in a protocol for synchronization of ovulation and timed AI in lactating dairy cows. Theriogenology. 1998. V. 50(8). P. 1275-1284.
6. Gabriel, H.G., Wallenhorst, S., Dietrich, E., Holtz, W. The effect of prostaglandin F2α administration at the time of insemination on the pregnancy rate of dairy cows. Anim Reprod Sci. 2011. T. 123(1-2). P. 1-4.
7. Gümen A, Seguin B. Ovulation rate after GnRH or PGF2α administration in early postpartum dairy cows. Theriogenology. 2003. V. 60(2). T. 341-348.
8. Kauffold, J., Gommel, R., Failing, K., Beynon, N., Wehrend, A. Postinsemination treatment of primiparous and multiparous cows with cloprostenol failed to affect ovulation and pregnancy rate in dairy cattle. Theriogenology. 2009. V. 72(5). P. 741-746.
9. Keskin, A., Yilmazbaś-Mecitoglu, G.Ü.L.N.A.Z., Gumen, A., Karakaya, E., Darici, R., Okut, H. Effect of hCG vs. GnRH at the beginning of the Ovsynch on first ovulation and conception rates in cyclic lactating dairy cows. Theriogenology. 2010. V. 74(4). P. 602-607.
10. Lajili H., Humblot P., Thibier M. Effect of PG F2 alpha treatment on conception rates of dairy cows treated with a GnRH agonist 12 to 14 days after artificial insemination. Theriogenology. 1991. V. 36(2). P. 335-3347.
11. Leonardi, C.E.P., Pfeifer, L.F.M., Rubin, M.I.B., Singh, J., Mapletoft, R.J., Pessoa, G.A., Bainy, A.M., Silva, C.A.M. Prostaglandin F2α promotes ovulation in prepubertal heifers. Theriogenology. 2012. V. 78(7). P. 1578-1582.
12. Liu, T.C., Chiang, C.F., Ho, C.T., Chan, J.P.W. Effect of GnRH on ovulatory response after luteolysis induced by two low doses of PGF2α in lactating dairy cows. Theriogenology. 2018. V. 105. P. 45-50.
13. Lopez-Gatius, F., Yaniz, J.L., Santolaria, P., Murugavel, K., Guijarro, R., Calvo, E., Lopez-Bejar, M. Reproductive performance of lactating dairy cows treated with cloprostenol at the time of insemination. Theriogenology. 2004. V. 62(3-4). P. 677-689.
14. Mohammadi, A., Seifi, H.A., Farzaneh, N. Effect of prostaglandin F2α and GnRH administration at the time of artificial insemination on reproductive performance of dairy cows. Vet Res Forum. 2019. V. 10 (2). P. 153–158.
15. Momcilovic, D., Archbald, L.F., Walters, A., Tran, T., Kelbert, D., Risco, C., Thatcher, W.W. Reproductive performance of lactating dairy cows treated with gonadotrophin-releasing hormone (GnRH) and/or prostaglandin F2a (PGF2a) for synchronization of estrus and ovulation. Theriogenology. 1998. V. 50(7). P. 1131-1139.
16. Neglia, G., Natale, A., Esposito, G., Salzillo, F., Adinolfi, L., Campanile, G., Francillo, M., Zicarelli, L. Effect of prostaglandin F2α at the time of AI on progesterone levels and pregnancy rate in synchronized Italian Mediterranean buffaloes. Theriogenology. 2008. V. 69(8). P. 953-960.
17. Peters M.W., Pursley J.R. Fertility of lactating dairy cows treated with Ovsynch after presynchronization injections of PGF2α and GnRH. J Dairy Sci. 2002. V. 85(9). P. 2403-2406.
18. Pfeifer L.F., Leonardi C.E., Castro N.A., et al. The use of PGF2α as ovulatory stimulus for timed artificial insemination in cattle. Theriogenology. 2014. V. 81(5). P. 689-695.
19. Pfeifer, L.F.M., Rodrigues, W.B., da Silva, K.C., Anache, N.A., Castro, N.Á., Castilho, E.M., Nogueira, E. Different protocols using PGF2α as ovulation inducer in Nelore cows subjected to estradiol-progesterone timed AI based protocols. Theriogenology. 2018. V. 120. P. 56-60.
20. Randel, R.D., Lammoglia, M.A., Lewis, A.W., Neuendorff, D.A., Guthrie, M.J. Exogenous PGF2α enhanced GnRH-induced LH release in postpartum cows. Theriogenology. 1996. V. 45(3). P. 643-654.
21. Roelofs, J., Lopez-Gatius, F., Hunter, R.H.F., Van Eerdenburg, F.J.C.M., Hanzen, C.H. When is a cow in estrus? clinical and practical aspects. Theriogenology. 2010. V. 74. P. 327–344.
22. Sauls, J.A., Voelz, B.E., Mendonça, L.G.D., Stevenson, J.S. Additional small dose of prostaglandin F2α at timed artificial insemination failed to improve pregnancy risk of lactating dairy cows. Theriogenology. 2018. V. 110. P. 27-33.
23. Vasconcelos, J.L.M., Silcox, R.W., Rosa, G.J.M., Pursley, J.R., Wiltbank, M.C. Synchronization rate, size of the ovulatory follicle, and pregnancy rate after synchronization of ovulation beginning on different days of the estrous cycle in lactating dairy cows. Theriogenology. 1999. V. 52(6). P. 1067-1078.
24. Weems, C.W., Weems, Y.S., Randel, R.D. Prostaglandins and reproduction in female farm animals Vet J. 2006. V. 172. P. 206-228.
25. Yilmazbaś-Mecitoglu, G., Karakaya, E., Keskin, A., Gumen, A., Koc, V., Okut, H. Comparison of synchronisation and fertility after different modifications of the ovsynch protocol in cyclic dairy cows. Acta Vet Hungarica. 2014. V. 62(1). P. 64-73.

Received 19 June 2022
Accepted 6 December 2022

Effect of Vitrification on Canine Sperm Parameters Using Coconut Water Extender and Egg Yolk as Cryoprotectant

Boyana Ivanova, Anton Antonov

Department of Obstetrics, Reproduction and Reproductive Disorders, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, 6000, Bulgaria

Keywords: *dog sperm, vitrification, egg yolk, coconut water.*

Abstract*. Our study was aimed to evaluate the effect of vitrification on canine sperm parameters using a coconut water extender with an addition of egg yolk as a cryoprotectant. Semen collection was done separately by manual stimulation from twelve healthy adult dogs. Only the second fraction of the ejaculate was used in this study, which was evaluated for volume, concentration, vitality, total and progressive motility, kinetic parameters and morphology. Semen was diluted with a coconut water extender (50% (v/v) coconut water, 25% (v/v) distilled water and 25% (v/v) 5% anhydrous monosodium citrate solution) with an addition of 20% (v/v) egg yolk and fructose at 1% until final concentration of 100 × 10^6 spermatozoa/mL. After equilibration at 5°C for 60 minutes, semen was vitrified by the "direct dropping method" into liquid nitrogen in spheres with a volume of 30 µL. After a week of storage, the spheres were warmed as three of them were dropped into 0.5 mL of CaniPlus AI (Minitüb, Germany) at 42°C for 2 minutes and evaluated about the same parameters. The results showed that vitrification produced a statistically lower percentage of vital sperms, normal morphology and total motility ($P < 0.05$), but progressive motility and most of velocity parameters (VCL, VSL, VAP, LIN, ALH and BCF) did not differ ($P > 0.05$) compared to fresh semen samples. In conclusion, our results demonstrate that vitrification with a coconut water extender with an addition of 20% egg yolk as a cryoprotectant affects the quality of canine sperms, but may be useful as a successful alternative to conventional cryopreservation. Further research on the spermatozoa vitrification technique on enhancement in cooling and warming should be conducted and investigated.*

Introduction

In recent years, there has been a demand on research for different methods for canine semen preservation. There have been many trials based on type of preservation, various component extenders, equilibration and warming procedures. Cryopreservation of spermatozoa is a method for assisted reproductive biotechnology, useful for extending their lifespan and viability, which increases reproductive capacity of male organisms (Gharajelar, 2016). Conventional cryopreservation uses a slow-gradual freezing method and has moderately poor post-thawed semen quality. Vitrification, on the other hand, uses an ultra-rapid freezing method for solidifying liquid into the glassy state by direct immersion into liquid nitrogen (LN_2) without ice crystallization. The method is widely used for embryo, oocyte or tissue storage (Isachenko, 2004; Rosato, 2013), and during the last decade, it has been successfully performed in different mammalian species as an option for sperm preservation; however, in dogs, there have been fewer investigations until now (Sánchez, 2011; Kim, 2012; Gharajelar, 2016; Caturla-Sánchez, 2018; Pipan, 2020; Galarza, 2021; Antonov & Ivanova, 2022). As a novel method, sperm vitrification protocols still require improvement and standardization for increasing post thaw sperm survival.

During the semen cryopreservation process, a cold shock phenomenon may occur, which can reduce the spermatozoa motility and viability (Enciso, 2006). Thus, the addition of cryoprotectants is mandatory to minimalize cryodamage of the spermatozoa. Most often glycerol is added to the extenders as a permeable cryoprotectant, which prevents intracellular ice crystals formation, but it has proven to be toxic for the cells (Curry, 2000; Holt, 2000). Egg yolk as a non-permeable cryoprotector, is also widely used in conventional semen cryopreservation. Since its discovery as a component of a cooling extender, egg yolk has been widely used in mammalian sperm cryopreservation to protect sperm from initial cold shock (Layek, 2016; Abdel-Aziz, 2019). Moreover, many studies have verified the benefits of egg yolk-based extenders for canine semen cryopreservation (Martinez-Rodriguez, 2020; Bencharif, 2020). Egg yolk contains LDLs, which can prevent cholesterol efflux and lower tyrosine-containing protein phosphorylation, thereby inhibiting sperm capacitation (Mahiddine & Kim, 2021). The addition of egg yolk greatly increases the viscosity of the solution, which prevents water precipitation and formation of intracellular and extracellular ice crystals (Isachenko, 2011). In the literature, there are limited

Corresponding to Boyana Taneva Ivanova, Department of Obstetrics, Reproduction and Reproductive Disorders, Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria.
Email: iboyana@ymail.com

data about the possible potential of egg yolk to preserve important physiological parameters of canine sperms during ultra-rapid cryopreservation.

Many researchers have focused on using antioxidants as preservatives in extenders to reduce the negative effect of oxidative stress on semen and to protect spermatological indices. A great number of studies have documented that adding antioxidants to the extender could be beneficial in preserving the sperm parameters after thawing in animals such as canines (Neagu, 2010; Lucio, 2016; Caturla-Sanchez, 2018). A viable alternative extender is the green coconut water based one. Coconut water as a whole seems to be suitable for a canine semen extender due to isotonic, not toxic, cheap, effective, and simple to use properties (Cardoso, 2003). It is a natural buffer and contains essential constituents with high antioxidant properties (Silva, 2009; Mantena, 2003; Cardoso, 2003, 2006).

The purpose of the current study was to evaluate the effect of vitrification on canine semen parameters using a green coconut water based extender with the addition of egg yolk and performing a simple cryopreservation method which can be applied for routine clinical use.

Materials and Methods

Experimental animals and initial semen quality

Twelve privately owned, clinically healthy male dogs, aged between 3 and- 6 years, were included in this study. They were presented at the University Veterinary Hospital of the Faculty of Veterinary medicine, Trakia university, Stara Zagora, Bulgaria. Semen samples from these dogs were previously conventionally cryopreserved and found to be with good cryotolerance. The experiment was conducted according to the recommendations of the Local Animal Ethics Committee and regulation for human attitude and animal protection. Dog owners were informed about the procedures and signed a written consent before semen collection. Inclusion criteria were that the ejaculates should have ⩾ 70% motile spermatozoa and ⩾ 70% morphologically normal spermatozoa.

Semen collection and evaluation

Semen was collected separately by digital manipulation into a pre-warmed sterile plastic vial. The procedure was performed by the same operator to eliminate variation due to the different collection technique and in the presence of a teaser bitch to provide stimulation. Only the second ejaculation fraction was used in the study, and immediately after collection, the vial was transferred to the laboratory for the preliminary analysis. Semen was evaluated for volume, concentration, vitality, kinematic parameters and morphology.

A pre-warmed graduated glass pipette was used to measure the semen volume.

Sperm concentration and velocity were analyzed by CASA System Sperm Class Analyser (SCA) (Microptic, S.L., Barcelona, Spain) with software analytical module Motility and Concentration, using a Makler counting chamber with volume of 10 µL samples. The CASA parameters were adjusted to accommodate canine semen according to protocols already in place. Examination was performed on a minimum of 30 optic fields. The assessed CASA parameters were: sperm concentration (× 10^6/mL), total motility (TM, %), progressive motility (PM, %), VCL (curvilinear velocity, µm/s), VAP (average path velocity, µm/s), VSL (straight line velocity, µm/s), LIN (linearity, %), STR (straightness rate, %), ALH (lateral head displacement amplitude, µm) and BCF (beat cross frequency, Hz).

Sperm morphology was evaluated by Sperm Class Analyser (SCA) (Microptic, S.L., Barcelona, Spain) with software analytical module Morphology. Smeared 10 µL semen samples were prepared on a clear glass slide and stained with SpermBlue (Microptic, Spain) for 2 minutes. The slide was analyzed by SCA with a minimum of 50 optic fields.

The sperm vitality was assessed by smearing on a slide a mixture of a 5 µL semen sample and 5 µL eosin-nigrosine stain. A minimum of 200 sperm cells were counted under a light microscope and oil immersion, magnified by 400×. Spermatozoa stained pink or red were identified as non- vital, and those unstained remaining white - as vital.

Extender preparation and semen dilution

After the initial processing, the second fraction was diluted with a green coconut water based extender until final concentration of 100 x 10^6/mL. Base vitrification media (BVM) was first prepared using 50% (v/v) coconut water, 25% (v/v) distilled water and 25% (v/v) solution of 5% anhydrous monosodium citrate. The extender consisted of BVM with an addition of 20% (v/v) egg yolk and 1% fructose. The extended semen was then equilibrated at 5°C for 1 hour.

Vitrification and thawing

The vitrification technique was performed according the description of Shah (2019) for human sperm. Sperm samples of 30 µL were dropped with micropipette from a 10 cm height upon a stainless steel strainer, which was previously placed into a styrofoam box and submerged in liquid nitrogen (LN_2). After solidification, the sperm pellets were transferred into pre-cooled cryotubes and placed in liquid nitrogen for a week.

The thawing process consisted of adding three sperm pellets to 0.5 mL of CaniPlus AI (Minitub, Germany) that had been pre-warmed in a water bath at 42°C for 2 minutes and then transferred at 37°C. After warming, the sperm parameters were immediately evaluated as described above.

Statistical analysis

The results were processed by statistical program Statistica version 7.0 (Stat-Soft., 1984-2000 Inc.,

Tulsa, OK, USA). All data are presented as mean ± standard deviation (SD) and were analyzed using ANOVA for repeated measures and compared using the Tukey's test. Value for $P < 0.05$ was considered significant.

Results

The volume of the second semen fraction was 0.95 ± 0.27 mL and the concentration was- 973 ± 175.04×10^6 spermatozoa/mL. The other evaluated parameters and the effect of vitrification on canine semen using the coconut water extender with the addition of egg yolk are presented in Table 1.

Fresh semen samples showed significantly higher ($P < 0.05$) vitality than those which were vitrified. A similar tendency was observed in total motility. Progressive motility was also improved by the vitrification process, but the levels were not significantly different ($P > 0.05$) with the fresh semen samples. After vitrification, there were also changes in most of the sperm velocity parameters; however, they were not significantly different ($P > 0.05$) than the fresh samples before cryopreservation.

The evaluation of sperm morphology showed significant variations ($P < 0.05$) between the fresh and vitrified samples. The major alterations found after vitrification were a detached head, a coiled and bent tail.

Discussion and Conclusion

In recent years, there has been a demand for research focused on canine sperm cryopreservation in order to increase the reproductive capacity of stud dogs. Conventional freezing methods usually result in high percent sperm mortality and morphological damage (Falah, 2020). However, cryopreservation by direct plunging of cells into liquid nitrogen (vitrification) has its own unique characteristics. The decisive factor in successful cryopreservation is avoiding intracellular crystallization, which is incompatible with living systems. A "popular" point of view holds that vitrification is the solidification without formation of hexagonal (big, lethal) intracellular crystals by extreme increase in viscosity during cooling (Merino, 2011).

Extenders tend to be extremely important for successful cryopreservation and choosing the proper one is an important part of semen processing (Peterson, 2007; Ogbu, 2014). A lot of commercial extenders for dog semen preservation, which consist of different chemical combinations, are available, but most of them could be replaced using alternative sources, including such as those of animal or plant origin (Bustani & Baiee, 2021). Our choice to use a coconut water based extender relies on the cheap, easy to find ingredient, which is also an excellent antioxidant and is in accordance with the "green trend" of recent years. The quality of preserved canine semen after devitrification in the present study exceeded any previously reported results (Sánchez, 2011; Kim, 2012; Gharajelar, 2016; Caturla-Sánchez, 2018; Pipan, 2020; Galarza, 2021; Antonov & Ivanova, 2022).

According to Gharajelar (2016), egg yolk is a common part of semen diluents with a protective effect on spermatozoa against cold shock during freezing and thawing, which also acts as an energy source and protectant at the level of the cell membrane (Sánchez, 2011). In the scientific literature, the best canine sperm vitality and total motility reported after vitrification were with a TRIS based extender (Pipan, 2020), and a previous study of our team using a coconut water extender, which showed even better results (Antonov & Ivanova, 2022). In both investigations, the extenders contained 1% soy lecithin and 0.25M sucrose as cryoprotectants. In the present investigation, we replaced them with egg yolk and found improved quality of preserved semen after devitrification, so it might be concluded that it can effectively preserve important physiological parameters of canine sperm during ultra-rapid cryopreservation.

Motility is one of the most important features of a fertile spermatozoa (Partyka, 2012). In previously reported results for conventional freezing, wide variability is observed, with studies reporting sperm motility ranging between 33% (Peña, 1998) and 70% (Ström, 1997). Concannon and Battista (1989) suggest that 30–50% sperm motility in frozen semen is considered acceptable and motility above 50% is ideal for artificial insemination with canine frozen semen. Our result showed that despite the significant difference in total motility between the fresh and vitrified sample, the mean average percent of sperm motility after devitrification is above mentioned

Table 1. Parameters of fresh and vitrified canine semen samples (n = 12) using the coconut water extender

	Vitality, %	Total motility, %	Progressive motility, %	VCL, µm/s	VSL, µm/s	VAP, µm/s	LIN, %	STR, %	ALH, µm	BCF, Hz	Normal morphology, %
Fresh semen	94.45 ± 1.59	87.76 ± 1.94	52.11 ± 2.89	191.1 ± 26.60	129.2 ± 16.60	146.3 ± 15.10	68.50 ± 8.36	88.93 ± 4.31	5.11 ± 0.71	25.1 ± 3.60	84.08 ± 5.56
Vitrified semen	67.22 ± 4.02 a	58.13 ± 5.61 a	48.98 ± 1.59	179.4 ± 17.3	123.8 ± 19.7	144.3 ± 13.3	67.97 ± 5.54	87.01 ± 3.57 a	5.01 ± 0.89	21.1 ± 2.8	73.67 ± 6.11 a

Data are expressed as mean ± SD. The values in a row marked with a superscript differ at $P < 0.05$.

levels and thus can be successfully used for artificial insemination.

In our research we found that there were no significant differences ($P > 0.05$) between the velocity parameters (VAP, VSL, VCL) and BCF of fresh and vitrified samples, which is in agreement with previously reported results of our team (Antonov & Ivanova, 2022). Their evaluation is the most useful method for comparing semen from fertile and infertile dogs (Domoslawska, 2013), because they are important for the progression of sperms into cervical mucus and penetration of zona pellucida of oocytes (Verstegen, 2002). According to the reported results, sperm vitrification yields a high survival rate of fertile canine spermatozoa after warming.

Our study demonstrates that canine sperm vitrification in a coconut water extender with an addition of 20% egg yolk could be successful for routine clinical use as alternative to conventional cryopreservation. This ultra-rapid freezing method is a much faster, simpler and cheaper method, which could prevent the high spermatozoa mortality rate observed in conventional freezing. Additionally, we believe that coconut water could successfully replace some of the expensive chemical ingredients of semen extenders and has a positive impact on the environment. Therefore, further research on fertility studies should be conducted and investigated to detect true measure of successful dog sperm vitrification with a coconut water extender.

Our results demonstrate that when a coconut water extender with an addition of 20% egg yolk as a cryoprotectant is used, vitrification affects the quality of canine spermatozoa, but could provide quality results near the conventional freezing method.

Acknowledgments

We would like to show apreciation for the support offered by all the owners of dogs, included in this study.

References

1. Abdel-Aziz, S. A., Saadeldin, I. M., Ba-Awadh, H., Al-Mutary, M. G., Moumen, A. F., Alowaimer, A. N., Abdalla, H. Efficiency of Commercial Egg Yolk-Free and Egg Yolk-Supplemented Tris-Based Extenders for Dromedary Camel Semen Cryopreservation. Animals (Basel). 2019. 9(11). P. 999.
2. Antonov, A., Ivanova, B. Effect of equilibration process on canine spermatozoa after vitrification using coconut water extender with addition of soy lecithin and sucrose as nonpermeable cryoprotectants. Spermova. 2022. 12(1). P. 27-32.
3. Bencharif, D., Dordas-Perpinya, M. Canine semen cryoconservation: Emerging data over the last 20 years. Reprod Domest Anim. 2020. 2. P. 61-65.
4. Bustani, G. S., Baiee, F. H. Semen extenders: An evaluative overview of preservative mechanisms of semen and semen extenders. Veterinary World. 2021. 14(5). P. 1220-1233.
5. Cardoso, R. C. S., Silva, A. R., Silva, L. D. M. Comparison of two dilution rates on canine semen quality after cryopreservation in a coconut water extender. Animal Reproduction Science. 2006. 92(3-4). P. 384-391.
6. Cardoso, R. C. S., Silva, A. R., Uchoa, D. C., Silva, L. D. M. Cryopreservation of canine semen using a coconut water extender with egg yolk and three different glycerol concentrations. Theriogenology. 2003. 59. P. 743-751.
7. Caturla-Sánchez, E., Sánchez-Calabuig, M., Gutiérrez, J. F. P., Cerdeira, J., Castaño, C., Santiago-Moreno, J. Vitrification of dog spermatozoa: Effects of two cryoprotectants (sucrose or trehalose) and two warming procedures. Cryobiology. 2018. 80. P. 126–129.
8. Concannon, P. W., Battista, M. Canine semen freezing and artificial insemination. In: Kirk RW, editor. Current veterinary therapy X: small animal practice. Philadelphia: WB Saunders. 1989. P. 1247–59.
9. Curry, M. R. Cryopreservation of semen from domestic livestock. Rev Reprod. 2000. 5(1). P. 46-52.
10. Domosławska, A., Zduńczyk, S., Niżański, W., Janowski, T. Assessment of semen quality in infertile dogs using computer-assisted sperm analysis by the Hamilton-Thorne Semen Analyser. Bull Vet Inst Pulawy. 2013. 57. P. 429-432.
11. Enciso, M., López-Fernández, C., Fernández, J. L., García, P., Gosálbez, A., Gosálvez, J. A new method to analyze boar sperm DNA fragmentation under bright-field or fluorescence microscopy. Theriogenology. 2006. 65 (2). P. 308-316
12. Falah, B., Bassam, A. A-W., Asaad, A. A., Lee, L. Yu., Wan-Nor, F. Abd. W. H., Ghadeer, S. B. Effect of vitrification on spermatozoa quality in bull semen. Eurasia J Biosci. 2020. 14. P. 3897-3904.
13. Galarza, D. A., Landi, G., Mejía, E., Samaniego, J. X., Mendez, S., Soria, M. E., Taboada, J., Sanchez-Calabuig, M. J., Castano, C., Santiago-Moreno, J. Cryopreservation of dog epididymal spermatozoa by conventional freezing or ultra-rapid freezing with nonpermeable cryoprotectant. Cryobiology. 2021. 103. P. 15-21.
14. Gharajelar, S. N., Sadrkhanloo, R. A., Onsori, M., Saberivand, A. A comparative study on the effects of different cryoprotectants on the quality of canine sperm during vitrification process. Vet Res Forum. 2016. 7(3). P. 235–239.
15. Holt, W. V. Fundamental aspects of sperm cryobiology: The importance of species and individual differences. Theriogenology. 2000. 53(1). P. 47-58.
16. Isachenko, E., Isachenko, V., Sanchez, R., Katkov, I., Kreienberg, R. Cryopreservation of spermatozoa: Old routine and new perspectives. In Principles and Practice of Fertility Preservation. Cambridge: University Press. 2011. P. 176–198.
17. Isachenko, V., Isachenko, E., Katkov, I., Montag, M., Dessole, S., Nawroth, F., Van Der Ven, H. Cryoprotectant-free cryopreservation of human spermatozoa by vitrification and freezing in vapor: effect on motility, DNA integrity, and fertilization ability. Biol Reprod. 2004. 71(4). P. 1167-73.
18. Kim, S., Lee, Y., Yang, H., Kim, Y - J. Rapid freezing without cooling equilibration in canine sperm. Anim. Reprod. Sci. 2012. 130. P. 111–118.
19. Layek, S. S., Mohanty, T. K., Kumaresan, A., Parks, J. E. Cryopreservation of bull semen: Evolution from egg yolk based to soybean based extenders. Anim Reprod Sci. 2016. 172. P. 1-9.
20. Lucio, C. F., Regazzi, F. M., Silva, L. C. G., Angrimani, D. S. R., Nichi, M., Vannucchi, C. I. Oxidative stress at differentstages of two-step semen cryopreservation procedures in dogs. Theriogenology. 2016. 85. P. 1568-1575.
21. Lucio, C., Silva, L. C. G., Regazzi, F. M., Angrimani, D. S. R., Nichi, M., Assumpção, M. E. O., Vannucchi, C. I. Effect ofreduced glutathione (GSH) in canine sperm cryopreservation: In vitro and in vivo evaluation. Cryobiology. 2016. 72. P. 135-140.
22. Mahiddine, F. Y., Kim, M-J. Overview on the Antioxidants, Egg Yolk Alternatives, and Mesenchymal Stem Cells and Derivatives Used in Canine Sperm Cryopreservation. Animals. 2021. 11(7). P. 1930.
23. Mantena, S. K., Badduri, S. R., Siripurapu, K. B., Unnikrishnan, M. K. In vitro evaluation of antioxidant properties of Cocos nucifera Linn. water. Nahrung. 2003. 47. P. 126-131.
24. Martinez-Rodriguez, J. A., Carbajal, F. J., Martinez-De-Anda, R., Alcantar-Rodriguez, A., Medrano, A. Melatonin added to freezing diluent improves canine (Bulldog) sperm cryosur-

vival. Reprod Fertil. 2020. 1(1). P. 11-19.
25. Merino, O., Risopatrón, J., Sánchez, R., Isachenko, E., Figueroa, E., Valdebenito, I., Isachenko, V. Fish (Oncorhynchus mykiss) spermatozoa cryoprotectant-free vitrification: Stability of mitochondrion as criterion of effectiveness. Anim. Reprod. Sci. 2011. 124. P. 125–131.
26. Neagu, V. R., García, B. M., Sandoval, C. S., Rodríguez, A. M., Ferrusola, C. O., Fernández, L. G., Tapia, J. A., Peña, F. J. Freezing dog semen in presence of the antioxidant butylated hydroxytoluene improves post thaw spermmembrane integrity. Theriogenology. 2010. 73. P. 645-650.
27. Ogbu, C. C., Ugwu, S. O., Ezebili, C. V. Use of coconut milk as an extender for cock semen. Res Opin Anim Vet Sci. 2014. 4(10). P. 571–577.
28. Partyka, A., Niżański, W., Ochota, M . Methods of Assessment of Cryopreserved Semen, Current Frontiers in Cryobiology, Prof. Igor Katkov (Ed.). 2012. ISBN: 978-953-51-0191-8.
29. Peña, A. I., Barrio, F., Quintela, L. A., Herradón, P. G. Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. Theriogenology. 1998. 50. P. 163-174.
30. Peterson, K., Kappen, M. A., Ursem, P. J., Nothling, J. O., Colenbrander, B., Gadella, B. M. Microscopic and flow cytometric semen assessment of Dutch AI-bucks: effect of semen processing procedures and their correlation to fertility. Theriogenology. 2007. 67(4). P. 863–871.

Received 20 December 2022
Accepted 28 December 2022

31. Pipan, Z., M., Casal, M. L., Šterbenc, N., Virant Klun, I., Mrkun, J. Vitrification Using Soy Lecithin and Sucrose: A New Way to Store the Sperm for the Preservation of Canine Reproductive Function. Animals. 2020. 10. P. 653.
32. Rosato, M. P., Iaffaldano, N. Cryopreservation of rabbit semen: Comparing the effects of different cryoprotectants, cryoprotectant-free vitrification, and the use of albumin plus osmoprotectants on sperm survival and fertility after standard vapor freezing and vitrification. Theriogenology. 2013. 79 (3). P. 508-516.
33. Sánchez, R., Risopatrón, J., Schulz, M., Villegas, J., Isachenko, V., Kreinberg, R., Isachenko, E. Canine sperm vitrification with sucrose: Effect on sperm function. Andrologia. 2011. 43. P. 233–241.
34. Shah, D., Rasappana, S., Karthik, G. A simple method of human sperm vitrification. MethodsX. 2019. 6. P. 2198-2204.
35. Silva, P., Bamunuarachchi, A. Manufacture of carbonated tender coconut water and development of a process for the utilization of coconut flesh. Asian Journal of Food and Agro-Industry. 2009. 2. P. 210-213.
36. Ström, B., Rota, A., Linde-Forsberg, C. In vitro characteristics of canine spermatozoa subjected to two methods of cryopreservation. Theriogenology. 1997. 48. P. 247-256.
37. Verstegen, J., Iguer-Ouada, M., Onclin, K. Computer Assisted Semen Analyzers in Andrology Research and Veterinary Practice. Theriogenology. 2002. 57. P. 149-179.

A Study of Stability and Antimicrobial Efficacy of a New Model Teat Dip Solution Containing Lactic Acid

Modestas Kybartas, Rita Šiugždinienė, Marius Virgailis, Raimundas Mockeliūnas
Lithuanian University of Health Sciences, Veterinary Academy, Microbiology and Virology Institute, Kaunas, Lithuania

Keywords: *teat dip solution, lactic acid, xanthan gum, stability, viscosity, antimicrobial efficacy.*

Abstract. *The aim of this study was to make a new teat dip formula containing lactic acid and evaluate its stability and antibacterial activity by physico-chemical and microbiological tests.*

*After selecting the concentrations of the thickener xanthan gum (0.67%) and lactic acid (6.3%), a teat dip solution was composed, which contains teat skin saving components: glycerol, sorbitol, oat and calendula extracts. According to the recipe, a teat dip solution of orange colour, homogeneous appearance, and a specific lactic acid smell was composed, the viscosity of which was 930–940 mPa*s., pH 2.7. Product physical, chemical properties and stability for 24 months were confirmed by real-time and accelerated stability studies. The teat dip solution remains homogenous, orange colour with a typical lactic acid smell, and did not become cloudy or flaky after 24 months following production. Viscosity reduction was determined after 6 months 33 mPa*s, i.e. it was 3.4%, while after 24 months, it was 12.7%. The measured pH value remains stable between 2.6 and 2.8 ($P > 0.05$). An antimicrobial study was performed according to EN 1656 by 80%, 50% and 10% concentrations with reference Staphylococcus aureus, Streptococcus uberis and Escherichia coli strains. The teat dip solution showed bactericidal efficiency (log $R > 5$) against S. uberis and E. coli reference strains. An antimicrobial effect to S. aureus was insufficient and the product was not effective against this bacteria. Teat dip at 10% did not show an antimicrobial effect to all tested strains.*

According to these data, the new model teat dip solution containing lactic acid, teat skin saving substance and thickener xanthan gum retains unchanged physical parameters and shows bactericidal activity against the reference E. coli and S. uberis strains.

Introduction

The bacterial colonization on teat skin is an important source for intramammary infection (Hassan et al., 2016). Good sanitation practices can reduce the number of bacteria on teat skin and improve the milk quality, especially the pre-milking and post-milking teat dip (Kučević et al., 2013). Teat disinfection is an important step in the control of mastitis within a dairy herd (Breen, 2019; Fitzpatrik et al., 2021). Depending on the hygiene and sanitary condition of the farm and the method of udder preparation, bacterial contamination of raw milk can decrease by 90%, and the cases of mastitis by 50–75%. Many studies have shown that well-performed teat hygiene reduces the spread of microorganisms that cause inflammation of the mammary gland, and the quality of milk improves. Various sprays, cleaners and teat dip solutions are used today for this purpose. The most widely used procedure is teat dipping. Post-milking dip solutions must have antimicrobial activity, cover the teat surface well and stay on it, moisturize without skin irritation (Oliver & Murinda, 2012; Mišeikienė et al., 2015). Therefore, products intended for cow udder and teat antiseptic – biocides – must contain a bactericidal, disinfecting substance, skin-protecting components and a thickener – a viscosity-forming substance. The following active substances can be found in veterinary biocides: chlorhexidine, iodine and its compounds, lactic acid, peracetic acid, hydrogen peroxide, and natural polymers guar or xanthan gum are used as thickening components (Nickerson, 2001; Fitzpatrick & Garvey, 2019; Chotigarpa & Lampang, 2019). There are certain requirements for teat dip solutions which are intended for the disinfection of cow udders. One of the most important is the viscosity. After applying this product to the nipple skin, a continuous film should be formed; its purpose is the nipple canal protection from the entry of infectious microorganisms (Garvey & Curran, 2016). The viscosity of solutions can be adjusted by selecting different types of thickeners, so natural thickeners are more common in veterinary medicine pharmacy as well as food industry (Alves, 2020).

Teat dip solutions must contain a disinfectant that does not harm the animal or veterinary and husbandry staff. Lactic acid is widely used in veterinary medicine because it is active against gram-positive and gram-negative bacteria and is not a toxic or dangerous substance. Teat dip solutions based on lactic acid are less harmful to the animal's skin. According to some studies, teat dip solutions which contain 5% lactic acid have noticeable antimicrobial activity against *E. coli*. The previous results have shown that the lactic acid inhibited the growth of other Gram-negative

Correspondence to Modestas Kybartas, Microbiology and Virology Institute of Lithuanian University of Health Sciences.
E-mail: kmodestas@yahoo.com

bacteria such as *Shigella* sp., *Salmonella* Enteritidis, and *Listeria monocytogenes* at 0.5% (In et al., 2013; Wang et al., 2015). Fitzpatrick et al. (2021) also say that, for streptococcal isolates, the product (5% w/w lactic acid) resulted in the numerically largest bacterial reduction of 70.1%.

An important feature of veterinary biocides is that the stability of the solution formula should stay throughout the product's shelf life (European chemicals agency, Guidance on the Biocidal Products Regulation). It means that the teat dip solution should retain stability of physical, chemical properties and show no changes of active substance. Lack of stability leads to diminishing antimicrobial properties.

The aim of this study was to create a new formula teat dip and antimicrobial efficacy and stability evaluation of the new model teat dip solution by physico-chemical and microbiological tests.

Materials and methods

The investigation was carried out in the Institute of Microbiology and Virology (LUHS) and Good Laboratory Practice laboratory (Vimodrone, Italy). A solution formula was developed by selecting and changing the concentration of xanthan gum and dyes so that teat dip properly covered the teat skin, persisted on the teat a required time and properly coloured the teat skin after dipping. The teat dip solution with lactic acid was produced using pharmacological substances (see Table 1 for composition) according to the technology where dry plant extracts and dyes are dissolved in a portion of purified water and filtered. The filtrate is mixed with sorbitol and glycerine compound and stirred until the solution becomes clear completely. Xanthan gum with the rest of the purified water is added with intensive mixing, and the suspension is homogenized to uniform consistency by a homogenizer (IKA T25 Ultra Turrax). Lactic acid (active substance) is added, and the solution is mixed again with the help of a laboratory mixer (IKA Eurostar 20 digital) to a homogeneous mass. This product is left in a sealed container and, after 24 hours, physico-chemical parameters are determined. The stability testing was carried out according to the guidelines on stability testing of cosmetic products (Colipa, 2004) and guidelines for assessing shelf life using real-time and accelerated stability tests (Magari, 2003). In the real-time study, the product was stored under recommended conditions, and changes of product properties were observed during time intervals (after 24 hours, 6 months, 12 months, and 24 months). When testing the teat dip in an accelerated way, it was placed in stressful conditions:

Table 1. The new model teat dip solution composition (100 g of the final product)

Pharmacological composition/ Chemical compound	Producer	CAS Number	Molecular mass	Compound, g	Notes
L-(+)-lactic acid $C_3H_6O_3$ Also indicated as l-(+)-2-hydroxypropanoic acid	Purac Biochem, The Netherlands	79-33-4	M = 90 g/mol	6.3	pH adjustment and disinfectant
Xanthan gum $C_{35}H_{49}O_{29}$ (monomer)	CP Kelco France, France	11138-66-2	M = 181.21 g/mol	0.67	Thickener
Dye CI 15985 Disodium 6-Hydroxy-5-[(4-Sulphonatophenyl)Azo]Naphthalene-2-Sulphonate	Neelikon Food Dyes & Chemicals Ltd, India	2783-94-0	M = 452.37 g/mol	0.03	Dye
Calendula officinalis dry extract (Extract Ratio 5:1)	Gonmisol, Spain	84776-23-8	The extract is the mixture of different substances (no molecular mass)	0.03	Antioxidant and anti-inflammatory effect
Avena sativa dry extract (Extract Ratio 4:1)	nVH Italia srl, Italy	84012-26-0	The extract is the mixture of different substances (no molecular mass)	0.02	Antioxidant, moisturizing effect
Glycerol 1,2,3-propantriol, $C_3H_5(OH)_3$	Aarhus Karshams Sweden AB, Sweden	56- 81-5	M = 92 g/mol Purity – 100%	9.00	Skin emollient
Sorbitol $H(CHOH)_6H$	Cargill Deutschland GmbH, Germany	50-70-4	M = 182.17 g/mol	1.00	Moisturizer
Water, H_2O		7732-18-5	M = 18.01 g/mol	Up to 100 g final product	Diluent

higher temperature, more intense freezing-heating cycle. The accelerated stability test program was carried out after 24 hours of manufacture, and after 2 months. Tests evaluate chemical, physical stability and sensory properties: colour, smell, homogeneity.

Samples of the developed teat dip containing lactic acid were used for further stability studies. Samples were selected and taken in accordance with LST EN ISO 9001 (Quality Management System) and LST EN ISO 227716 (Good Manufacturing Practices for Cosmetic Products) standards. Viscosity was measured at 20°C with a rotary (NDJ-1, COMECTA SA) viscometer. The pH measurement was carried out by a standard potentiometric test method with a laboratory pH-meter (InoLab pH 7310). Homogeneity, smell, and colour were determined by a visual method in the Light Cabinet (Byko-Spectra basic).

The microbiological examination was carried out according to MP-S_SVP-6:2020 (Edition I) and the European Pharmacopoeia (7th edition, 2.6.12). Bacteria colonies were counted on Mueller-Hinton Agar II, (BBL, Cockeysville, USA) after incubation at 35°C for 24 hours in aerobic conditions. The total number of aerobic microorganisms CFU/g in the test sample was calculated according to the formula and the final result was given as the number of microorganisms (CFU)/g: $N = \sum C/V \times 1.1 \times d$, kr: $\sum$C is the sum of the colonies counted in the two evaluated plates from two serial (one by one) dilutions.

Bactericidal activity of the teat dip containing lactic acid was determined according to the standard EN: 1656/2010/AC: 2011 at Good Laboratory Practice laboratory (Vimodrone, Italy). Briefly: 80%, 50%, and 10% concentration teat dip was tested on *S. aureus* ATCC 6538, *S. uberis* ATCC 19436, *E. coli* ATCC 10536. Test conditions: temperature 30 ± 1°C. Exposure time: 5 min. The nutrient medium Tryptone Soya Agar (TSA) was used in these tests. The indicator of biocide activity against the microorganisms used in the study is expressed as a logarithm. A teat dip solution is considered as bactericidal active if the logarithm value obtained during the test exceeds 5, i.e. log R > 5.

The final product was tested for contamination of *S. aureus, P. aeruginosa* and *C. albicans*.

Statistical analysis. Obtained research results were statistically processed using statistical data analysis packages: SPSS 19.0, Microsoft Office Excel 2007. Statistically reliable differences in arithmetic means when $P < 0.05$ between two groups were determined.

Results

The manufacturer's specification for xanthan gum as a thickener has a wide concentration range from 0.5% to 2.5%. According to the limits set by the supplier, the tests were started from the lowest concentration, and it was increased until the required viscosity value was reached and the minimum amount of the thickener was used.

Thus, the determination of the xanthan gum composition started from the lowest amounts, and during the tests, the amount of xanthan gum was increased to 0.67%. We found that this concentration of a thickener was sufficient for the product to meet the viscosity requirements. After laboratory tests, the final teat dip solution viscosity was set to 930–940 mPa*s. After a series of tests, according to the recipes (Table 1), a teat dip solution of orange colour, homogeneous appearance, specific lactic acid smell and pH 2.7 was created. Quality assurance tests were made for possible variation of pH, viscosity, sensory properties (colour, odour and homogeneity) and microbiological parameters.

The tested teat dip solution remained homogeneous throughout the whole stability testing period, bright orange in colour, characteristic of a lactic acid smell. It did not become cloudy, and did not fade after 6, 12, and 24 months after production (Table 2). Changes in the teat dip solution with lactic acid sensual properties were not detected.

Measuring the pH value showed that it remained stable ($P > 0.05$). Evaluation in a real-time programme showed that the pH value did not change statistically significantly. The pH value was in the range of 2.6–2.8, so it was found that the teat dip solution met the initially set requirements. After 24 months, the average value of pH decreased slightly – 0.01 ($P > 0.05$) in the samples from the three tested series in the real-time stability program. After correlation analysis evaluation, it was found that there were statistically significant linear relationships between pH in all teat dip solutions lots. The pH value stability test showed that the teat dip solution containing lactic acid properties did not change significantly ($P > 0.05$) during the 24-months stability testing period. The obtained data are presented in Table 2.

Teat dip solutions viscosity decreased insignificantly during the real-time stability program ($P > 0.05$). A decrease of viscosity was determined after 6 months to 33 mPa*s, compared with the manufactured product: it was 3.4% lower than after production after 6 months, 7.5% after 12 months, and 12.7% after 24 months (Table 2).

The microbiological quality of the new formulated teat dip solutions containing lactic acid was assessed after preparation (emulsion) and the samples were stored and tested at intervals specified in the real stability program (Table 2).

High manufacturing standards of hygiene and components with antimicrobial activity ensure that microbial growth was mostly inhibited in the newly manufactured teat dip solution (Table 2). Total aerobic bacteria count was 1.0×10^1 CFU/g (mL). Microorganisms such *P. aeruginosa, S. aureus, C. albicans* in 0.1g of the tested final teat dip solution samples were not detected. During the accelerated stability program, colour, homogeneity, and odour of the new formulated teat dip solution remained

Table 2. The new model teat dip solution containing lactic acid stability data

Time of evaluation	Homogeneity	Colour	Smell	pH	Viscosity, mPa*s	Total count of aerobic bacteria CFU/g (mL)	*P. aeruginosa*, *S. aureus*, *C. albicans*
Batch No. 1							
After 24 h	Homogeneous	Orange	Specific lactic acid	2.7	940	$< 1.0 \times 10^1$	Unidentified
After 6 months	Homogeneous	Orange	Specific lactic acid	2.6	910	$< 1.0 \times 10^1$	Unidentified
After 12 months	Homogeneous	Orange	Specific lactic acid	2.7	870	$< 1.0 \times 10^1$	Unidentified
After 24 months	Homogeneous	Orange	Specific lactic acid	2.6	820	$< 1.0 \times 10^1$	Unidentified
Batch No. 2							
After 24 h	Homogeneous	Orange	Specific lactic acid	2.8	940	$< 1.0 \times 10^1$	Unidentified
After 6 months	Homogeneous	Orange	Specific lactic acid	2.8	900	$< 1.0 \times 10^1$	Unidentified
After 12 months	Homogeneous	Orange	Specific lactic acid	2.8	870	$< 1.0 \times 10^1$	Unidentified
After 24 months	Homogeneous	Orange	Specific lactic acid	2.6	830	$< 1.0 \times 10^1$	Unidentified
Batch No. 3							
After 24 h	Homogeneous	Orange	Specific lactic acid	2.6	930	$< 1.0 \times 10^1$	Unidentified
After 6 months	Homogeneous	Orange	Specific lactic acid	2.6	900	$< 1.0 \times 10^1$	Unidentified
After 12 months	Homogeneous	Orange	Specific lactic acid	2.7	860	$< 1.0 \times 10^1$	Unidentified
After 24 months	Homogeneous	Orange	Specific lactic acid	2.6	830	$< 1.0 \times 10^1$	Unidentified

unchanged (Table 3). After the centrifugation tests (speed 45 rpm, time 8 min), the investigated samples of the product did not change; they remained stable during the entire study. Samples are considered stable if the product phase does not separate during centrifugation at the specified mode. At this stage of testing, the total number of aerobic microorganisms in 1 g (mL) did not exceed the permitted amounts, no microbial strains of *P. aeruginosa*, *S. aureus*, and *C. albicans* were detected. The accelerated stability program did not detect possible microbiological contamination.

No pH value deviation from the initial value was observed (Table 3) when samples were stored at a lower temperature. It can also be stated that no change in pH value occurred in the samples during freezing/heating cycles and these data remain stable; no statistically reliable decrease in pH value ($P > 0.05$) was found. From the data presented in Table 3, it can be seen that the harder experimental conditions did not have a statistically significant effect on the pH value; the pH value varies in stable and appropriate characteristic limits of the product. During this study, antibacterial activity (*in vitro*) of teat dip solutions was determined by the ability to reduce the total number of microorganisms used in testing. Results were evaluated according to the standard.

The teat dip solution antimicrobial efficiency test was carried out by evaluating bactericidal tests against reference strains *S. aureus S. uberis*, and *E. coli*, according to the EN 1656 standard. The teat dip solution is considered as bactericidal effective if the logarithm value obtained during the test exceeds 5. The test was performed with 80%, 50% and 10% teat dip solution concentrations. Bactericidal testing showed that 80% and 50% teat dip solution concentrations were bactericidal active against *S. uberis* ATCC 19436 and *E. coli* ATCC 10536 strains (log R > 5, Table 4). After diluting the teat dip solution to 10%, it was not effective in the performed study.

Discussion

The new formulated teat dip solution contains components such a thickener, an antimicrobial active ingredient, moisturizing, teat skin-friendly ingredients, and water. When developing new products and preparing chemical mixtures, it is important that the product is not harmful to the animal and friendly to the environment. For teat dip solutions and other skin surface disinfectants, classification of the chemical mixture is very important. Therefore, during the creation of a new formula teat dip solution, the

Table 3. The new model teat dip solution containing lactic acid data during accelerated stability program

Time of evaluation	Homogeneity	Colour	Smell	pH	Total aerobic microorganisms count CFU/g (mL)	Centrifugation, speed 45 rpm, time 8 min
Batch No. 1						
After manufacturing	Homogenic	Orange	Specific lactic acid	2.7	$< 1.0 \times 10^1$	Stabile
Heating/cooling cycles (10 d. at +4°C; 10 d. +45°C; 10 d. +4°C; 10 d. +45°C; 10 d. 15°C)	Homogenic	Orange	Specific lactic acid	2.7	$< 1.0 \times 10^1$	Stabile
After 2 months. keep at +45°C	Homogenic	Orange	Specific lactic acid	2.6	$< 1.0 \times 10^1$	Stabile
Batch No. 2						
After manufacturing	Homogenic	Orange	Specific lactic acid	2.8	$< 1.0 \times 10^1$	Stabile
Heating/cooling cycles (10 d. +4°C; 10 d. +45°C; 10 d. +4°C; 10 d. +45°C; 10 d. 15°C)	Homogenic	Orange	Specific lactic acid	2.7	$< 1.0 \times 10^1$	Stabile
After 2 months at +45° C	Homogenic	Orange	Specific lactic acid	2.8	$< 1.0 \times 10^1$	Stabile
Batch No. 3						
After manufacturing	Homogenic	Orange	Specific lactic acid	2.6	$< 1.0 \times 10^1$	Stabile
Heating/cooling cycles (10 d. +4°C; 10 d. +45°C; 10 d. +4°C; 10 d. +45°C; 10 d. 15°C)	Homogenic	Orange	Specific lactic acid	2.7	$< 1.0 \times 10^1$	Stabile
After 2 months at +45°C	Homogenic	Orange	Specific lactic acid	2.6	$< 1.0 \times 10^1$	Stabile

classification of the used thickener and antimicrobial substance was taken into account according to the European Parliament and Council Regulation (EC) No. 1272/2008. Xanthan gum is a natural polymer and does not pose a risk to animal health or the environment under this regulation. Also, lactic acid is recognized as safe for use in animal husbandry for surface disinfection.

Disinfectant effect and sufficient thickness are important in biocidal teat dip solutions. Viscosity should be sufficient to form a non-drop emulsion when the teat dip solution is applied to the skin of the teat. Therefore, viscosity is an important physico-chemical parameter of the teat dip solution, reflecting the quality of the product (Elella, 2020; Petri, 2015). In our study, we paid high attention to obtain the required viscosity. In the study, we chose the natural material that forms the viscosity in the new formula product. Xanthan gum is sufficient when inserted into the product at 0.67%. The product viscosity was statistically reliably stable throughout the entire study period, i.e., 24 months after manufacture.

A disinfectant is a necessary component in teat dip. In veterinary and animal husbandry, one of the most widely used biocides is lactic acid. Lactic acid is recognized by the US and the European Union Commission as a safe food additive for health and its quantities are unlimited. This compound is environmentally friendly and exhibits antimicrobial activity (Storton, 2010). Also, the 2017 EU regulation

Table 4. Antimicrobial activity (log R) of the new model teat dip solutions containing lactic acid (80%, 50%, 10%) according to standard UNI EN 1656

Test microorganisms	Test suspension	Results		
		80%	50%	10%
S. aureus ATCC 6538	10^{-6}: >330–>330* 10^{-7}: 46–35* N: 4.05 x 10^{8} No: 4.05 x 10^{7} log No: 7.61	Vc: >330–>330 Na > 3300 log Na > 3.52 log R < 4.09 Not active	Vc: >330–>330 Na > 3300 log Na > 3.52 log R < 4.09 Not active	Vc: >330–>330 Na > 3300 log Na > 3.52 log R < 4.09 Not active
S. uberis ATCC 19436	10^{-6}: >330–>330* 10^{-7}: 42–30* N: 3.60 x 10^{8} No: 3.60 x 10^{7} log No: 7.56	Vc: <14–<14 Na < 140 log Na < 2.15 log R > 5.41 Active	Vc: <14–<14 Na < 140 log Na < 2.15 log R > 5.41 Active	Vc: >330–>330 Na > 3300 log Na > 3.52 log R < 4.04 Not active
E. coli ATCC 10536	10^{-6}: >330–>330* 10^{-7}: 44–49* N: 4.65 x 10^{8} No: 4.65 x 10^{7} log No: 7.67	Vc: <14 – <14 Na < 140 log Na < 2.15 log R < 5.52 Active	Vc: <14–<14 Na < 140 log Na = 2.15 log R = 5.52 Active	Vc: >330–>330 Na > 3300 log Na > 3.52 log R < 4.15 Not active

* number of colonies (CFU) in test suspension dilutions; N = number of CFU/mL of the test suspension; Vc = viable count; Na = number of CFU/mL of the test mixture; R = reduction in viability. No – number of CFU/mL in the test mixture (diluted).

(https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32017R2002) confirmed that lactic acid can be used in different types of veterinary products intended for animal skin or surface disinfection. Lactic acid products have been shown to have antibacterial activities against Gram-positive and Gram-negative bacteria (Boomsma et al., 2015). These ingredients are used in food and cosmetics as preservatives and also have low toxicity; lactic acid is less sensitive to skin irritation (Alsaheb et al., 2015). Many commercial teat antiseptic products have various active ingredients including iodine, hydrogen peroxide, chlorine, and chlorhexidine. Some may cause skin irritation and bacterial resistance (Sadakane et al., 2015). Therefore, pre-parations of natural products are desirable. The study of Chivero and Gohtani (2015) states that teat dip solutions with lactic acid protect the teat canal from possible infections or inflammations, and the moisturizer film remains effective until the first washing of the skin.

Our newly formulated teat dip solution showed a bactericidal effect. It was confirmed by *in vitro* study against reference isolates. Our study according to the EN 1656 standard showed bactericidal activity at 80% and 50% concentration to reference cultures, as log R > 5 was obtained. World scientists have analyzed the antibacterial effectiveness of teat dip containing lactic acid. Extensive research has been conducted (Fitzpatrick et al., 2021). These researchers tested products containing lactic acid as the main active ingredient. Lactic acid concentrations in these products ranged from 1.76% w/w to 8% w/w. For streptococcal isolates, the product with 5% w/w lactic acid resulted in a numerically largest bacterial reduction of 70.1%. The product with 2% lactic acid combined with 0.6% w/w chlorhexidine obtained the largest bacterial reduction of 100% against staphylococcal isolates. The product with 1.6% w/w lactic acid combined with hydrogen peroxide was found to result in bacterial reductions of 89.9% and 59.4% for streptococcal and staphylococcal isolates, respectively. This study suggests that some teat disinfectant products achieve a higher reduction in bacterial levels against different specific bacterial groups on teat skin than other teat disinfectant products. However, Chotigarpa (2019) found that no relationship was observed between a higher concentration of active ingredient and increased effectiveness. The scientist notes that the use of a lactic acid-based teat disinfectant reduced the bacterial load on the teat skin and decreased the prevalence of mastitis due to coliforms such as *E. coli*. Important studies on reduced susceptibility to lactic acid teat disinfectants have not been published yet; only resistance mechanisms of lactic acid bacteria coping with acid stress have been observed (Wang, 2018; Schwenker, 2022).

In assessing the quality of pharmaceutical products, stability of physio-chemical parameters is important. When conducting stability studies for biocidal products, it is important to determine whether the quality of disinfectant products is sufficient during the entire shelf life, whether the product maintains its original physical and chemical properties. In case of a decrease of viscosity, film would not form on the skin, which ensures the lowest disinfectant properties by the inability to keep the active substances where they are needed. The importance of stability studies is also defined in Regulation (EU) of the European Parliament and Council no. 528/2012, Title 2 of Annex III, which states that in order to register a

biocidal product in the European Union, it is necessary to submit the results of stability studies at low and ambient temperatures, according to which the shelf life of the biocidal product is determined. For this purpose, two types of stability programs were implemented: real-time and accelerated stability. In real-time stability studies, the product was stored under recommended conditions and monitored for changes in product properties. When testing the product in an accelerated way, the product is placed under stressful conditions: +45°C temperature, heating-freezing cycle. Both real-time and accelerated stability program tests are designed to reliably assess the following aspects: stability and physical integrity of products, chemical and microbiological stability. After carrying out stability tests of the samples and evaluating the obtained results, it was observed that the teat dip solution maintained sufficient stability when stored at low and room temperature; the pH and viscosity results of the samples also showed the stability of the composition. During the stability study, it was found that the storage conditions did not significantly affect the physical product appearance and chemical characteristics of the new formulated teat dip solution.

Microbiological quality control of pharmaceutical products is necessary to ensure their safety for the environment, the animal and health of the husbandry service personnel. Microbiological contamination of the new formulated teat dip solution was not detected by real and accelerated product stability programs. At accelerated stability program conditions, microbiological contamination did not initiate.

Conclusions

After determining the appropriate thickener xanthan gum and lactic acid concentrations, a new teat dip solution was formulated that also contains glycerol, sorbitol, oat and calendula extracts.

At real and accelerated stability program, the teat dip solution kept unchanged physical parameters such as colour, appearance, homogeneity, smell and viscosity. The pH of the teat dip solution during the study varied slightly.

The teat dip solution showed bactericidal activity against reference *E. coli* and *S. uberis* bacteria (log R > 5). The antimicrobial effect to *S. aureus* was insufficient and the considered product was not effective against this bacteria.

Conflict of Interests

The authors declare that there is no conflict of interests.

References

1. Alsaheb R.A.A., Aladdin A., Othman N.Z., Malek R.A., Leng O.M., Aziz R., Enshasy H.A.E. Lactic acid applications in pharmaceutical and cosmeceutical industries. J. Chem. Pharm. Res. 2015. 7(10):729–735.
2. Alves F.R. Applications of Natural, Semi-Synthetic, and Synthetic Polymers in Cosmetic Formulations, Cosmetics. 2020. vol. 7. p. 75.
3. Boomsma B., Bikker E., Lansdaal E., Stuut P. L-lactic acid a safe antimicrobial for home and personal care formulations. Sofw J. 2015. 10(141):2–5.
4. Breen J. The Importance of Teat Disinfection in Mastitis Control. Livestock. 2019; 24:122–8.
5. Chivero P., Gohtani S. Effect of xanthan and guar gums on the formation and stability of soy soluble polysaccharide oil-in-water emulsions, Food Research International. 2015. vol. 70. p. 7–14.
6. Chotigarpa R., Lampang N.K. Antiseptic effect of natural teat dip containing lactic acid against mastitis-causing Escherichia coli, Veterinary World. 2019. vol. 12 (3). p. 397-401.
7. Colipa guidelines „Guidelines on stability testing of cosmetic products", 2004.
8. Commission Implementing Regulation (EU) 2017/2002 of 8 November 2017 approving L(+) lactic acid as an existing active substance for use in biocidal products of product-types 2, 3 and 4 (Text with EEA relevance. https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32017R2002.
9. Elella A.H.M. Xanthan gum-derived materials for applications in environment and ecofriendly materials: A review, Journal of Environmental Chemical Engineering. 2020. DOI:10. 1016/j. jece. 2020.104702.
10. EN 1656:2009/AC:2010 – Chemical desinfectants and antiseptics – Quantitative suspension test for the evaluation of bactericidae activity of chemical disinfectant and antiseptics used in veterinary field. Test methods and requirements (Phase 2/step 1).
11. European chemicals agency, Guidance on the Biocidal Products Regulation, vol. 2, version 3.0. 2018. P. 67.
12. European Pharmacopoeia (7th edition, 2.6.12).
13. Fitzpatrick S.R., Garvey M. Screening commercial teat disinfectants against bacteria isolated from bovine milk using disk diffusion. Veterinary World. 2019. vol. 12. P. 629-637.
14. Fitzpatrick S. R., Garvey M., Flynn J., O'Brien B., Gleeson D. The effect of disinfectant ingredients on teat skin bacteria associated with mastitis in Irish dairy herds. Irish Veterinary Journal. 2021. V. 74, N:1. P. 2-12.
15. Garvey M., Curran D. Efficacy testing of teat dip solutions used as disinfectants for the dairy industry: Antimicrobial properties, International Journal of Dairy Technology. 2016. vol. 69. P. 179-187.
16. Hassan R., Kudi C.A., Chiezey N.P., Rekwot P.I., Egbodo B.E., Samuel F.U., Maikaji F.S., Bello T.K. Mastitis causing pathogens within the environment and teats of milking cows in Zaria, Kaduna state. J. Anim. Prod. Res. 2016. 28(1):8–13.
17. In Y.W., Kim J.J., Kim H.J., Oh S.W. Antimicrobial activities of acetic acid, citric acid and lactic acid against *Shigella* species. J. Food. Saf. 2013. 33(1). P. 79–85.
18. Kučević D., Plavšić M., Trivunović S., Radinović M., Kučević D.S. Effect of post-milking teat dipping on hygienic quality of cow's milk. Biotechnol. Anim. Husbandry. 2013. 29(4). P. 665–673.
19. LST EN ISO 227716 (Good Manufacturing Practices for Cosmetic Products) standart.
20. Magari R.T. „Assessing Shelf Life Using Real-Time and Accelerated Stability Tests", BioPharm International. 2003.
21. Mišeikienė R., Rudejevienė J., Gerulis G. Effect of premilking antiseptic treatment on the bacterial contamination of cow teats' skin Bulgarian Journal of Veterinary Medicine. 2015. N. 2. P. 159–166.
22. Nickerson S.C. Choosing the Best Teat Dip for Mastitis Control and Milk Quality. Proceedings NMC-PDPW Milk Quality Conference. 2001. P. 43–54.
23. Oliver S.P., Murinda S.E. Antimicrobial Resistance of Mastitis Pathogens. Veterinary Clinics: Food Animal. 2012. V. 28. P. 165-185.
24. Petri F. S. Xanthan gum: A versatile biopolymer for biomedical and technological applications, J. Appl. Polym. Sci., 2015. vol. 132. DOI: 10.1002/APP.42035.

25. Regulation (EU) No 1272/2008 of the European Parliament and of the Council.
26. Regulation (EU) No 528/2012 of the European Parliament and of the Council.
27. Sadakane K., Ichinose T. Effect of the hand antiseptic agents benzalkonium chloride, povidone-iodine, ethanol, and chlorhexidine gluconate on atopic dermatitis in NC/Nga mice. *Int. J. Med. Sci.* 2015. 12(2). P. 116–125.
28. Schwenker J.A., Schotte U., Hölzel C.S. Minimum inhibitory concentrations of chlorhexidine- and lactic acid-based teat disinfectants: An intervention trial assessing bacterial selection and susceptibility. Journal of Dairy Science. V.105, 2022, p. 734-747.
29. Storton M., Exarchakis J., Waters T, Hao Z. Lactic acid quantitation in hand dishwashing liquid using an HILIC-UV methodology. Journal of separation science. 2010. Vol. 33, P. 982-987.
30. Wang C., Chang T., Yang H., Cui M. Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella* Enteritidis *Escherichia coli* and *Listeria monocytogenes*. *Food Control.* 2015. 47(1). P. 31–236.
31. Wang C., Cui Y., Qu X. Mechanisms and improvement of acid resistance in lactic acid bacteria. Arch. Microbiol., 200 (2018). P. 195-201.

Received 21 October 2022
Accepted 1 December 2022

Veterinarija ir Zootechnika

Volume 80(2), 2022
Supplement

2nd international scientific conference
Lithuanian University of Health Sciences,
Veterinary Academy, Faculty of Animal Sciences.
2022 November 16. Kaunas

LIVESTOCK PRODUCTION: RECENT TRENDS AND FUTURE PROSPECTS

Abstracts

CEREAL BY-PRODUCT VALORIZATION BY APPLYING ENZYMATIC TREATMENT AND FERMENTATION MODELS

Elena Bartkienė[1,2], Vita Lėlė[1,2], Vytautė Starkutė[1,2], Eglė Zokaitytė[1,2], Vadims Bartkevics[3]

[1]Department of Food Safety and Quality, Veterinary Faculty, Lithuanian University of Health Sciences, Mickevičiaus str. 9, LT-44307 Kaunas, Lithuania,
[2]Institute of Animal Rearing Technologies, Faculty of Animal Sciences, Lithuanian University of Health Sciences, Mickevičiaus str. 9, LT-44307 Kaunas, Lithuania,
[3]Institute of Food Safety, Animal Health and Environment "BIOR", Lejupes iela 3, Zemgales priekšpilsēta, Riga, LV-1076, Latvia

Corresponding author: elena.bartkiene@lsmu.lt

The by-products of the cereal industry are potential resources for functional compounds. They can also be used as a solid substrate for the cultivation of biomass of desirable beneficial microorganisms. In addition to degradation properties (compared to pure enzymes), selected viable microorganisms may possess desirable antimicrobial and microbiota modeling properties *in vivo*. Wheat and barley processing generates a large amount of by-products, and the latter are very good producers of enterolignans *in vitro*, compared to other cereals. In addition to lignans and enterolignans, alkylresorcinols are also present in the outer layer of the cereal. Finally, the outer layer is a good source of many phytochemicals, but the highly complex insoluble structure of bran reduces the accessibility of these valuable compounds. Therefore, the application of enzymatic treatment and fermentation can be considered potentially useful to effectively utilizing cereal by-products. The objective of this study was to apply enzymatic treatment and lactic acid fermentation models to the valorization of cereal by-products. In addition, to increase the sustainability of the process, the possibility of using the potato juice for the propagation of technological microorganisms was tested. It was found that the potato juice is a suitable substrate for the cultivation of *P. acidilactici*, as the juice has more than 9.6 $\log_{10}$ CFU/mL of viable lactic acid bacteria cells. The fermented juice powder (stabilized by spray drying) also had a number of viable lactic acid bacteria greater than 7.0 $\log_{10}$ CFU/g after 12 months of storage. The changes in the microbial profile and the chemical composition of treated cereal by-products showed that microbial treatment increases biosafety and reduces mycotoxin content of grain by-products. In addition, the use of selected lactic acid bacteria strains for the fermentation of cereal by-products increases the concentration of matairesinol and secoisolariciresinol. Finally, fermentation of cereal by-products with selected technological strains can be recommended as an effective method for more sustainable and higher value raw material production.

Keywords: fermentation, enzymatic treatment, cereal outer layer, functional compounds.

Acknowledgments:
The authors gratefully acknowledge the COST Action CA18101 'SOURDOugh biotechnology network towards novel, healthier, and sustainable food and bIoproCesseS' and the EUREKA Network Project E!13309 "SUSFEETECH" (No. 01.2.2-MITA-K-702-05-0001).

EVALUATION OF MILK YIELD AND MILKABILITY TRAITS OF DIFFERENT GENOTYPE COWS DURING LACTATION

Lina Anskienė[1], Renata Japertienė[1], Sigitas Japertas[2]

[1]Department of Animal Breeding, Lithuanian University of Health Sciences, Faculty of Animal Husbandry Technology. Tilžės str. 18, LT-47181, Kaunas, Lithuania,
[2]Public Institution Practical Training and Trial Center of Lithuanian University of Health Sciences. Akacijų str. 2, Giraitės village, LT-54310, Kauno distr., Lithuania

Corresponding author: lina.anskiene@lsmu.lt

Dairy herds in Europe and the United States of America were selected for high milk production under intensive farming conditions. Even under optimal management conditions, selection for the increase of milk yield has reduced dairy cattle health and reproductive efficiency worldwide [1]. This raises doubts whether these high-cost genotypes are suitable for organic farming systems [2], [3]. The evaluation of milk production and milkability traits of different genotype Lithuanian black and white cattle population (n = 528) was carried out by the data of state enterprise Agricultural Information and Rural Business Center and in a herd of an organic farm. The aim of this study was to investigate the milk yield and milkability traits during lactation of Lithuanian Black and White and Holstein cows with cows of different genotypes. The milk yield, milking speed, the highest milk flow, and milking time were investigated during the study. Evaluated traits were measured with DeLaval electronic milk meter, "Apro Windows" software. All records were obtained during 305 days of lactation. Statistical data analysis was conducted using SPSS 25.0 (SPSS, Inc., Chicago, IL, USA) software. The data were presented using descriptive statistics and normal distribution analysis methods, Kolmogorov–Smirnov test. The Pearson correlation (r) was determined to define the linear relationship between investigated traits from Apro Windows software. Multiple comparisons were calculated using the Tukey test. We observed that the highest milk yield and the highest milk flow rate were detected in cows with Lithuanian Black and White breed genotype (Lithuanian Black and White, Lithuanian Black and White x Holstein, Lithuanian Black and White x Holstein x Lithuanian Black and White) during all stages of lactation. Milking time of these cows during the first two stages of lactation was longer, compared to cows of other genotypes ($P < 0.05$). An average positive correlation was estimated between the stage of lactation and milk yield (r = 0.397), high milk flow (r = 0.331), ($P < 0.001$). We estimated that of all fixed effects (stage of lactation, genotype, interaction of stage of lactation with genotype) the biggest influence on milk yield, highest milk flow, milking speed, milking time was produced by the stage of lactation ($P < 0.001$); a genotype showed the highest impact only on milking time ($P < 0.01$)

Keywords: genotype, milk yield, milk flow, milking time.

References:

[1] Oltenacu P.A. and Broom D.M. The impact of genetic selection for increased milk yield on the welfare of dairy cattle. Journal of Animal Welfare, 2010. 19, 39–49.
[2] Nauta W.J., Veerkamp R.F., Brascamp E.W. & Bovenhuis H. Genotype by environment interaction for milk production traits between organic and conventional dairy cattle production in The Netherlands. Journal of Dairy Science, 2006, 89, 2729-2737.
[3] Horn M., Steinwidder A., Podstatzky L., Gasteiner J., Zollitsch W. Comparison of two different dairy cow types in an organic, low input milk production system under Alpine conditions. Journal of Agriculture and Forestry Research, 2012, 362 (3), 22–325.

CANNABIDIOL MEDIATE ASSOCIATION BETWEEN CAECA BACTERIAL ACTIVITY AND BREAST MEAT VOLATILE COMPOUNDS IN CHICKENS

Paweł Konieczka[1,2], Iwona Wojtasik-Kalinowska[3], Andrzej Poltorak[3], Misza Kinsner[1], Dominika Szkopek[1], Bartosz Fotschki[4], Jerzy Juśkiewicz[4], Joanna Banach[5], Monika Michalczuk[6]

[1]The Kielanowski Institute of Animal Physiology and Nutrition Polish Academy of Sciences, Jabłonna, Poland,
[2]University of Warmia and Mazury in Olsztyn, Olsztyn, Poland,
[3]Department of Technique and Food Development, Warsaw University of Life Sciences, Warsaw, Poland,
[4]Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Olsztyn, Poland,
[5]Institute of Natural Fibres and Medicinal Plants – National Research Institute, Poznań, Poland,
[6]Department of Animal Breeding, Institute of Animal Sciences, Warsaw University of Life Sciences, Warsaw, Poland

Corresponding author: p.konieczka@ifzz.pl

Currently, great attention has been put to investigating the use of bioactive substances in poultry diet to improve birds' welfare and ensure final product safety and quality. One phytogenic substance that manifests the potential to beneficially modulate gut health and functionality is cannabidiol (CBD) obtained from hemp, which has recently attracted increasing attention. Therefore, in the present experiment, we investigated the effects of CBD on meat volatile organic compounds (VOCs) and gut microbiota activity in chickens reared under optimal (with no induced inflammation) conditions or subjected to *C. perfringens*- or *E. coli*-induced inflammation. Samples of breast meat were obtained from chickens (Ross 308 male broilers) allocated considering average body weight into 6 treatments, each containing 34 birds. The birds in the control (CON) group were fed a basal diet over the entire an experimental period. The birds in the CBD treatment received diet as CON but supplemented (on top) with 30 g/kg *C. sativa* extract, while the birds in the CON+*C. perfringens* and CON+LPS groups (positive control groups) were fed the CON diet but they were subjected to a *C. perfringens* and LPS challenge, respectively. Chickens in the CBD+*C. perfringens* and CBD+LPS groups received the same diet as the birds in the CBD treatment group in addition to being subjected to the respective challenge factor (*C. perfringens* or LPS). Results indicated that CBD supplementation counteracted the formation of breast meat spoilage VOCs, including alcohols, trimethylamine and pentanoic acid, in the challenged birds, partly by decreasing caecal putrefactive SCFA production. Meat VOC/caecal SCFA relationships differed according to the applied challenge; however, CBD attenuated the effects of *C. perfringens* infection better than the effects of the LPS challenge on meat VOCs. The present study demonstrated the existence of a close association between caecal bacterial activity and chicken meat VOCs based on the production of SCFAs. CBD supplementation decreased the formation of putrefactive fatty acids, which resulted in decreased production of spoilage VOCs in the meat. Due to the complexity of pathogen–host interactions, more research is needed to investigate CBD potential in modulating the health status of the host.

Keywords: cannabidiol, *C. perfringens*, *E. coli*, chickens.

This work was supported by the National Science Centre, Grant No. 2018/29/B/NZ9/01351.

COMPARATIVE ANALYSIS OF TWO CONFORMATION SCORING SYSTEMS OF POLISH WARMBLOOD MARES ASSIGNATED AS JUMPING AND DRESSAGE TYPE

Alicja Borowska[1], Dorota Lewczuk[2]

[1]Department of Genetics and Animal Breeding, Poznań University of Live Science, ul. Wojska Polskiego 28; 60-637 Poznań, Poland,
[2]Department of Biotechnology and Nutrigenomics, IGAB PAS, Jastrzębiec, ul. Postępu 36A, 05-552 Magdalenka, Poland

Corresponding author: alicja.borowska@up.poznań.pl

The selection of horses is based primarily on conformation evaluation. Over the years, the evaluation methods have been improved, and finally, in many sport horse breeding populations, linear scoring is used. This study aimed to compare differences between jumping and dressage types of warmblood horses according to two systems used simultaneously in Poland (point evaluation on the scale 0–100 pkt [1] and linear profiling [2]). Additionally, a comparison of basic measurements (height at withers, circumference of the trunk, and cannon circumference) was conducted. The data coming from 1500 mares were used. The point scaling for 10 traits was analyzed separately and as a general result. The linear scoring was transformed into the 1–40 points scale for the statistical analysis with a mean value of 20 points [3]. The analysis of variance using the MIXED procedure of the SAS program was conducted with the statistical model including the following fixed effects: sport type, breeding district, and breed. The sire effect was used as a random effect. Correlations between different traits were also calculated using the CORR procedure. Horses of different sports types differ in the circumference of the trunk ($P = 0.0013$), forelimbs point evaluation $P = 0.0274$), and walk point evaluation ($P = 0.0368$). Dressage horses were stronger in the trunk, with a higher evaluation of forelimbs and walk points. Linear conformation profiling analyses showed differences between both types of horses at the position of the neck ($P = 0.0373$), the position of the shoulder ($P = 0.0036$), the line of loins ($P = 0.0142$), the shape of the croup ($P = 0.0054$), the stance of the pastern ($P = 0.0266$) and quality of legs ($P = 0.044$). Differences observed for these traits were for both types of horses on the same side of the mean (< 20, > 20). Linear movement profiling showed differences between both types of horses only for the trait balance of the canter ($P = 0.0103$). This trait was less balanced for jumping horses. The traits repeatabilities calculated from the sire effect were low for the basic measurements (0.13–0.30), very low for point evaluation (0.02–0.11), and divergent for linear profiling – general (0.12–0.51) and descriptive (0.03–0.12) – traits. The Pearson correlations between the general marks of the linear evaluation and the 100-point scale were positive and showed a similar direction of the assessment, while the correlations between the 100-point assessments and detailed linear traits of conformation were mostly low negative. According to obtained results, the diversification of horses in jumping and dressage types is small and should be investigated further to obtain the required horse specialization.

Keywords: horse, conformation type, linear profiling, scaling.

References:

[1] Lewczuk D., The effect of sire's breed on three body measurements and body conformation score in Polish Halfbred Horse at the beginnings and on the present-day of the breed Anim. Sci. Pap. Rep, 2005, 23, 171-179.
[2] Duensing J., et al. Implementation and Prospects of Linear Profiling in the Warmblood Horse Journal of Equine Veterinary Science, 2014, 34, 360-368.
[3] Ducro B.J., et al. Genetic relations of movement and free-jumping traits with dressage and show-jumping performance in competition of Dutch Warmblood horses Livestock Science, 2007, 107, 227-234.

THE EFFECT OF A LOW OR HIGH-FAT DIET AND SUPPLEMENTATION WITH VARIOUS FORMS OF CHROMIUM ON IMMUNOLOGICAL PARAMETERS IN THE RAT'S ORGANISM

Anna Stępniowska[1], Krzysztof Tutaj[1], Przemysław Sołek[1], Bartosz Fotschki[2], Jerzy Juśkiewicz[2], Paulius Matusevicius[3], Katarzyna Ognik[1]

[1]Department of Biochemistry and Toxicology, Faculty of Animal Science and Bioeconomy, University of Life Sciences in Lublin, Akademicka 13, 20–950 Lublin, Polan,
[2]Division of Food Science, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10-748 Olsztyn, Poland,
[3]Lithuanian University of Health Sciences, Tilžės 18, 47181 Kaunas, Lithuania

Corresponding author: paulius.matusevicius@lsmu.lt

The aim of the study was to determine how the administration of a low-fat or high-fat diet supplemented with various forms of chromium to rats affects the immune response in blood plasma. The study schema consisted of two periods, i.e. initial and experimental, 9 weeks each. During the initial 9-week period, the rats aged 6 weeks were randomly assigned to the control group (n = 12) fed standard low-fat C diet and the HF group (n = 72) fed high-fat diet. After the initial period, the rats from the control group were fed the same standard diet for subsequent 9 weeks of the experimental period. The HF rats were then randomly divided into 6 groups with n = 12 per one group. The M group was subjected to the standard low-fat diet, the F group was further fed for subsequent 9 weeks the high-fat diet, the MP group was fed a standard low-fat diet with supplementation of chromium picolinate, the MN group was fed a standard low-fat diet with supplementation of chromium nanoparticles, the FP group was subjected to a high-fat diet with chromium picolinate supplementation, and the FN group was fed a high-fat diet with nanoparticle chromium supplementation. The amount of chromium administered to each rat was 0.3 mg/kg body weight (BW), selected according to the EFSA NDA Panel [2014]. Plasma levels of interleukins (IL-2, IL-6), immunoglobulins (IgA, IgG, and IgM), as well as TNF-α, acute phase protein CRP and ceruloplasmin-Cp were determined. Compared to the control diet (C), the use of a high-fat diet (group F) in the feeding of rats resulted in lower plasma levels of CRP, IgA ($P < 0.001$, respectively). Shortening the duration of the high-fat diet (group M) resulted in an increase in plasma IL-2 levels and a decrease in plasma CRP levels ($P < 0.001$, respectively). Two-way ANOVA analysis showed interactions of diet x chromium addition for IL-2, CRP and Cp ($P < 0.001$, respectively) and IL-6 ($P = 0.032$). The occurrence of the interaction indicates that the studied parameter was influenced by both diet and chromium addition. However, it was found that chromium addition in the form of nanoparticles resulted in lower levels of IgG ($P < 0.001$) and TNF-α ($P = 0.003$). In conclusion, it was found that in the case of prolonged use of a high-fat diet, no elevation of inflammatory parameters was observed, but the addition of chromium in the form of nanoparticles to a high-fat diet, although it did not increase plasma CRP levels, caused an adverse increase in the pro-inflammatory cytokine IL-6. Such an effect was not observed in the case of a shorter use of a high-fat diet with the addition of chromium nanoparticles. On the other hand, in the case of a long-term use of a high-fat diet, the addition of chromium in the form of picolinate favorably reduced plasma levels of CRP and Cp.

This work was supported by the National Science Centre, Grant No. 2020/39/B/NZ9/00674.

SOIL-DERIVATED PRODUCTS IN POULTRY PRODUCTION

Agila Dauksiene[1,2], Dovile Klupsaite[1], Romas Gruzauskas[4], Vytaute Starkute[1,3], Vita Lele[1,3], Paulina Zavistanaviciute[1,3], Erika Mozuriene[1], Egle Zokaityte[1,3] and Elena Bartkiene[1,3]

[1]Institute of Animal Rearing Technologies, Faculty of Animal Sciences, Lithuanian University of Health Sciences, Tilžės str. 18 Kaunas, Lithuania,
[2]Department of Anatomy and Physiology, Faculty of Veterinary Medicine, Lithuanian University of Health Sciences, Tilžės str. 18 Kaunas, Lithuania,
[3]Department of Food Safety, Faculty of Veterinary Medicine, Lithuanian University of Health Sciences, Tilžės str. 18 Kaunas, Lithuania,
[4]Department of Food Science and Technology, Faculty of Chemical Technology, Kaunas University of Technology, Radvilėnų str. 19 Kaunas, Lithuania

Corresponding author: agila.dauksien@lsmu.lt

In recent decades, various zootechnical feed additives, that affect good health, improve digestibility and productivity of broiler chickens have been formulated and applied [1]. In the frame of sustainable poultry production, solid-derivated substances as feed additives for poultry have become more promising. Humic acids (HAs) are a naturally occurring component of soil, which is formed by the decomposition of organic matter, especially plants [2]. HAs are involved in metabolic processes in animals, interact with many compounds and structures (organic and inorganic molecules, minerals), thereby promoting the better absorption of minerals necessary to the host [3; 4]. The following study was performed to investigate the effect of dietary HAs supplementation on growth performance, blood indices and *Tibia* development of broiler chickens. The broilers were assigned to 2 treatments: 1 (control group) = basal diet, 2 (HAs group) = basal diet with 2 g/kg of feed HAs. Chicks were raised from 1 to 42 days of age. A corn-soybean meal-based diet (basal diet) was formulated according to the nutritional requirements prescribed in the *Ross* nutrition specification (2019) and NRC (2004). SPSS software version 15.0 (SPSS, Chicago, IL, USA) was used for statistical analysis. Differences were classified by the Duncan multiple comparison test. Results were considered statistically significant at $P \leqslant 0.05$. The final body weight (FBW) of broilers increased by 1%, feed conversion ratio (FCR) by 0.6% and mortality by 2% were decreased ($P \geqslant 0.05$). The blood cholesterol, HDL-cholesterol and LDL-cholesterol concentrations were lower ($P \geqslant 0.05$) in broiler chickens with HAs. The results showed that the addition of HAs had a positive impact on the broilers' blood Ca, P, Fe levels, compared with the control group ($P \geqslant 0.05$). The *Tibia* lengh, weight and strenght showed responses to HAs addition to the feed of broiler chickens ($P \geqslant 0.05$). The results of this study indicate that HAs can be used in broiler feeds. It showed tendencies to increase productivity, blood Ca, P and Fe concentrations and better *Tibia* development of broiler chickens.

Keywords: humic acids, productivity, blood indices, bone development, broiler chickens.

References:

[1] Pirgozliev V., et al., Feed additives in poultry nutrition. Bulgarian Journal of Agricultural Science 2019, 25, 8-11.
[2] Saleh A. A., et al., Effect of dietary supplementation of humic acid and lincomycin on growth performance, nutrient digestibility, blood biochemistry, and gut morphology in broilers under clostridium infection. Journal of Applied Animal Research 2022, 50:1, 440-452.
[3] Bezuglova O., Molecular structure of humus acids in soils. J. Plant Nutr. Soil Sci. 2019, 182, 676–682.
[4] Bezuglova O., et al., Application of Humic Substances in Agricultural Industry. Agronomy 2022, 12, 584.

DOES 43RF-AMIDE MODULATE THE PITUITARY GONADOTROPHE CELLS SECRETORY ACTIVITY IN SHEEP? PRELIMINARY RESULTS

Kinga Pałatyńska, Bartosz Jarosław Przybył, Michał Szlis, Bartłomiej Wysoczański, Anna Wó

The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences; Jabłonna, Poland

Corresponding author: b.przybyl@ifzz.pl

Peptides involved in the regulation of food intake, synthesized in the central nervous system, are often involved in the controlling of the gonadotrophic axis activity. Premises exist that 43RF-amide (43RFa) may be simultaneously engaged in the functioning of the complex neurohormonal network responsible for the maintenance of the organism energy homeostasis and the regulation of reproductive processes. The aim of this study was to verify the research hypothesis, which assumes that 43RFa (orexigenic neuropeptide, belonging to RF-amide peptides) can be engaged in the modulation of the pituitary gonadotrophes secretory activity in sheep. The experiment was performed on sexually mature Polish Merino sheep (n = 48). Animals were divided into three groups. The following intracerebroventricular infusions were performed: control group (Ringer-Locke solution), group I (43RFa in dose 10μg/480μL/day), and group II (43RFa in dose 50μg/480μL/day). After the experiment, the animals were slaughtered: the pituitaries were stored for Real Time RT qPCR or immunohistochemistry and plasma samples were stored for radioimmunoassay analysis. Preliminary results showed that central infusion of 43RFa at dose of 50μg/480μL/day increase *fshβ* mRNA expression in pituitary cells. At the same time, no changes in FSH blood concentration were noted after 43RFa treatment in any groups of sheep. Furthermore, received results showed no changes in *lhβ* gene expression as well as LH concentration in blood plasma in all investigated groups of sheep. Based on the presented results it can be concluded that 43RFa can modulate the FSH, but not LH secretory activity in pituitary cells in sheep but further studies, especially immunohistochemical determinations, need to be done to confirm those observations.

Keywords: sheep reproduction, FSH, LH, orexigenic neuropeptide.

The study was financed by National Science Centre, Poland, grant PRELUDIUM 17; no. 2019/33/N/NZ9/00287

FACTORS DETERMINING GOAT MILK AMINO ACIDS AND FATTY ACIDS

Evaldas Šlyžius[1], Lina Laučienė[2], Lina Anskienė[1], Birutė Šlyžienė[3], Renata Bižienė[4]

[1]Lithuanian University of Health Sciences, Faculty of Animal Science, Department of Animal Breeding, Kaunas, Lithuania,
[2]Lithuanian University of Health Sciences, Faculty of Veterinary Medicine, Department of Food Safety and Quality, Kaunas, Lithuania,
[3]Small Community "Research Gamma", Kaunas, Lithuania,
[4]Lithuanian University of Health Sciences, Faculty of Animal Science, Institute of Biology Systems and Genetic Research, Kaunas, Lithuania

Corresponding author: evaldas.slyzius@lsmu.lt

The aim of this study was to estimate the influence of factors on milk amino acids and fatty acids of the dairy goats. The research was carried out at the Lithuanian dairy goat farm with Saanen and Alpine goat breeds (n = 97). Analyses of the amino acids and fatty acid composition of goat milk were carried out with SHIMADZU gas chromatographer. Statistical analysis was conducted with statistical package SPSS 25.0 (SPSS, Inc., Chicago, IL, USA). Results were considered to be reliable when $P < 0.05$. The present study showed that the milk yield was higher in Saanen goats, but their milk was significantly lower in fat and protein content than that of Alpine goats ($P < 0.05$). The quantity of all individual amino acids and their groups (essential (EAA), nonessential (NEAA), total (TAA), and branched-chain (BCAA)) was significantly higher in Alpine compared to Saanen goat milk ($P < 0.01$). An analysis of amino acids in milk from different seasons showed that the highest content of both essential and nonessential amino acids was found in the grazing season ($P < 0.05$), except for arginine and alanine acids the content of which was slightly higher during the housing period and for glycine acid the content of which was the same in both seasons. The individual fatty acids ranged between goat breeds while the total amount of SFA, UFA, and MUFA was not significantly different between the breeds ($P < 0.05$). An analysis of fatty acids in milk from different LPL genotype showed that the highest content of fatty acids was determined in the CG genotype of the LPL gene (on average 11.77% higher than in the CC and GG genotypes) of goats ($P < 0.01$). The milk of CC and GG genotypes was similar for MCFA, LCFA, SFA, UFA, MUFA and PUFA percentages. Meanwhile, the CG genotype milk had significantly more MCFA and SFA, and less UFA, MUFA and PUFA than the CC and GG genotype milk ($P < 0.05$). The highest content of short-chain and medium-chain fatty acids was found in the grazing season, and that of long-chain fatty acids – in the housing season goat milk ($P < 0.05$). Analysis of variance revealed that the goat breed, LPL genotype and season showed a statistically reliable impact on amino acid and fatty acid content ($P < 0.01$).

Keywords: goat, milk, amino acids, fatty acids.

TRENDS IN THE COURSE OF PARTURITION OF POLISH HOLSTEIN-FRIESIAN COWS

Dariusz Piwczyński, Beata Sitkowska, Magdalena Kolenda

Bydgoszcz University of Science and Technology, Faculty of Animal Breeding and Biology, Department of Animal Biotechnology and Genetics / 85-084 Bydgoszcz, Poland

Corresponding author:darekp@pbs.edu.pl

The ease of the course of labour contributes to low calf mortality, thus improving the economic performance of dairy farms. In Poland, the course of labour is expressed on a 6-point scale: CE1 – independent delivery (performed by natural forces), CE 2 – easy delivery (completed with little human interference), CE3 – difficult delivery (completed with the use of more force than normally), CE4 – complicated delivery (related to surgery, damage to a cow or calf), CE5 – miscarriage, and CE6 – caesarean section. In numerous studies, however, a simplified classification is used, i.e., easy (CE1 + CE2) and difficult (CE3 + CE4, CE6) [1]. The aim of the research was to analyse the trends in the delivery of Holstein-Friesian cows (from 2014 called Polish Holstein-Friesian) of the Black and White variety used in 9 highly productive herds. A total of 39,346 cows calving in 2008–2021 were included in the study. Cows were controlled in terms of the course of the delivery expressed in the bipartite scale (easy, difficult). Moreover, data on the milk yield of cows for full lactation, the body weight of calves after birth with division into pregnancy size (1–3), and perinatal mortality (the number of stillborn calves and those who died in the perinatal period) were recorded. The statistical analyses were performed separately for the group of heifers/primiparous (37.317 cows) and multiparous cows (28.779 cows). Development tendencies in terms of the controlled traits were established using regression analysis. Based on the research carried out in 2008–2021, it was shown that the share of easy births among heifers was 94.65%, and the perinatal mortality of their offspring was 5.29%. The corresponding rates for multiparous cows were 96.97% and 2.77%, respectively. It should be emphasised that the level of both these features in 2008–2021 was stable ($P > 0.05$). Moreover, a favourable trend was found in the scope of milk yield: in the group of primiparous cows, it increased annually by over 34 kg, and in multiparous cows by 82 kg. At the same time, the increase in the body weight of calves from single pregnancies born to heifers (by 0.11 kg/year) and multiparous cows (by 0.07 kg /year) was observed. It was found that in 2008–2021, the share of easy deliveries increased insignificantly, which was accompanied by a systematic increase in the lactation milk performance of heifers and multiparous cows.

Keywords: reproduction, delivery type, mortality, calf body weight.

References:

[1] Piwczyński D., Sitkowska B., Brzozowski M., Bogucki M., Wójcik P., Survival of Polish Holstein-Friesian cows to second, third and fourth lactation in conventional and automatic milking systems. Ann. Anim. Sci. 2021, 21(3), 1081-1101.

GENE AND HORMONE IMMUNOCASTRATION IN SMALL RUMINANTS

Özdal Gökdal

Aydın Adnan Menderes University, Çine Vocational School, Department of Plant and Animal Production, Aydın, Türkiye

Corresponding author: ogokdal@adu.edu.tr

Castration is carried out in order to stop the production of male hormones and spermatozoa production by removal of testicles or by in situ destruction of testicular function. Nevertheless, physical castration has negative effects on some characteristics such as feed efficiency, growth rate and carcass characteristics. Besides, its painful effects and different levels of stress in animals are questioned in terms of animal welfare. Immunization against reproductive hormones could be an alternative means of surgical castration methods for various practical and experimental reasons [1–5]. The hormone immunocastraton is based on inducing antibodies against gonadotropin releasing hormone (GnRH). Immunization against GnRH has been described as one of the effective means to reduce reproductive functions in farm animals [6]. Gene immunization is a novel immunization method that is performed by constructing a plasmid encoding an exogenous gene and injecting it into an animal [7]. In this review, gene and hormone immunocastration in sheep and goats were evaluated in terms of reproductive characteristics, growth, carcass, meat quality and feed efficiency.

Keywords: DNA, GnRH, castration, sheep, goat.

References:

[1] ***Ülker*** H., et al., The effect of immunization against LHRH on body growth and carcass characteristics in Karakaş ram lambs. Small Ruminant Research 2002, 45 (3), 273-278.
[2] ***Ülker*** H., et al., LHRH Fusion Protein Immunization Alters Testicular Development, Ultrasonographic and Histological Appearance of Ram Testis. Reproduction in Domestic Animals 2009, 44, 593-599.
[3] Cui, S., et al., Sexual development and the effects of active immunization against GnRH in Chinese Tanyang ram lambs. Anim. Reprod.Sci., 2003, 77, 129-139.
[4] Adams, E.T. Using gonadotropin-releasing hormone (GnRH) and GnRH analogs to modulate testis function and enhance the productivity of domestic animals. Anim.Reprod.Sci., 2005, 88, 127-139.
[5] Rault J.L., et al., Castration induced pain in pigs and other livestock. Appl.Anim.Beh.Sci., 2011, 135, 214-225.
[6] Gökdal, Ö., et al., The effects of immunological castration against GnRH with recombinant OL protein (Ovalbumin-LHRH-7) on carcass and meat quality characteristics, histological appearance of testes and pituitary gland in Kıvırcık male lambs. Meat science 2010, 86 (3), 692-698.
[7] Wassie T., et al., Immunization against Kisspeptin-54 perturb hypothalamic–pituitary–testicular signaling pathway in ram lambs. Theriogenology 2019, 125, 193-202.

EFFECT OF EARLY ADMINISTRATION OF AN ANTIBIOTIC OR FEEDING A DIET CONTAINING A COCCIDIOSTAT ON THE LEVELS OF MINERALS IN YOLK SAC AND BLOOD IN TURKEYS

Radosław Smagieł[1], Ewelina Cholewińska[1], Magdalena Krauze[1], Anna Stępniowska[1], Dariusz Mikulski[2], Katarzyna Ognik[1], Jan Jankowski[2]

[1]University of Life Sciences in Lublin, Department of Biochemistry and Toxicology, Akademicka 13, 20-950 Lublin,
[2]University of Warmia and Mazury in Olsztyn, Department of Poultry Science and Apiculture, Oczapowskiego 5, 10-719 Olsztyn, Poland

Corresponding author: radoslaw.smagiel@up.lublin.pl
magdalena.krauze@up.lublin.pl

The study verified the hypothesis that that early administration of the antibiotic or vaccinations may affect the levels of minerals in the yolk sac and blood plasma of turkeys. The experiment was conducted on female turkeys in a two-factor design (2 × 4), which included 2 groups of birds (vaccinated, unvaccinated) and 4 treatments (CON, MON, ENR, DOX). Half of the birds were vaccinated against aMPV and NDV on day 1 and against ORT on day 28. MON turkeys were given the coccidiostat monensin in the feed for 56 days. ENR and DOX turkeys received enrofloxacin or doxycycline for the first 5 days of life. Birds in the control group (CON) received no coccidiostat or antibiotics. On days 1, 3 and 5 of the birds' lives, yolk sacs were collected post mortem from 21 birds in the group (3 birds from the repetition). On days 1, 3, 5, 7 and 56, blood was collected from 21 birds in the group to assess Ca, P, Mg, Cu, Zn, and Fe levels. Two-way ANOVA showed antibiotic x vaccine interactions for levels of Ca, P, Mg, Zn, Cu and Fe ($P < 0.001$, respectively) in yolk sacs taken from both 3 and 5-day-old turkeys. The antibiotic x vaccine interaction was also found for Ca, Cu levels ($P < 0.001$ respectively) in blood plasma collected from 3, 5 and 7-day-old turkeys. For P levels ($P < 0.001$) in blood plasma, antibiotic x vaccine interactions were found in 3, 5 and 56-day-old turkeys. For Fe levels ($P < 0.001$) in blood plasma, antibiotic x vaccine interactions were found in 5, 7 and 56-day-old turkeys, while for Zn levels ($P < 0.001$) in blood plasma, antibiotic x vaccine interactions were found in 3, 7 and 56-day-old turkeys. The recorded interactions indicate that the tested levels of mineral indicators in yolk sacs and blood were affected by both the antibiotic and vaccine used. Only the application of MON resulted in a reduction of Ca levels ($P = 0.031$) in the blood plasma of 56-day-old turkeys. In contrast, vaccination of turkeys resulted in an increase in plasma P levels ($P = 0.025$) in 7-day-old turkeys, and Ca levels ($P < 0.001$) in 56-day-old turkeys. Short-term administration of the antibiotic has no significant effect on the levels of Ca, P, Mg, Cu, Zn, and Fe in the yolk sac and blood plasma of growing turkeys. On the other hand, long-term administration of MON may result in decreased Ca levels in the blood plasma.

Keywords: antibiotics, yolk sac, blood, minerals.

This work was supported by the National Science Centre in Poland, Grant No. 2020/39/B/NZ9/00765.

RELATIONSHIP BETWEEN B-CASEIN GENOTYPES (A1A1, A1A2, AND A2A2) AND COAGULATION PROPERTIES OF MILK AND THE FATTY ACID COMPOSITION AND SENSORY CHARACTERISTICS OF DAIRY PRODUCTS (SOFT CHEESE, SOUR CREAM, AND BUTTER)

Kristina de Vitte[1], Sigita Kerzienė[2], Jolita Klementavičiūtė[3], Marius de Vitte[4], Vaida Dilbiene[1], Rolandas Stankevičius[1]

[1]Department of Animal Nutrition, Lithuanian University of Health Sciences, Faculty of Animal Husbandry Technology. Tilžės str. 18, LT-47181, Kaunas, Lithuania,
[2]Department of Animal Breeding, Lithuanian University of Health Sciences, Faculty of Animal Husbandry Technology. Tilžės str. 18, LT-47181, Kaunas, Lithuania,
[3]Institute of Animal Rearing Technologies, Faculty of Animal Sciences, Lithuanian University of Health Sciences, Mickevičiaus str. 9, LT-44307 Kaunas, Lithuania,
[4]Coventry University, Priory St, Coventry CV15FB, England, United Kingdom

Corresponding author: kristina.de.vitte@lsmu.lt

Milk products are a source of lipids, carbohydrates, proteins, minerals, and vitamins. Although milk is a staple in the diets of many cultures, the demands for specific milk products differ between consumers (some are allergic to cow's milk proteins or incapable of digesting lactose). However, such consumers are not without options, and multiple studies suggest that milk containing only A2 beta-casein may be an alternative. [1] concluded that people with lactose intolerance reported less abdominal pain while consuming milk containing only A2 beta-casein. Furthermore, a review by [2] concluded that A2 beta-casein milk is a potential remedy for people with a milk protein allergy. We hypothesized that because the *CSN2* genotype (A1A1, A1A2, and A2A2) influences milk's biochemical composition, it should also influence the final product. The study aimed to investigate the viability of breeding crossbred dual-purpose Simmental cows exclusively towards the A2 variant beta-casein by investigating the influence of the *CSN2* genotypes, with a focus on the A2A2 genotypes' influence on the coagulation traits, fatty acid composition, and sensory characteristics of different milk products (soft cheese, sour cream, and butter). We assigned 15 crossbred cows (1⁄2 Swedish red X 1⁄4 Simmental X 1⁄2 Holstein Friesian) into three groups (A1A1, A1A2, and A2A2) of five cows per group. Milk samples for milk products (soft cheese, sour cream, and butter) production were taken following the ISO 707:2008 protocol [3]. Milk coagulation properties were determined by exposing milk samples to rennet enzyme solution. Rennet coagulation time and curd-firming rate were fastest in A2A2 milk and slowest in A1A1 milk. Accordingly, curd firmness was highest in A2A2 milk and lowest in A1A1 milk. A1A1 milk products had higher levels of monounsaturated fatty acids and lower levels of saturated fatty acids. Polyunsaturated fatty acids were mainly found in A2A2 milk products and were lowest in A1A2 products. Although *CSN2* genotypes influenced milk coagulation traits and fatty acid content of milk products, they did not influence the latter's sensory traits.

Keywords: cow milk, MUFA, PUFA; SFA, *β*-casein, CSN2.

References:

[1] Ramakrishnan, M., Eaton, T. K., Sermet, O. M., & Savaiano, D. A. (2020). Milk containing A2 β-casein only, as a single meal, causes fewer symptoms of lactose intolerance than milk containing A1 and A2 β-caseins in subjects with lactose maldigestion and intolerance: A randomized, double-blind, crossover trial. Nutrients, 12 (12), 3855. [2] Park, Y.
[2] W., & Haenlein, G. F. (2021). A2 bovine milk and caprine milk as a means of remedy for milk protein allergy. *Dairy*, *2*(2), 191-201.
[3] ISO 707/IDF 50C:2008. 2008. Milk and milk products — Guidance on sampling.

BOVINE COLOSTRUM EXERTS IMMUNOMODULATORY ACTIVITY ON INNATE IMMUNE SYSTEM AND INTESTINAL EPITHELIUM BY PASSIVELY TRANSFERRED PROTEINS

Ramunė Grigalevičiūtė[1,3], Rita Plančiūnienė[2], Vilma Zigmantaitė[1], Audrius Kučinskas[1], Paulius Matusevičius[3], Povilas Kavaliauskas[1,4,5,6]

[1]Biological research center, Lithuanian University of Health Sciences, Kaunas, Lithuania,
[2]Institute of Microbiology and Virology, Lithuanian University of Health Sciences, Kaunas, Lithuania,
[3]Department of Animal Nutrition, Lithuanian University of Health Sciences, Kaunas, Lithuania,
[4]Joan Sanford I. Weill Cornell Medical Colege, New York, United States,
[5]Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, United States,
[6]Institute of Infectious Diseases and Pathogenic Microbiology, Prienai, Lithuania.

Corresponding author: ramune.grigaleviciute@lsmu.lt

Bovine colostrum (BC) is the first milk produced by lactating cows after parturition. BC is rich in numerous bioactive components that ensure the nutrition of a neonate calf, confers passive protection against pathogens and promotes the gut colonization with beneficial microbiota. Despite the numerous efforts to understand BC effects on the development of neonates, very little is known about the complex protein composition of BC. In order to better understand the BC proteome and changes at different time points, BC was collected at 1–4 hours postpartum. BC samples collected from animals (n = 10) at each time point were pooled together. The BC serum was produced by gradient centrifugation and total proteins were digested into peptides using trypsin, alkylated and subjected to mass spectrometry. Proteins were identified using the reference genome of Bos taurus. In total, 553 proteins were successfully identified in BC samples collected over 4 hours postpartum. Identified proteins represented coagulation factors (factor V-XII), growth factors (IGF, EGF, GMS), immune markers (CD177, CD44, CD5, CD81), enzymes (MPO, ENO1) and others. Carboxipepridase 2 (CPN2) was only detected 4 hours BC and was absent in the 1–3 hour samples. Moreover, collagen alpha-1 was only detected at 1 hour and was absent in 2–4 hour BC samples. Finally, BC was highly enriched by numerous innate immune response proteins, such as antimicrobial peptides (Cathelicidin 1-9, complement), lipocalin, TNF receptor superfamily proteins and others. Bovine colostrum secreted during the first four hours postpartum is rich in numerous nutritious and immunomodulatory proteins. The high amounts of antimicrobial peptides, immunoglobulins and protective factors confer passive protection for calves. Further studies are needed to better understand these proteins role during calves growth and development.

Keywords: bovine colostrum, intestinal homeostasis, immunomodulation.

References:

[1] H. M. Hammon, W. Liermann, D. Frieten, C. Koch. Review: Importance of colostrum supply and milk feeding intensity on gastrointestinal and systemic development in calves. Animal, Volume 14, Supplement 1, 2020, 133-143.
[2] A. J. Fischer, C. Villot, J. K. van Niekerk, T. T. Yohe, D. L. Renaud, and M. A. Steele. Invited Review: Nutritional regulation of gut function in dairy calves: From colostrum to weaning. Animal Science, 35(5): 498–510. Published online 2019 Oct 9. doi: 10.15232/aas.2019-01887.

INFLUENCE OF LACTIC ACID BACTERIA AND ROSEMARY ESSENTIAL OIL ON ILE DE FRANCE LAMB BREED MEAT (*MUSCULUS GLUTEUS*) QUALITY PARAMETERS

Vilija Buckiuniene, Dovile Klupsaite, Paulina Zavistanaviciute, Vytaute Starkute, Vita Lele, Jolita Klementaviciute, Elena Bartkiene

Institute of Animals Rearing Technology, Faculty of Animal Science, Lithuanian University of Health Sciences, Kaunas, Lithuania

Corresponding author: Vilija.Buckiuniene@lsmu.lt

The aim of this study was to evaluate *Lactobacillus paracasei* (LUHS244), rosemary essential oil (0.1% v/v) and their combination to treat Ile de France lamb meat. Changes in the microbiological profile and physiochemical parameters of meat were evaluated after 24 h of treatment at 4°C. The experiment resulted in significantly lower mold/yeast ($P < 0.05$), water-holding capacity (by 49–63%), and coking loss (by 9.05–11.31%) in all trial groups. Moisture content in all groups had a tendency to decrease from 7.09% to 8.49%. Polyunsaturated fatty acid content increased in all experimental groups. The content of malondialdehydes increased in all experimental groups from 4.7 to 10.1 times ($P < 0.05$) compared to the control group. For the cholesterol content in lamb meat, the highest effect was determined where meat was marinated only with rosemary essential oil (it decreased by 22% ($P < 0.05$)). Treatments significantly affected some biogenic amines. Sensory analysis showed that raw lamb meat colour was more acceptable after all treatments, while the odour acceptability was ranked highest after combined treatment. In concludsion, this treatment showed that it could be used to improve microbiological safety and some quality characteristics, increasing PUFA content of lamb meat.

Keywords: lamb meat quality, lactic acid, essential oil.

LAYING HENS' AGE INFLUENCE ON EGG QUALITY AND CONSUMER ACCEPTABILITY

Šarūnė Aranauskaitė, Asta Racevičiūtė-Stupelienė, Monika Nutautaitė, Vilma Vilienė

Institute of Animal Rearing Technologies, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181 Kaunas, Lithuania

Corresponding author: Sarune.Aranauskaite@lsmu.lt

From a nutritional perspective, eggs are particularly important since they include essential lipids, proteins, vitamins, minerals, and trace elements. They also provide a moderate calorie source (about 140 kcal/100 g), have a wide range of culinary applications, and are affordable as they are the second-cheapest animal source of zinc and calcium and the lowest-cost source of proteins, vitamin A, iron, vitamin B12, riboflavin, and choline [1]. However, egg producers should be aware of the age at which laying hens produce eggs of the finest quality. Therefore, the aim of this study was to compare the nutritional value of eggs produced by laying hens of different age groups. Research was performed on eggs produced by these laying hen age groups: 1-year-old (LH1), 2-year-old (LH2), and 3-year-old (LH3). Laying hens were raised under the same feeding and housing conditions. The following indicators were determined in fresh and stored (for 28 days) eggs: height of egg, yolk, and albumen; Haugh unit; yolk colour intensity; eggshell thickness; yolk and albumen pH; dry matter (DM), fat, ashes, and protein contents in the yolk; the sensory profile of fresh eggs. When compared to LH1 fresh eggs, the LH2 fresh eggs were found to be 16% higher and to have substantially redder (a*) egg yolks ($P < 0.05$). The weight of the egg yolk was found to be highest in LH3 and lowest in LH1 ($P < 0.05$). Comparing LH2 to LH1, the ratio of egg yolk to egg mass was 42% higher ($P < 0.05$). LH1 had the lowest egg albumen index, while LH3 had the highest. Compared to LH3 eggs, the albumen index in LH1 eggs was even 49% lower ($P < 0.05$). However, as compared to LH2 eggs, the egg yolk index for LH1 eggs had the greatest value (26%) ($P < 0.05$). Meanwhile, fresh egg albumen's height was 31% higher in LH2 than in LH3 ($P < 0.05$). When comparing distinct age groups of laying hens, no significant variations in the chemical composition of the eggs were discovered. After storage, egg weight in LH2 was 24% higher than it was in LH1 ($P < 0.05$). The egg yolk index after storage was 9% higher in LH1 than in LH3, and the egg mass to yolk ratio was 37% greater in LH2 than in LH1 ($P < 0.05$). When analysing eggshell strength, it was found to be 19% higher in LH1, compared to the eggs of LH3 ($P < 0.05$). After evaluating the sensory profile and consumer acceptability of the egg albumen, it was found that the albumen in LH1 was 10% harder, compared to LH2 ($P < 0.05$); the general flavour intensity of the yolk was most noticeable in the eggs of LH3, and the least in LH1 ($P < 0.05$). However, LH2 yolk residual flavour intensity was 13% lower than in LH3 ($P < 0.05$). According to the recent study's findings, in order to produce fresh, high-quality eggs that consumers would be satisfied with, it is appropriate to regularly replace the flock of farmed laying hens with younger ones.

Keywords: laying hens, influence of age, egg quality, comparative analysis.

References:

[1] Drewnowsk A. The Nutrient Rich Foods Index helps to identify healthy, affordable foods. The American Journal of Clinical Nutrition 2010, 91, 1095s-1101s.

REDOX STATUS OF RATS FED A DIET CONTAINING VARIOUS SOURCES OF DIETARY FIBER AND COPPER NANOPARTICLES

Aleksandra Marzec[1], Radosław Smagieł[1], Ewelina Cholewińska, Magdalena Krauze, Bartosz Fotschki[2], Jerzy Juśkiewicz[2], Paulius Matusevicius[3], Katarzyna Ognik[1]

[1]Department of Biochemistry and Toxicology, Faculty of Animal Science and Bioeconomy, University of Life Sciences in Lublin, Akademicka 13, 20–950 Lublin, Poland,
[2]Division of Food Science, Institute of Animal Reproduction and Food Research of the Polish Academy of Sciences, Tuwima 10, 10-748 Olsztyn, Poland,
[3]Lithuanian University of Health Sciences, Tilžės str. 18, 47181 Kaunas, Lithuania

Corresponding author: Paulius.Matusevicius@lsmu.lt

The aim of this study is to verify the statement that the metabolic effect of copper nanoparticles (Cu-NPs) is strongly reliant on the physiological function of different dietary fibre types. We hypothesized that a dietary combination of Cu-NPs with either a control inert (cellulose) or a prebiotic (inulin) or a viscous (pectin) or a bulking (psyllium) fibre would stimulate immune response of rats. Rats were fed for 6 weeks a standard diet with dietary addition of two Cu-NP dosages (recommended and two times higher; 6.5 and 13 mg/kg diet) and combined with different types of dietary fibre; 6% of a diet (control – cellulose, inulin with a prebiotic effect, psyllium with a bulk effect, pectin with a viscous effect; 10 groups, n = 10 per group). Blood was collected from 10 rats from the group for the determination of immunological parameters. Nanoparticles increase the stimulation of the immune system, but determining whether this effect should be considered beneficial requires a wider range of research. Plasma levels of superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), glutathione (GSH and GSSG) were determined in the blood plasma of rats by Elisa assays. It was found that compared to a diet containing cellulose, replacing it with pectin or inulin or psyllium resulted in lower SOD levels ($P < 0.001$). A fiber x Cu-NP dose interaction was noted for MDA, GSSG ($P < 0.001$ both). The interaction for MDA was due to the fact that in diets containing cellulose and pectin, increasing Cu from 6.5 mg/kg to 13 mg/kg in the diet had no effect on MDA levels, while in diets containing inulin resulted in an increase in MDA levels, and in diets containing psyllium in a decrease of this parameter. The interaction for GSSG was due to the fact that in diets containing cellulose or pectin or psyllium, increasing the Cu dose from 6.5 mg/kg to 13 mg/kg had no effect on the level of oxidized glutathione, while in diets containing inulin, increasing the Cu dose resulted in a decrease in the level of this parameter. It was established that it is beneficial to use fiber in the diet of rats in the form of inulin, especially with an increased dose of copper nanoparticles (13 mg/kg), as lipid oxidation can be reduced in this way.

This work was supported by the National Science Centre, Grant No. 2021/41/B/NZ9/01104.

DIVERSE DIFFERENTIATION PATTERNS BETWEEN RABBIT PREIMPLANTATION EMBRYOS DEVELOPED IN VITRO VERSUS IN VIVO

Anna Chołoniewska[1,2], Jan Chołoniewski[3], Elżbieta Wenta-Muchalska[1], Katarzyna Filimonow[1], Anna Piliszek[1]

[1]Department of Experimental Embryology, Institute of Genetics and Animal Breeding of the Polish Academy of Sciences, Jastrzębiec, Poland,
[2] Department of Neurogenetics and Functional Genomics, Mossakowski Medical Research Institute of the Polish Academy of Sciences, Warsaw, Poland,
[3]Faculty of Physics, Center of Excellence for Complex Systems Research, University of Technology, Warsaw, Poland

Corresponding author: a.piliszek@igbzpan.pl

Proper differentiation and segregation of the first three cell lineages in the mammalian embryo – epiblast (EPI), primitive endoderm (PrE) and trophectoderm (TE) – play a crucial role in embryo development. The knowledge about specification processes and timing is mostly based on the mouse model studies. Here, we focus on non-rodent model: rabbit. Previously, we analyzed specification of rabbit EPI, PrE and TE at different time points in *in vivo* preimplantation rabbit embryos. Here we compare the timing and distribution of these lineages in *in vitro* cultured embryos with embryos obtained *in vivo*, to determine how culture conditions affect the differentiation potential of a rabbit embryo. Popielno breed rabbit zygotes (19 hours *post coitum*, hpc) were cultured *in vitro* in commercially available sequential culture media: 1) G-1™ PLUS/G-2 PLUS™ (Vitrolife); 2) ORIGIO® Cleav/ORIGIO® Blast (CooperSurgical); and single step media, 3) Global®Total®, and 4) SAGE 1-step™ (CooperSurgical). Embryos cultured in single step RDH medium served as *in vitro* culture control. After culture, 120 hpc embryos (5 dpc) were fixed and immunostained. Cell number and specific lineage differentiation were assessed based on localization of lineage-specific transcription factors: CDX2 for TE, SOX17 for PrE and SOX2 for EPI. Cell lineage distribution in *in vitro* cultured embryos was compared to *in vivo* embryos from stage 3 dpc to 5 dpc. In all analyzed culture media, more than 80% of the rabbit zygotes reached the blastocyst stage. Nonetheless, the pattern of differentiation significantly varied between the analyzed media. Moreover, *in vitro* cultured rabbit embryos had significantly fewer cells and fewer differentiated cells compared to *in vivo* ones. Our results show that 5 dpc *in vitro* cultured embryos are developmentally delayed. After culture in Global medium, the embryos show a similar distribution of trophectoderm and primitive endoderm lineages as 3.25 dpc *in vivo* derived embryos. Culture in ORIGIO or RDH medium results in the expression pattern more typical for 3.75 dpc *in vivo* embryos. All the presented results were statistically significant.

Keywords: rabbit, preimplantation embryo, differentiation, *in vitro* culture.

DOES CORRELATION BETWEEN LENGTH OF INFECTION AND ACUTE PHASE PROTEINS GENE EXPRESSIONS IN SOMATIC CELLS FROM MILK OF SRLV-INFECTED DAIRY GOATS EXIST?

Daria Urbańska[1], Justyna Jarczak[2], Michał Czopowicz[3], Jarosław Kaba[3], Emilia Bagnicka[1]

[1]Institute of Genetics and Animal Biotechnology PAS, Postepu 36A, Jastrzębiec, Poland,
[2]Nencki Institute of Experimental Biology, PAS, Ludwika Pasteura 3, Warszawa,
[3]Warsaw University of Life Sciences, Institute of Veterinary Medicine, Nowoursynowska 159 C, Warsaw, Poland

Corresponding author: Daria.m.urbanska@gmail.com

Caprine arthritis-encephalitis (CAE) is a result of a small ruminant lentivirus (SRLV) infection. One of the CAE symptoms can be mastitis. Thus, CAE is an economic problem [1]. Acute-phase proteins (APPs) participate in immune defense during various infections. Their activation is very important to the reinstatement of homeostasis. In goats, seven proteins belong to APPs: serum amyloid (*SAA*), haptoglobin (*Hp*), ceruloplasmin (*Cp*), C-reactive protein (*CRP*), alpha-1 acid glycoprotein (*AGP*), fibrinogen (*Fb*), and α-lactalbumin (*LALBA*) [2, 3, 4]. The goal of the study was to estimate the correlation between the length of infection and expressions of APPs genes in milk somatic cells (MSC) of SRLV-seropositive goats as well as correlation with milk traits being indicators of mastitis such as MSC count (MSCC), total protein and lactose contents. The study was conducted on 12 dairy goats (n = 6 Polish White Improved, n = 6 Polish Fawn Improved) being between 2nd and ⩾ 4th parity, naturally infected with SRLV and confirmed at least twice using ELISA test, but without clinical CAE symptoms. The milkwas collected five times during lactation (day 1, 30, 60, 120, 180 day). The gene expression was measured using the qPCR method with *cyclophilin A* as a reference. Pearson correlation with PROC CORR of SAS package was used to estimate the associations. The duration of the SRLV infection was not correlated with expressions of any studied APPs gene. However, the longer the infection lasts, the higher the MSCC (0.47, $P < 0.0001$) and total protein (0.39, $P = 0.0006$) were, but not lactose contents. Moreover, correlations between *AGP* and *Cp* (0.41, $P = 0.0019$), *AGP* and *CRP* (0.66, $P < 0.0001$), *AGP* and *Hp* (0.63, $P < 0.0001$), *Cp* and *CRP* (0.36, $P = 0.0048$), *Cp* and *Hp* (0.41, $P = 0.0010$), *Cp* and *LALBA* (–0.57, $P < 0.0001$), and *CRP* and *Hp* (0.65, $P < 0.0001$) gene expressions were found. No correlation between the length of infection and APPs gene expressions may suggest that the length of infection in asymptomatic goats does not trigger an acute immune response in the udder. However, increased MSCC and total protein content in parallel to infection may, however, indicate inflammation but probably subclinical and mild. The relationship between expressions of some APPs gene probably indicated synergism or antagonism (Cp and LALBA) in their activity.

Keywords: SRLV, goat, milk somatic cell, acute phase proteins genes, correlations.

INFLUENCE OF PUERPERAL METRITIS ON THE RECOVERY OF THE ESTROUS CYCLE AFTER CALVING IN MODERN DAIRY COWS

Vilmantas Juodžentis[1,2], Giedrius Palubinskas[2], Jūratė Šiugždaitė[3], Vytuolis Žilaitis[1], Henrikas Žilinskas[1]

[1]Large Animals Clinic, Faculty of Veterinary, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, Kaunas, Lithuania,
[2]Animal Breeding Department, Faculty of Animal Sciences, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181, Kaunas, Lithuania,
[3]Department of Veterinary Pathobiology, Faculty of Veterinary, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181, Kaunas, Lithuania

Corresponding author: vilmantas.juodzentis@lsmu.lt

The aimwas to determine how puerperal metritis influences the recovery of estrous cycle in modern dairy cows. The study was carried out on lactating Holstein cows from a commercial dairy farm located in Lithuania. The cows were housed in freestall barns with access to fresh water ad libitum and were fed a total mixed ration supplemented with the concentrate based on milk yield. The cows were selected between day 5 to 14 after calving (day 0 = day of calving). The cows were divided into two different groups: multiparous cows after puerperal metritis treatment (M (n = 34)) and multiparous cows without signs of puerperal metritis (H (n = 38)). All 72 cows were divided into groups after their first ovulation: HSO (n = 29) – cows without signs of puerperal metritis and with a single ovulation; MSO (n = 21) – cows after puerperal metritis treatment with a single ovulation; HDO (n = 9) – cows without signs of puerperal metritis and with a double ovulation; and MDO (n = 13) – cows after puerperal metritis treatment with a double ovulation. The changes in ovaries were examined using a digital diagnostic ultrasound scanner (Dramiński iScan, Dramiński S.A., Olsztyn, Poland) at a frequency of 7.5 MHz, using a linear rectal transducer. The first dominance of the follicle postpartum was recorded when at least one of the follicles reached 8.5 mm in diameter. To detect follicle ovulation, the cows were monitored by ultrasound machine three times a week (Monday, Wednesday, Friday). Ultrasonography was started on day 5 postpartum and was continued until the follicle ovulation was diagnosed. The statistical analysis was performed using computer software (SPSS Inc., Chicago, IL, USA) SPSS 22.0. A probability below 0.05 was considered reliable.

The mean time to the first follicle deviation postpartum was longer in the MSO group compared with the HSO group, 8.9 ± 1.6 and 6.8 ± 1.8 days postpartum, (P = 0.002). The same tendency was observed in the MDO and HDO groups, 9.5 ± 1.3 and 7.0 ± 1.4 days postpartum, (P = 0.002). We found that in the HDO group the first dominant wave follicle's ovulation was more frequent when in the MDO group, 55.6% and 23.1%, (P = 0.027). Moreover, the HDO group cows ovulated their follicle during the first follicular wave faster compared to the MDO group (11.4 ± 2.7 day and 20 ± 1 day, (P = 0.01). Also, cows of the HDO group had a smaller diameter of the ovulatory follicles compared to the cows of the MDO group (15.3 ± 1.9 mm and 17.3 ± 1.7 mm, P = 0.04).

In conclusion, dairy cows which have had puerperal metritis need more time until the first follicle deviation postpartum. Also, healthy cows have a higher frequency for double ovulation in the first dominant wave postpartum.

Keywords: metritis, postpartum, ovulation, follicle.

RELATIONSHIP BETWEEN LACTATION NUMBER, MILK YIELD AND COMPOSITION IN DAIRY COWS

Jūratė Rudejevienė[1*], Ramutė Mišeikienė[2]

[1]Dr. L.Kriauceliunas Small Animal Clinic, Veterinary Academy, Faculty of Veterinary Medicine, Lithuanian University of Health Sciences, Tilžės str. 18, Kaunas, Lithuania,
[2]Institute of Animal Rearing Technologies, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, Kaunas, Lithuania

Corresponding author: jurate.rudejeviene@lsmu.lt

Milk production increases 30% from the first to fifth lactation, but the percentage increase progressively decreases with age. The milk yield are increased with an increasing lactation number, which may be due to increasing development and size of the udder with a resulting increase of secretory cells. Other reasons for high milk yield may be increased parity, which plays a significant role in the control of the tissue mobilization between the primiparous and multiparous cows, and includes increasing body weight of dairy cattle over that of first lactation. The lower milk yield in first lactation cows, because their mammary gland and mammary vein not well developed [1]. Milk composition also changes over lactation [2]. The aim of this work was to investigate the relationship between lactation with milk yield and composition in dairy cows. The study was carried out on 80 Holstein lactating dairy cows, in accordance with the Law on the Care, Keeping and Use of Animals of the Republic of Lithuania. Milk production was determined by control milking. Milk composition (milk proteins, milk fat, lactose concentration and somatic cell count) was determined in the laboratory at Joint Stock Company "Pieno tyrimai". According to the lactation number, cows were divided into 5 groups: 1st lactation (n = 15), 2nd (n = 17), 3rd (n = 14), 4th (n = 18) and 5th (n = 16). The statistical analysis was performed using computer software SPSS 22. Data was statistically significant when $P<0.05$. During the study, results showed that the fattest milk was in 4th lactation cows, which was 4.49 ± 0.05%. The leanest milk was in the milk of 1st lactation cows, 4.31 ± 0.05% ($P< 0.05$). Milk whiteness differed slightly in all lactation cows. The highest milk protein content was found in lactating milk of 2nd and 5th lactation cows(3.28 ± 0.03%). The highest amount of lactose in milk was found in the milk of 1st and 4th lactation cows (4.73 ± 0.02%). The highest amount of milk was milked from 4th lactation cows (31.77 ± 1.19 kg), and the smallest in 5th lactation cows (28.21 ± 2.03 kg, $P > 0.05$). Examination of the influence of lactation on the number of somatic cells demonstrated that the SLS in the milk of 1st lactation cows was the lowest in comparison with other lactations of cows and amounted to 232.40 thousand / mL. ± 44.33 thousand / mL, which is 11% less than in the milk of 2nd lactation cows. The milk of 5th lactation cows had the highest number of somatic cells in comparison with other lactations and amounted to 328.38 thousand / mL ± 53.83 thousand / mL ($P < 0.05$).

Keywords: milk yield, composition, lactation, cows.

References:

[1] Vijayakumar M., et al. The effect of lactation number, stage, length, and milking frequency on milk yield in Korean Holstein dairy cows using automatic milking system. Asian-Australas J Anim Sci. 2017, 30,1093-1098.
[2] Ariza J.M.,et al. J. Scientific reports. 2019, 9, 1-7.

IDENTIFICATION AND PREVALENCE OF MILK PROTEIN B-CASEIN A1 AND A2 GENETIC VARIANTS IN THE DAIRY CATTLE POPULATION

Kristina Morkūnienė, Renata Bižienė, Simona Birutė Ašmonaitė, Nijolė Pečiulaitienė, Ramutė Mišeikienė, Laimutis Kučinskas

Institute of Biology Systems and Genetics, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181 Kaunas, Lithuania.

Corresponding author:kristina.morkuniene@lsmu.lt

The aim of this work was to identify prevalence of milk protein β-casein A1 and A2 variants in dairy cattle population. β-casein is encoded by exon 7 of the CSN2 gene. The protein encoded by the gene consists of 209 amino acids (molecular weight ~24 kDa) and has a high degree of polymorphism [1, 2]. Mutation of proline (CCT) at position 67 to histidine (CAT) in the original A2 genetic variant of the *Bos genus* 5–10 thousand years ago gave rise to the A1 variant [3]. The structural change between the A1 and A2 variants resulted in the two β-casein variants being hydrolyzed differently in the human gastrointestinal tract. The peptide bond between proline and isoleucine in the A2 variant is more resistant to enzymes than the bond between histidine and isoleucine in the A1 variant [2]. This means that milk containing variant A1 is much more easily hydrolyzed by enzymes in the gastrointestinal tract and secretes higher levels of opioid protein β-casomorphin-7 (BCM-7), which can adversely affect human health [4, 5]. During this work, in total, 208 samples were tested from eight dairy cattle breeds: Danish Black-and-White (22), Danish Red (18), Holstein (32), Lithuanian White Back (34), Lithuanian Black-and-White (31), Lithuanian Grey (32), Lithuanian Red (28), and Swedish Black-and-White (11). Genetic testing was done using the allele specific oligonucleotide primer PCR (ASO-PCR) method. All three possible genotypes of β-casein A1 and A2 variants – A1A1, A1A2 and A2A2 – were detected in the studied breeds, except for the Danish Red and the Lithuanian Black and White. Comparing all eight studied breeds, the highest frequency of the A2 allele was in the Danish Red and the lowest in the Lithuanian Grey. The highest frequency of the heterozygous A1A2 genotype was found in the Lithuanian Grey, and the lowest in the Danish Red. Due to the high frequencies of the A1 allele, homozygous A1A1 and heterozygous A1A2 genotypes, the consumption of milk from Lithuanian Grey cattle may increase or promote the risk of the development of diseases due to the release of BCM-7 during digestion. Finally, it can by stated, that Lithuanian Black and White cattle mostly give milk containing A2 variant, which after hydrolysis produces four times less opioid-like protein β-casomorphin-7 (BCM-7) and thus is safer for human consumption.

Keywords: β-casein, dairy cattle, CSN2 gene, ASO-PCR.

References:

[1] Kamiński S, et al., Polymorphism of bovine beta-casein and its potential effect on human health. J Appl Genet. 2007, 48(3), 189-98.
[2] Nguyen DD, et al., Formation and Degradation of Beta-casomorphins in Dairy Processing. Crit Rev Food Sci. 2015, 55(14), 1955-67.
[3] Duarte-Vázquez MA, et al., Production of Cow's Milk Free from Beta-Casein A1 and Its Application in the Manufacturing of Specialized Foods for Early Infant Nutrition. Foods. 2017, 6(7), 50-64.
[4] ul Haq MR, et al., Impact of Milk Derived β-Casomorphins on Physiological Functions and Trends in Research: A Review. Int J Food Prop. 2014, 17(8), 1726-41.
[5] Caroli AM, et al., Detecting β-Casein Variation in Bovine Milk. Molecules. 2016,21(2), 141-7.

EFFECTS OF AGE AND GENDER OF HORSE AND RIDER IN HIGH LEVEL SHOW JUMPING COMPETITION

Katarzyna Becker[1], Dorota Lewczuk[2]

[1]Bydgoszcz University of Science and Technology (Politechnika Bydgoska), Mazowiecka 28, 85-084 Bydgoszcz, Poland,
[2]Department of Biotechnology and Nutrigenomics, PAS, Jastrzębiec, ul. Postępu 36A, 05-552 Magdalenka, Poland

Corresponding author: d.lewczuk@igbzpan.pl

In many countries, women are constituted a large majority of riders, especially in recreation riding or low-class competition. In higher classes, the gender structure is quite different [1]. The financial success is also lower in higher classes in horses ridden by women. On the other side, the horse's age and gender are significant effects on show jumping results [2]. According to the literature, the relationships between horses and people depend on gender [3]. The stated hypothesis is that the results in high-class show jumping will be comparable between people's gender after corrections for additional influences like the number of starts or horse age and gender. The data were collected on the final jump-off during the Polish Show Jumping Championships during the years 2016–2020. Because of the limited amount of riders in the jump-off, the number of observations was 50. The observations of 29 riders and 40 horses were divided according to gender and age. The analyzed observations belong to stallions (30%), mares (24%), geldings (46%), men (92%) and women (8%). The following age groups were created: people below 30 years (36%), 30–39 years (46%), 40 years and above (18%); horses below 10 years (28%), 10–12 (38%), and above 12 (34%). Preliminary analysis showed that almost all subclasses had observations. Analysis of the results overall showed that, among general points, place and time, the general point trait has to be transformed by the SQR function (root extraction) to be normally distributed. The statistical analysis of variance was conducted using the MIXED procedure from the SAS program with the model including a random rider-horse pair effect and fixed effects of rider and horse age and gender effects, as well as the year of competition. Horse age and gender did not affect competition results. The rider's age was statistically significant for location ($P = 0.003$) and points ($P = 0.04$), but not the time of jump-off. The placing is better with the rider's age, and the amount of points is lower for the oldest group of riders (however, differs only with the youngest age group). The rider's gender was statistically significant for placing in competition ($P = 0.01$) probably because of some differences in timing as differences in points were not noted. The hypothesis on equal results between the results of people of different genders is not supported. However, it is worth noting that 75% of women's records were noted for the oldest horses. This percentage was much lower for men (30%). Such characteristics may be gender-specific. Because of the limited amount of data, the study should be continued with a larger amount of data.

Keywords: horse, show jumping, age, gender.

References:

[1] Coulter K., et al. Horse Power: Gender, Work, and Wealth in Canadian Show Jumping. In Gender and equestrian sport 2013, pp.165-181.
[2] Whitaker T.C., et al., The influence of horse gender on eventing competition performance. Comprative Exercise Physiology 2008, 5,67-71.
[3] Birke l., Brandt K. Mutual corporeality: Gender and human/horse relationships. Women's Studies International Forum 2009, 32, 3, 189-197.

AQUAPORINS EXPRESSION AND SPERM METABOLOME FOR THE EVALUATION OF BULL SEMEN QUALITY

Katarzyna Michałek[1], Elena Bartkiene[2], Patrycja Oberska[1], Maciej Murawski[3], Dariusz Gączarzewicz[4], Marta Marynowska[1], Andrzej Syczewski[5]

[1]Department of Physiology, Cytobiology and Proteomics, West Pomeranian University of Technology in Szczecin, Poland,
[2]Department of Food Safety and Quality, Lithuanian University of Health Sciences, Kaunas, Lithuanian,
[3]Department of Animal Nutrition, Biotechnology and Fisheries, University of Agriculture in Krakow, Poland,
[4]Department of Animal Reproduction, Biotechnology and Environmental Hygiene, West Pomeranian University of Technology in Szczecin, Poland,
[5]Genetic and Animal Husbandry, Szczecin, Poland

Corresponding author: kmichałek@zut.edu.pl.

It is well known that standard semen analysis is a basic test that is used to determine male fertility in farm animals, including cattle. However, sensitivity of this test does not meet challenges associated with the modern animal reproduction, and it leads to economic loss, especially, when cryopreserved sperm is used [1]. In the view of the above, new fertility-related biomarkers are urgently needed for diagnosis and prognosis of male fertility potential [2]. In our project, we hypothesize that aquaporins (AQPs) and related *in situ* metabolic processes can be integrated as sensitive markers for bull fertility and production of high quality semen. The present study includes a total of 20 cryopreserved ejaculates, each coming from a separate healthy and sexually mature Polish Holstein-Friesian, black and white bull. The ejaculates will be classified into two groups: high-quality semen (n = 10) and low-quality semen (n = 10), based on sperm motility, viability, morphology, DNA defragmentation and sperm apoptosis. Subsequently, semen samples will be processed (i) for analysis of the location and expression of AQPs, and (ii) for analysis of the selected physicochemical markers (free amino acid and fatty acid profile, biogenic amines, cholesterol and malondialdehyde concentration). Research carried out to date has confirmed the presence of AQP3 and AQP7 in bovine sperm. Both proteins belong to the subfamily of AQPs called aquaglyceroporins and are probably involved in the transport of glycerol into sperm cells for energy production, elimination of reactive oxygen species and regulation of sperm volume.

Keywords: water channel, metabolomics, male reproduction, sperm, biomarker.

References:

[1] Oberska P., Michałek K. Aquaporins: New markers for male (in)fertility in livestock and poultry?. Animal Reproduction Science 2021, 231, 106807.
[2] Michałek K., et al. In search of new potential markers for male fertility and semen quality control. aquaporins in reproductive system and metabolomic profiling of semen. Journal of Pharmacology and Physiology 2021, 72, 3, 309-319.

This work is financed by OPUS 22 research grant no. 2021/43/B/NZ9/00204, obtained from the National Science Center in Poland.

IMPROVED CHROMATOGRAPHIC METHOD FOR QUANTIFYING FATTY ACIDS IN PIG ERYTHROCYTES AS A BIOMARKER OF DIETARY FAT SUPPLEMENTATION

Marian Czauderna[1], Monika Karpińska[1], Jarosław Woliński[1], Kamil Zaworski[1], Małgorzata Białek[1], Stefan Pierzynowski[2,4] and Kateryna Pierzynowska[1,2,3,4]

[1]The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland,
[2]Department of Biology, Lund University, Sölvegatan 35, 22362 Lund, Sweden
[3]Department of Biology, Institute Rural Medicine, ul. Jaczewskiego 2, 20-090 Lublin, Poland
[4]SGP + Group Consortium, Alfågelgränden 24, 23132, Trelleborg, Sweden

Corresponding author: m.bialek@ifzz.pl

Erythrocytes (red blood cells, RBCs) are one of the major cellular components of blood. They are small, round, and biconcave, they lack nucleus and contain haemoglobin, which binds oxygen, and to the lesser extent carbon dioxide. RBCs are covered with a membrane containing nearly equal amounts of proteins and lipids, which are either phospholipids or neutral lipids, mostly unesterified cholesterol (Chol) located in between phospholipids [1]. Changes in lipid composition of membranes result in an impairment of their deformability and, thus, affect their functional characteristics. RBCs are particularly suitable for studies on membrane lipid composition, as due to the lack of internal organelles, the majority of their lipids are attributed to the membrane. Factors such as a diet, lifestyle, aging, and diseases may alter the fatty acid (FA) composition of the RBC membrane, and, thus, the FA profile may be used as an indicator of dietary interventions and various states [2]. In animal sciences, membrane lipidomics studies are still scarce [3], partially due to the some methodological issues. RBCs membranes are very fragile, and both chemicals and sample preparation conditions (time, temperature) may alter the lipid profile and hence reduce the usefulness of the RBC as a biomarker. The aim of this study was to improve methods of saponification and methylation of FA present in RBCs membrane of pigs' blood samples, followed by gas chromatography-mass spectrometry analysis (GC-MS). Blood samples (~2 mL) were collected from crossbred ((Polish Landrace × Yorkshire) × Hampshire)) male piglets (*Sus scrofa domesticus*), fed high fat diet supplemented with 5 g of fish oil (FO). RBCs were collected by centrifugation of blood collected into heparinized tubes and thrice saline rinse of the residue. RBCs samples were subjected to saponification for 2, 10 and 20 minutes at 95°C to choose a suitable time period, then to mild base and acid methylation followed by GC-MS analysis. The shortest time (2 min) of saponification resulted in significantly higher concentrations of arachidonic, eicosapentaenoic, docosapentaenoic, docosahexaenoic, C24:5 n-3 and C24:6 n-3 FAs in RBCs. The longest time (20 min) of saponification significantly reduced concentrations of all FA whereas time of saponification shorter than 2 minutes did not ensure visible dissolution of RBCs. Moreover, they were also visible after mild base and acid methylation. The improved saponification method (95°C for 2 min) is an appropriate analytical procedure of sample preparation for GC-MS analysis of FA profile in RBCs.

Keywords: fatty acids, gentle saponification, mild methylation, erythrocytes, piglet.

References:

[1] Vahedi A., et al. Quantitative analysis of red blood cell membrane phospholipids and modulation of cellmacrophage interactions using cyclodextrins. Scientific Reports 2020, Sep 15;10(1):15111.
[2] Song Y., et al. Red blood cell triglycerides—a unique pool that incorporates plasma-free fatty acids and relates to metabolic health. Journal of Lipid Research 2021, 62:100131.
[3] Prasinou P., et al. The Erythrocyte Membrane Lipidome of Healthy Dogs: Creating a Benchmark of Fatty Acid Distribution and Interval Values. Frontiers in Veterinary Sciences 2020, 7:502.

LIQUID CHROMATOGRAPHIC METHOD FOR ANALYSIS OF CANNABINOIDS LEVEL IN HEMP SAMPLES AND EGG YOLKS

Marian Czauderna[1], Věra Skřivanová[2], Milan Marounek[2], Małgorzata Białek[1] and Agnieszka Białek[1,3]

[1]The Kielanowski Institute of Animal Physiology and Nutrition, Polish Academy of Sciences, 05-110 Jabłonna, Poland,
[2]Institute of Animal Science, Department of Nutrition Physiology and Animal Product Quality, CZ-104 00, Praha-Uhříněves, Czech Republic,
[3]University of Economics and Humans Sciences, Okopowa 59, 01-043 Warsaw, Poland

Corresponding author: m.czauderna@ifzz.pl

The diet composition, especially added supplements, can be an effective factor in increasing the nutritive value of animal products from the aspect of human health. Currently, cannabinoids (CBDs) derived from hemp could be used as physiologically valuable dietary supplements for farm animals (like poultry, ruminants, pigs or rabbits) [1]. CBDs have a wide spectrum of biological activity, including anti-oxidant and anti-inflammatory activity and anti-necrotic protective effects, as well as displaying a favourable safety and tolerability profile in humans. Interestingly, hemp seeds added to tap water and feed have increased bone strength of farm animals, which is of great practical importance. Thus, hemp rich in CBDs (with trace levels of psychoactive tetrahydrocannabinol (THC)) added to diets appears to be of particular interest for farm animal feeding [2]. It is important to monitor the CBD profile in animal products due to the possible presence of psychactive CBDs (like Δ9-THC and Δ8-THC). Thus, the aim of our study was to develop the reversed-phase (RP) high performance liquid chromatographic method (HPLC) with photodiode array detection (DAD) for the determination of cannabinoids in hemp supplements, the diets enriched in CBDs and yolk and white of eggs of hens fed these diets. CBDs are lipophilic substances, so their transition to egg yolks and adipose tissues is possible. The determination of CBDs in animal products is important because some CBDs (like cannabidiol) alleviate the psychoactive effects of THC. The chromatographic separation of twelve non-psychoactive CBDs, two psychoactive CBDs (Δ9-THC and Δ8-THC) and free cholesterol in these samples was conducted using a Shim-Pak C18-column (2.2 μm; 75 mm × 3 mm) and two Phenomenex C18-columns (2.5 μm; 100 mm × 3 mm). The preparation of egg yolks for HPLC analysis consisted of four extractions with hexane. All hexane layers were combined, and hexane was removed under a stream of argon at ~45°C. The residue in a vial was dissolved in 1 mL of acetonitrile (ACN). The preparation of fine powdered hen diets, hemp and flax seeds for chromatographic analyses consisted of four extractions with hexane and four extractions with methanol. Hexane and methanol layers were combined, and solvents were removed under a stream of argon at ~45°C. The residue in a vial was dissolved in 1 mL of ACN. Then, biological samples and CBDs standards were analysed using a ternate gradient of ACN with formic acid (solvent A), solvent B consisted of water with formic acid, while solvent C was methanol; the maximum system pressure was 29.5 MPa. After detailed studies of the influence of column temperature, mobile phase compositions and the pH of methanol on resolution of CBDs, the use of three columns, 40°C elution temperature and methanol was chosen as optimum separation conditions for analysis of all assayed CBDc and cholesterol in egg yolk samples, feed, hemp seeds and flax seeds.

Keywords: Cannabinoids, liquid chromatography, hemp, farm animals, egg yolks.

References:

[1] Landa L., et al., Veterinarni Medicina. The use of cannabinoids in animals and therapeutic implications for veterinary medicine: a review. 2016, 61(3), 111–122; doi: 10.17221/8762-VETMED.

[2] Kis B., et al. Cannabidiol—from Plant to Human Body: A Promising Bioactive Molecule with Multi-Target Effects in Cancer International Journal of Molecular Sciences 2019, 20, 5905; doi:10.3390/ijms20235905.

BEEF AND DAIRY BULL SEMEN QUALITY PARAMETERS IN RELATION TO AMBIENT TEMPERATURE AND HEAT STRESS

Monika Veikutyte, Audrone Rekesiute, Rokas Macijauskas, Neringa Sutkeviciene

Lithuanian University of Health Sciences, Veterinary Academy, Tilžės str. 18, Kaunas, LT-47181, Lithuania

Corresponding author: neringa.sutkeviciene@lsmu.lt

High ambient temperature is considered to be one of the most important factors for subfertility in cattle in tropical or subtropical countries [1]. Bull spermatozoa are very susceptible to heat stress, so the current increase in global temperature is of concern for future livestock production [2]. Some differences in sperm quality between dairy and beef bulls are also described [3]. A further aim was to identify where potential differences in sperm quality occur between dairy and beef bull semen in relation to ambient temperature and heat stress. In this study, 233 fresh raw ejaculated bull semen samples (107 of dairy breeds and 126 of beef breeds), collected over a period of three years, were analyzed. After semen collection, the volume of ejaculate was fixed and NewBauer chamber was used to measure sperm concentration. Progressive as well as total sperm motility were measured via Sperm Class Analyzer software. The average age was 68.80 ± 8.92 months for beef and 49.77 ± 6.93 months for dairy bulls.

The data on the average monthly ambient temperature were obtained from the Lithuanian Hydrometeorological Service. The highest average ambient temperature was detected on summer season: June (17.84 ± 1.06°C), July (18.42 ± 0.74°C) and August (17.67 ± 2.71°C). Our results showed that the type of the bull has a significant influence on sperm motility parameters for the whole tested period. Dairy bulls showed a higher average of the total and progressive sperm motility rates (79.39 ± 15.02% and 59.31 ± 16.00%) ($P < 0.01$), while the average of the volume of the ejaculate (7.37 ± 2.26 mL) ($P > 0.05$) and sperm concentration (1836.12 ± 791.50 cells/mL) ($P < 0.01$) were higher in beef bull samples. During the summer, sperm motility parameters in the beef bull samples decreased significantly while the motility in dairy bull samples remained constant and did not differ from other seasons ($P < 0.01$). An increase in ejaculate volume and a decrease in concentration in dairy bull ejaculates were observed during the summer. Meanwhile, the beef bull samples in summer were more concentrated without a rise in the volume of the ejaculate. The analysis of influence of ambient temperature and heat stress on bull semen parameters showed that the spermatozoa of the beef bulls to compare with dairy bulls were more sensitive for high ambient temperature by decreasing their motility, which could be caused by higher sperm concentration in the ejaculate and an increase in oxidative stress.

Keywords: heat stress, cattle, fertility.

References:

[1] Rahman M. B., Schellander K., Luceño N. L. Heat stress responses in spermatozoa: Mechanisms and consequences for cattle fertility. Theriogenology 2018, 113;102–112.
[2] Morrell J.M. Heat stress and bull fertility. Theriogenology 2020, 153; 62–67.
[3] Morrell J.M., Valeanu A.S., Lundeheim N. and Johannisson A. Sperm quality in frozen beef and dairy bull semen. Acta Veterinaria Scandinavica 2018, 60:41.

STAT5 GENE (*6853C>T*) POLYMORPHISM AND ITS INFLUENCE ON THE CARCASS QUALITY IN BEEF CATTLE

Nijolė Pečiulaitienė, Ramutė Mišeikienė, Kristina Morkūnienė, Renata Bižienė, Laimutis Kučinskas

Institute of Biology Systems and Genetics, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181 Kaunas, Lithuania.

Ccorresponding author: Nijole.Peciulaitiene@lsmu.lt

Signal transducer and activator of transcription 5 (STAT5) is known as a main mediator of growth hormone (GH) action on target genes [1]. 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 [2]. 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 [3, 4]. The aim of this study was to investigate the prevalence of STAT5 gene (6853C>T) polymorphism and to determine its influence on the carcass quality in beef cattle. Samples of cattle hair follicles were collected from 85 bulls consisting of Angus (41), Limousin (19), Galloway (19) and Simmental (6) cattle. Hair samples and the data on carcass quality records were obtained from Šilutė control bulls feeding station. Bovine genomic DNA was extracted from hair follicles using Chelex DNA extraction method. Polymorphism of *STAT5* locus was identified using a PCR-RFLP method. PCR product of *STAT5* gene was digested with *AvaI (Eco88I)* restriction nuclease. Both C and T alleles of the *STAT5* gene were detected in Lithuanian beef cattle population. Frequency of C allele was found the highest and that of T allele the lowest. The C and T allele frequencies were 0.959 and 0.041, respectively. The *STAT5* gene CC genotype was the most common in the studied population (91–92%) followed by the CT genotype (9–8%), while the TT genotype was not found in the analyzed population. Evaluation of the observed and expected heterozygosity in the investigated group of animals demonstrated that the observed heterozygosity was found to be lower than expected, indicating an insufficient amount of genetic diversity in the loci studied; the difference was not statistically significant. According to SEUROP carcass classification system, most of the CT and CC genotypes bovine carcasses were classified as R (good) carcass conformation class and the 3rd (average) fat coverage class. However, results were not statistically significant (*P* value – 0.334, $P > 0.05$); thus, *STAT5* gene polymorphism has no influence on bovine carcass conformation and fat coverage. In conclusion, after evaluating the polymorphism of the *STAT5* gene (6853C> T) in the studied population of beef bulls bred in Lithuania, it was found that the frequencies of alleles and genotypes are unevenly distributed. Therefore, the low frequency of some of the obtained genotypes of polymorphism makes it difficult to determine the influence on the carcass quality traits of cattle. An increased sample size is required to obtain more confident results.

Keywords: cattle, STAT5 gene, polymorphism, PCR-RFLP.

References:

[1] Selvaggi, M.A.C., et al. Bovine STAT5A gene polymorphism and its influence on growth traits in Podolica breed. Animal Production Science 2015, 56(7), 1056-1060.
[2] Kiyici J.M., et al., Relationships between polymorphisms of growth hormone, leptin and myogenic factor 5 genes with some milk yield traits in Holstein dairy cows. International Journal Dairy Technology 2019, 72(1): 1- 7.
[3] Arslan K., et al., Investigation of STAT5A, FSHR, and LHR Gene Polymorphisms in Turkish Indigenous Cattle Breeds (East Anatolian Red, South Anatolian Red, Turkish Grey, Anatolian Black, and Zavot). Russian Journal of Genetics 2015:51:1088-95.
[4] Daldaban F., et al. Polymorphism of the ST olymorphism of the STAT5A and M T5A and MYF-5 genes in Anat YF-5 genes in Anatolian water olian water buffalo. Turkish Journal of Veterinary and Animal Sciences 2020, 44: 284-289

THE INFLUENCE OF RATION CHANGES IN THE DYNAMICS OF THE ACTIVITY OF BLOOD BIOCHEMICAL INDICATORS AND LIVER ENZYMES IN HEIFERS

Ruta Budreckiene[1*], Filip Samveljan[1], Jurgita Dailidaviciene[2], Sigita Kerziene[3]

[1]Department of Biochemistry, Medical Academy, Lithuanian University of Health Science, Tilžės str. 18, Kaunas, Lithuania,
[2]Department of Anatomy and Physiology, Veterinary Academy, Lithuanian University of Health Science, Tilžės str. 18, Kaunas, Lithuania,
[3]Department of Animal Breeding and Nutrition, Veterinary Academy, Lithuanian University of Health Science, Tilžės str. 18, Kaunas, Lithuania

Corresponding author: Ruta.Budreckiene@lsmu.lt

Cattle, like all other animals, need five main components in their diet: carbohydrates, proteins, minerals, vitamins and water (although the water is not a nutrient material, any diet or vitality is impossible without it) [1, 2]. Sometimes, it is difficult to ensure a proper diet and to match the ration exactly as required by the animals. In this case, feed additives are used to ensure a proper diet that fully meets the needs of the animal. Feeds may also be fortified with supplements if clinical symptoms appear to be present due to a lack of some materials in the diet. Proper selection of the supplement and its dose is very important, because too much of it can be harmful to animals: decreased productivity, impaired metabolism, the onset of pathological processes may occur [3]. The main tasks of this investigation were determination of the effect of dietary changes on the hepatic activity of the liver enzyme; analysis of the most important biochemical parameters of the blood, which are the most sensitive indicators for changes in the diet and how they relate to changes in liver enzymes; and calculation of the interactions and relationships between the obtained biochemical parameters. A particularly pronounced change was observed in GGT and ALP enzymes. The mean GGT concentration at the beginning of the study was 19.07 U/L, and at the end of the study (90 days), it reached 22.41 U/L (norm: 6–17.4 U/L), while the ALP at the beginning of the study was 160.07 U/L, and at the end, it was 224.31 U/L (norm: 0–488 U/L). The correlation coefficient of these enzymes is very strong – 0.97. Hypocalcaemia and hypomagnesaemia, which occurred throughout the study irrespective of changes in the diet, were investigated for other biochemical parameters, determining the cause and relationship between changes in liver enzymes. The calcium concentration on day 0 of the experiment was 5.61 mg/dL and on day 90, it was 6.2 mg/dL doses (norm: 8–11.4 mg/dL), magnesium concentration 0.98 mg/dL and 1.1 mg/dL, respectively (norm: 1.5–2.9 mg/dL). However, a significant increase in phosphorus concentration was observed throughout the study, hypophosphataemia was found to be 5.36 mg/dL on day 0 and increased to 8.22 mg/dL on day 90 (rate: 5.6–8.0 mg/dL). A positive correlation was found between the increase in protein concentrations and the increase in urea and protein uptake and creatinine change, respectively, by a factor of 0.97 and 0.64. It is also likely that due to the increased protein content and the resulting ammonia in the liver, GGT serum levels were also increased due to liver toxicity of ammonia, as the GGT and urea correlation coefficient is very strong and equal to 0.97.

Keywords: racion, proteins, minerals, liver enzymes, heifers, dynamics.

References:

[1] R. Blair, NUTRITION AND FEEDING, London, UK.: Cabi, 2011.
[2] P. McDonald, R.A. Edwards, J.F.D. Greenhalgh, C.A. Morgan, L. Sinclair ir R. W. , ANIMAL NUTRITION, Pearson, 2010.
[3] D. G. Hinton, Supplementary feeding of sheep and beef cattle, Collingwood: Landlinks Press, 2007.

INVESTIGATION OF *PIT1* GENE POLYMORPHISMS AND THEIR INFLUENCE ON MILK TRAITS IN DAIRY CATTLE

Renata Bižienė, Kristina Morkūnienė, Gintarė Kolaitytė, Ramutė Mišeikienė, Nijolė Pečiulaitienė, Laimutis Kučinskas

Institute of Biology Systems and Genetics, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181 Kaunas, Lithuania

Corresponding author: renata.biziene@lsmu.lt

Investigation of gene polymorphisms is very useful in determining the genetic potential of an animal [1]. The use of genomic or marker selected analysis in cattle breeding can effectively improve the quality of dairy products and better results of quantitative traits [2]. Previous studies in dairy cattle have shown that polymorphisms in the pituitrin-specific transcription factor (*PIT1*) gene are associated with traits of cattle milk productivity. The pituitrin-specific transcription factor (*PIT1* or *POU1F1*) gene is involved in the transcription of the growth hormone (GH) gene, the prolactin (*PRL*) gene, and the thyroid stimulating hormone-β (*TSHB*) gene. Pituitrin-specific transcription factor is also important for the differentiation and growth of adenohypophyseal somatotropic, lactotropic, and thyrotropic cells, pituitary development, and maintenance of function [3]. Blood samples were taken from 101 dairy cattle from various dairy farms in Lithuania. Data sets for second lactation production period from dairy cattle were analysed. In this study, two *PIT1* gene polymorphisms (c.1178A/G and c.545G/A) were analysed. Genotyping of polymorphisms was achieved using the PCR-RFLP method. There were three genotypes detected at *PIT1* gene polymorphism c.1178A/G, i.e., BB, AB and AA. The observed frequencies were 64.4% (BB), 27.7% (AB) and 7.9% (AA). The observed allele frequencies were B 78.2% and A 21.8%, respectively. In this polymorphism, heterozygosity was insufficient. There were three genotypes detected at *PIT1* gene polymorphism c.545G/A, i.e., GG, GA and AA. Their observed frequencies were GG 62.4%, GA 27.7% and AA 9.9%. The mutated allele A frequency was 23.8% and the wild type allele G frequency was 78.2%. In this polymorphism, heterozygosity was also insufficient. The investigational analysis of *PIT1* gene polymorphism c.1178A/G shows that B allele determines higher milk yield, while *PIT1* gene polymorphism c.545G/A allele A determines higher milk and protein yield. In conclusion, results from the analysis show that *PIT1* gene polymorphisms c.1178A/G and c.545G/A have a significant impact on the quantity of milk and protein yield.

Keywords: dairy cattle, *PIT1* gene, RFLP.

References:

[1] Tizioto PC, Decker JE, Taylor JF, Schnabel RD, Mudadu MA, Silva FL ir kiti. Genome scan for meat quality traits in Nelore beef cattle. Phys Genom. 2013, 45(1), 1012–1020.
[2] Kiyici JM, Arslan K, Akyuz B, Kaliber M, Gamze Aksel E, Cinar M. Relationships between polymorphisms of growth hormone, leptin and myogenic factor 5 genes with some milk yield traits in Holstein dairy cows. Int J Dairy Tech. 2018, 70, 1–7.
[3] Lan XY, Peñagaricano F, DeJung L, Weigel KA, Khatib H. Short communication: A missense mutation in the *PROP1* (prophet of *Pit1*) gene affects male fertility and milk production traits in the US Holstein population. J Dairy Sci. 2013, 96(2), 1255–1257.

EVALUATION OF MILK YIELD AND MILKABILITY TRAITS OF LITHUANIAN RED COWS WITH DIFFERENT GENOTYPES DURING LACTATION IN ORGANIC FARMS

Renata Japertienė[1], Lina Anskienė[1], Sigitas Japertas[2]

[1]Department of Animal Breeding, Lithuanian University of Health Sciences, Faculty of Animal Sciences, Tilžės str. 18, LT-47181, Kaunas, Lithuania,
[2]Practical training and trial center of Lithuanian University of Health Sciences, Akacijų str. 2, Giraitės village, LT-54310, Kauno distr., Lithuania

Corresponding author: renata.japertiene@lsmu.lt

Holstein cattle have been used for many decades for the breeding of other dairy cattle in order to increase milk production. Productivity of cows differs between organic and conventional herds; therefore, the ability of cows to adapt to an organic production environment has been questioned, whether these high-cost genotypes are suitable for organic farming systems. Organic dairy farming has grown, farmers have realised that many available conventional breeds of cows are not well adapted to new situations and that more robust cows are able to function well in the organic environment.

The aim of this study was to investigate the milk yield and milkability traits during lactation of Lithuanian red cows with different genotypes. The research was carried out in an organic farm in 2020 with dairy cows (n = 248) of Lithuanian red cattle population. The milk yield (MY), milking speed (MS), highest milk flow (HMF), and milking time (MT) were evaluated. Investigated traits were measured with DeLaval electronic milk meters, "Apro Windows" software. All records were between 5 and 330 days of lactation, with average 2.26 ± 0.44 of lactation. All cows had two milk-recording events per test day (morning and evening). Lactation of cows was divided into stages: (stage 1 – <= 60; stage 2 – 61–150; stage 3 – 151–240; an stage 4 – 241–330 days of lactation). The statistical analysis of data was performed using the SPSS 25.0 (SPSS Inc., Chicago, IL, USA) software.

The highest number of cows was with LRxRHxRH genotype, which accounted for 37.83% (χ^2 = 53.540, df = 1, $P < 0.001$) of all investigated cows. We observed that the highest MY and HMF in the organic farm was detected in cows with genotypes LRxAIxRH, LRxHxH and LRxRHxRH in the first and the second stages of lactation ($P < 0.05$). MT of LRxHxH, LRxRHxDR and LRxAIxRH genotype cows during all stages of lactation was longer, compared to cows of other genotypes ($P < 0.05$). We estimated that of all fixed effects the biggest influence on MS, HMF, MT ($P < 0.001$) and MY was by a genotype of cows ($P < 0.01$), while the stage of lactation showed the highest impact on MY, HMF, MT ($P < 0.001$) and MS ($P < 0.01$). Analysis of different genotypes of cows revealed that local breeds are well-adapted and more suitable for organic farming.

Keywords: genotype, milk yield, milking speed, milking time, stage of lactation.

INFLUENCE OF THE FIRST CALVING AGE ON THE COW PRODUCTIVITY INDICATORS

Ramutė Mišeikienė[1], Saulius Tušas[1], Jūratė Rudejevienė[2], Agata Balcevič[1]

[1]Institute of Animal Rearing Technologies, Lithuanian University of Health Sciences, Lithuania,
[2]Dr. L.Kriauceliunas Small Animal Clinic, Veterinary Academy, Lithuanian University of Health Sciences, Lithuania

Corresponding author: ramute.miseikiene@lsmu.lt

Age at first calving is considered an important predictor of subsequent milk yield and is an extremely important economic trait determining the profit of cow milk production [1, 2]. Cow longevity and lifetime performance traits are good indicators of breeding effectiveness and animal welfare. They are also interrelated with the economics of dairy herd [3]. Our study aimed to analyze and to determine the influence of the first calving age on cow productivity. In the investigated farm, the cows were raised under loose-housing conditions in a modern cold-type barn throughout the year. Cows were fed a complete mixed diet that met their physiological needs. For the study, 362 cows with their completed first lactation were selected. According to the age of the first calving, the cows were divided into 4 groups according to the calving age: up to 22 months, 22.1–24.0 months, 24.1–26.0 months, and over 26.1 months. For the analysis of the data, statistical indicators (arithmetic mean, standard error of mean and statistical reliability of the data (P) were calculated for each evaluated trait. The obtained results were considered statistically significant when $P < 0.05$. The first calving age of cows ranged from 20 to 29 months, with an average of 23.7 months. We found that 1483.16 kg ($P < 0.05$) more milk was produced in cows of 22.1–24 months of age compared to the cows up to 22 months of age, and 300.35 kg milk more compared to the cows over 26.1 months of the first calving age. The milk fat content of the first-calved cows that calved before the age of 22 months was 0.16% ($P < 0.05$) higher than in the 22.1–24-month-old group of cows with the lowest milk fat content of all groups of cows. Milk protein content was the highest in milk of cows of calving under 22 months and in cows from 26.1 months of age; it was 0.1% ($P < 0.05$) higher than in the group of cows aged 22.1–24 months and 0.06% higher than in cows aged 24.1–26 months. The number of somatic cells in milk increased with the increasing first calving age. The number of somatic cells in the milk of cows that calved at age from 26.1 months and over was 137.71 thousand/mL ($P < 0.05$) higher than in the group of cows up to 22 months of age.

Keywords: dairy cows, age at first calving, productivity.

References:

[1] Van Eetvelde M. et al. A large-scale study on the effect of age at first calving, dam parity, and birth and calving month on first-lactation milk yield in Holstein Friesian dairy cattle. Journal of Dairy Science. 2020, 103(12), 1515-1523.
[2] Torshizi M.E. Effects of season and age at first calving on genetic and phenotypic characteristics of lactation curve parameters in Holstein cows. Journal of Animal Science and Technology. 2016, 58, 1-8.
[3] Adamczyk K., Makulska J., Jagusiak W., Węglarz A. Associations between strain, herd size, age at first calving, culling reason and lifetime performance characteristics in Holstein-Friesian cows. Animal. 2017, 11(2), 327-334.

CHOLESTEROL DEFICIENCY HAPLOTYPE IMPACT ON MILK PRODUCTION IN LITHUANIAN HOLSTEIN COWS

Ramutė Mišeikienė, Nijolė Pečiulaitienė, Renata Bižienė, Kristina Morkūnienė, Laimutis Kučinskas

Institute of Biological Systems and Genetic Research, Lithuanian University of Health Sciences, Lithuania

Corresponding author: ramute.miseikiene@lsmu.lt

Cholesterol deficiency (CD) is an autosomal monogenic recessive defect in Holstein cattle [1]. Carriers of cholesterol deficiency haplotype (CDH) have been reported to have a significantly higher genetic merit for milk protein and fat, somatic cell score than non-carriers [2]. Our study examined the distribution of CDH in the *APOB* gene in Lithuanian Holstein cows and its impacts on milk production. A total of 80 cows were checked for the APOB mutation. Genomic DNR was isolated from the blood samples. All cows were diagnosed by the test described by Menzi et al. [3]. In this test, a combination of three allele-specific primers was allowed for genotype differentiation: a reverse common primer starting from a wild sequence and two forward primers discriminating the wild sequence from a mutant. The wild forward primer ensured amplification of 249 bp, and the mutant forward primer produced larger amplicon of 436 bp. Genotypes were determined using RFLP-PCR and detected by performing 2% agarose gel electrophoresis of PCR-RFIP samples and evaluating fragment sizes according to the molecular marker in UV light. Statistical analysis was performed using the statistical program SPSS. Using the Kolmogorov-Smirnov test, we found that the values of the studied symptoms were distributed. To evaluate the differences between genotypes, the means of the studied traits and the arithmetic errors of the means were calculated, and the Student criterion for independent samples was calculated to assess the statistical significance of the differences. Differences were considered statistically significant at $P < 0.05$. The average carrier frequency of analysed cows was 8.77%. Non-significant differences were found between milk production traits of carriers and non-carriers. The cholesterol deficiency haplotype status had a non-significant ($P > 0.05$) effect on milk fat ($P = 0.577$), milk protein ($P = 0.337$), milk lactose ($P = 0.82$) and milk somatic cell count ($P = 0.710$). Still, the analysis showed that the milk of heterozygous cows compared with non-carrier homozygous cows had about 0.16% less fat, 0.05% less protein, and 0.01% less lactose. The somatic cell count in carrier milk was about 30.9 thousand/mL less than in non-carrier Holstein cows.

Keywords: *APOB* gene, cattle, productivity.

References:

[1] Kamiński S., Ruść A. Cholesterol Deficiency – new genetic defect transmitted to Polish Holstein-Friesian cattle. Polish Journal of Veterinary Sciencs. 2016, 19(4), 885–887.
[2] Cole, J.B., Null, D.J., and VanRaden, P.M. Phenotypic and genetic effects of recessive haplotypes on yield, longevity and fertility. J. Dairy Sci.2020, 99(9): 7274–7288.
[3] Menzi F. et al. A transposable element insertion in APOB causes cholesterol deficiency in Holstein cattle. Animal Genetics. 2016, 47(2), 253-257.

BIOCHEMICAL PARAMETERS OF LIVER FUNCTION IN GUINEA PIGS FED WITH DIFFERENT DIETS

Judita Zymantiene, Rasa Zelvyte, Vaidas Oberauskas, Ugne Spancerniene, Kristina Musayeva, Antanas Sederevicius

Department of Anatomy and Physiology, Faculty of Veterinary Medicine, LSMU VA, Tilžės str. 18, Kaunas, Lithuania

Corresponding author: judita.zymantiene@lsmu.lt

Guinea pigs have a close resemblance to humans, including comparable plasma lipoprotein metabolism enzymes, a requirement for dietary vitamin C, similar gender plasma lipoprotein differences and equivalent responses to dietary interventions, exercise and drug treatment, toxicity tests, and can be used as a convenient animal model for human hepatic injury, hepatic steatosis and ultimately hepatic cirrhosis without undergoing any genetic manipulations [1, 2]. They are also used as models to investigate the auditory system, kidney function, osteoarthritis, nutrition, infectious diseases, and reproductive biology [1]. It is noteworthy that diet plays a vital role in maintaining animal health and could have a meaningful impact on liver functions. The present study aimed to assess the changes of some biochemical serum parameters associated with the liver function and to find out the correlation between these parameters using two different diets. Housing conditions and the experimental procedures of 12 short-haired guinea pigs served in this study were in line with EU Directive 2010/63/EU. Guinea pigs of group 1 (n = 6) were fed for six months with commercial pellets (free of genetically modified organisms sourced ingredients), whereas animals of group 2 (n = 6) got a diet containing genetically modified soybeans. The reductions in aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) of 16.59%, 6.60%, 23.72% and 32.00% were fixed in group 2, respectively, as compared with group 1. However, the albumin (Alb) value in group 2 showed an increment of 16.00% in comparison to the group 1. No marked changes between the groups were observed in total protein (T-Pro) values. A significant strong positive correlation was noted between AST and ALT (r = 0.80), AST and LDH (r = 0.88), ALT and LDH (r = 0.87) activity values, as well asbetween Alb and T-Pro values (r = 0.88) in group 1 ($P < 0.01$). In group 2, a significant ($P < 0.05$) positive correlation was determined between ALP and LDH (r = 0.48), ALT and LDH (r = 0.73) activity values, as well as between Alb and T-Pro values (r = 0.51). The correlations between ALT and ALP, ALP and LDH activity values in group 1, AST and ALT, AST and LDH, ALT and ALP activity values in group 2 were found to be statistically insignificant ($P > 0.05$). Based on these results, we concluded that liver enzymes activity declined from 6.60% to 32.00% in group 2, but corresponded to the established reference intervals for healthy guinea pigs; therefore group 2 may be fed the diet without restrictions for guinea pigs used in experimental studies.

Keywords: guinea pig, diet, serum parameters, liver.

References:

[1] Fernandez M. L., Volek J. S. Guinea pigs: a suitable animal model to study lipoprotein metabolism, atherosclerosis and inflammation. Nutr Metab . 2006, 27, 3-17.

[2] DeOgburn R., et al. Guinea pigs as models for investigating non-alcoholic fatty liver disease. Int Food Nutr Met 2016, 3, 309-333.

EFFECTS OF DIFFERENT TUNNELS ENRICHMENT ON BEHAVIOR AND PHYSIOLOGICAL PARAMETERS OF MOUSE

Judita Žymantienė[1], Vaidas Oberauskas[1], Rasa Želvytė[1], Antanas Sederevičius[1], Kristina Musayeva[1], Ugnė Spancernienė[1]

[1]Department of Anatomy and Physiology, Faculty of Veterinary Medicine, LSMU VA, Tilžės str. 18, Kaunas, Lithuania

Corresponding author: judita.zymantiene@lsmu.lt

There are many incongruencies between the natural adaptations of mice (*Mus musculus*) and the laboratory conditions in which they are typically housed [1]. Therefore, environmental enrichment maybe used to increase sensory/motor stimulation, increase natural and facilitate species-typical behavior, and provide the animals with some degree of control over their environment [2]. Recent evidence indicates that even the addition of simple forms of enrichment to standard laboratory cages may enhance mice well-being, including behavioral, physiological and neurochemical parameters, as shown by reduced abnormal repetitive behavior, reduced measures of anxiety, and/or reduced aggression and mortalities [3, 4]. A broad variety of enrichment items and materials (various innovative elements as tunnels, toys, lily pads, mouse wedge, tent, mezzanine, bouls, etc.) are available, but there is quite a high knowledge gap (whether they do not deteriorate the quality of scientific data) and further investigations are essential. There is mounting evidence that enrichment can differ in its effects on physiology and behavior between species and strains; therefore, it remains a major area of interest. Thus, we aimed to evaluate the effects of tunnel enrichment on several mouse physiological parameters and behavior. Eight-week-old mice of BALB/C strain (a total of 40 mice) were randomly selected and divided into 4 groups of 10. The holding facilities were kept at a relatively constant temperature (24 ± 2°C), humidity (55 ± 5%), and a 12-hour light-dark cycle. The mice were fed with commercial rodent diet containing 19.90% of crude protein, 12.50% of crude fat, 1.70% of crude fiber, cereals, trace elements and vitamins; they also received *ad libitum* water. We varied cage enrichment across four levels: (group 1, control) without enrichment object (no tunnel); (group 2) with an iron tunnel; (group 3) with a plastic tunnel; (group 4) with iron and plastic tunnels. A thermal imaging camera (FLIR T640) was used for measuring eyes and noses temperatures. A 15-minute period was chosen for assessment of behavioral patterns (rearing (R), grooming (G), heading up and rearing (HR), sitting (S), hunching (H), heading down (HE), and stopped huddled (ST)). The eye temperature of mice was 35.23 ± 0.53°C, 34.98 ± 0.71°C, 35.45 ± 0.58°C and 35.40 ± 0.47°C in groups 1, 2, 3 and 4, respectively. Cage enrichment with plastic tunnels increased eye temperature by 0.47°C comparing with cage enrichment of an iron tunnel. The nose temperature was 24.79 ± 1.78°C, 25.17 ± 1.74°C, 26.48 ± 2.09°C and 24.65 ± 1.21°C in groups 1, 2, 3 and 4, respectively. Statistically significant R behavior in mice was noticed among groups (χ^2= 1 4.09; P = 0.0028). The distribution of other behavioral patterns between the groups exhibited to be statistically insignificant. To summarize, we may state that cage enrichment with tunnels does not cause stress because temperature of eyes and noses (as indicative of the mice emotional state) remained stable, and enrichment did not arouse abnormal behavioral pattern.

Keywords: mouse, enriched environment, tunnels, temperature, behavior.

References:

[1] Latham N., Mason G. From house mouse to mouse house: the behavioural biology of free-living Mus musculus and its implications in the laboratory. Applied Animal Behaviour Science, 2004, 86, 261–289.
[2] Oatess T.L. et al. Effects of acrylic tunnel enrichment on anxiety like behavior, neurogenesis, and physiology of C57BL/6J Mice. J Am Assoc Lab Anim Sci, 2021, 60, 44-53.
[3] Bailoo J. D. et al. Effects of cage enrichment on behavior, welfare and outcome variability in female mice. Front. Behav. Neurosci., 2018, 12, 232.
[4] Hobbiesiefken U. et al. Evaluation of different types of enrichment – their usage and effect on home cage behavior in female mice. PLoS One, 2021, 16(12), e0261876.

NFKB AND *NFKBIA* GENE EXPRESSION ANALYSIS IN DAIRY CATTLE UDDER PARENCHYMA WITH CHRONIC MASTITIS CAUSED BY STAPHYLOCOCCAL INFECTION

Adrianna Szprynca[1], Ewelina Kawecka-Grochocka[1], Magdalena Zalewska[2], Magdalena Rzewuska[3],Tomasz Ząbek[4], Emilia Bagnicka[1]

[1]Institute of Genetics and Animal Biotechnology PAS, Jastrzębiec, Poland,
[2]University of Warsaw, Faculty of Biology, Warsaw, Poland,
[3]Warsaw University of Life Sciences, Institute of Veterinary, Warsaw, Poland,
[4]The National Research Institute of Animal Production, Balice, Poland

Corresponding author: a.szprynca@igbzpan.pl

The expression of cytokines, the main agents regulating inflammation, may be regulated by a transcription factor *NFκB* [1], which, in turn, regulates numerous innate and adaptive immunological processes and increases the expression of several pro-inflammatory genes. *NFκB* high activity in milk somatic cells (MSC) derived from udders with both acute (high activity) and chronic (wide range of activity from low to high) mastitis, but not from healthy ones, has been observed [2]. The study aim was to compare the expression of the *NFKBIA* and *NFκB* genes in dairy cow udder parenchyma infected with coagulase-positive (CoPS) or coagulase-negative staphylococci (CoNS) with healthy cows. A total of 36 samples were taken from udder quarters infected with CoPS (n = 12) or CoNS (n = 12) and healthy one derived from the whole healthy udders (control, H = 12). Animals were culled at the end of lactation (286 days; SD, 27) and showed either reproductive difficulties but had healthy udders, or chronic asymptomatic mastitis. RNA was extracted from frozen tissue samples. Only RNA samples with the integrity number (RIN) > 7.0 (the Bioanalyzer 2100, Agilent, Santa Clara, USA) were selected for further analysis. The RT-qPCR method was used to measure gene expression with *GAPDH* and *HPRT* as reference genes. The *NFKBIA* gene was higher expressed in CoPS than in the H group, whereas the *NFκB* gene was discovered to be the least expressed in this group. Both genes in the CoNS group were moderately expressed. An expression of *NFKBIA* gene is always associated with the expression of *NFκB*. *NFKBIA* gene had an increased expression probably to reduce the expression of *NFκB* to protect tissue from destruction, but further study is needed. Yang et al. (2008) [3] showed that during *E. coli*-induced mastitis *NF-κ*B signalling pathway is strongly activated, with elevated expression of this factor. The inhibition of NFKB expression by NFKBIA in subclinical mastitis caused by CoPS is evidenced by the lower level of *NFκB* mRNA and higher *NFKBIA* in the CoPS group than in H. These phenomena taking place in CoPS-infected tissues may represent the activation of tissue-protection mechanisms during chronic and subclinical staphylococcal mastitis.

Keywords: dairy cow, subclinical mastitis, expression.

References:

[1] Karin M., The Beginning of the End: IkB Kinase (IKK) and NF-kB Activation. Journal of Biological Chemistry, 1999, volume 274, issue 39, 27339-27342.
[2] Boulanger D., Bureau F., Mélotte D., Mainil J., Lekeux P., et al. Increased Nuclear Factor κb Activity in Milk Cells of Mastitis-Affected Cows. Journal of Daily Science, 2003, volume 86, issue 4, 1259-1267.
[3] Yang W, Zerbe H, Petzl W, Brunner RM, Günther J, Draing C, et al. Bovine TLR2 and TLR4 properly transduce signals from *Staphylococcus aureus* and *E. coli*, but *S. aureus* fails to both activate NF-κB in mammary epithelial cells and to quickly induce TNFα and interleukin-8 (CXCL8) expression in the udder. Molecular Immunology. 2008; volume 45, 1385–97.

QUALITY OF CHEESE MADE FROM EWE'S MILK DURING STORAGE

Raquel P. F. Guiné and Paula M. R. Correia

CERNAS-IPV Research Centre, Polytechnic Institute of Viseu, Campus Politécnico, Viseu, Portugal

Corresponding author: raquelguine@esav.ipv.pt

The extension of shelf-life is pivotal in the cheese industry, and the test of different conservation methods is, therefore, necessary. In this context, this work aimed at evaluating the effect of storage temperature and packaging material in ewe's cheeses quality along a period of 4 months' storage. Cheeses were produced from raw ewe's milk produced by animals of two Portuguese breeds (Bordaleira Serra da Estrela and Churra Mondegueira). They were stored under different conditions: under refrigeration and in a chamber with controlled temperature and RH, packed in vacuum and using two different plastic films. The ewe's cheeses were analysed in three different moments: on day 1 and again after 2 and 4 months. The samples were evaluated for moisture, water activity, colour, texture, and also sensory characteristics, involving a descriptive test (appearance, aroma, taste, texture, global appreciation) made by 25 untrained panellists.

The results showed that the cheeses stored in polyolefin film developed moulds after 20 days, because the moisture content and water activity were high at the beginning of storage, although they decreased along storage time, particularly for the cheeses that were not packed with plastic films. With respect to colour, the vacuum packed cheeses stored in the chamber presented a uniform colour along storage, with high lightness and low yellowness. The texture results revealed that the harder cheeses were those stored in the chamber after 4 months, while the stickier were the ones packed in plastic after the same 4 months. The sensorial tests showed that the refrigerated cheeses were the most appreciated, followed by those stored in the chamber with vacuum package. Because these cheeses are a soft paste cheese type, and this characteristic is pivotal for their appreciation and valorisation by consumers, the buttery characteristics were analysed and they showed high differences according to the storage conditions or type of package. In conclusion, the storage conditions and duration, as well as the packaging material, exert a great influence on the physical characteristics and sensorial properties of ewe's cheeses.

Keywords: texture, sensory quality, colour, storage conditions.

A COMPARATIVE ANALYSIS OF THE EXTERIOR, HORSE TEMPERAMENT, AND KEEPING CONDITIONS OF ŽEMAITUKAI HORSES RAISED ON DISTINCT (X AND Y) STUD FARMS

Vilma Vilienė, Asta Racevičiūtė-Stupelienė, Monika Nutautaitė, Jurgita Noreikaitė

Institute of Animal Rearing Technologies, Veterinary Academy, Lithuanian University of Health Sciences, Tilžės str. 18, LT-47181 Kaunas, Lithuania

Corresponding author: vilma.viliene@lsmu.lt

Each breed of horses has its own set of evaluation standards, and body composition has long been an essential indicator of working capacity. Aspects of body shape and size correlate directly with movement characteristics, making it easier to select horses for certain use [1, 2]. Therefore, this study was designed to perform a Žemaitukai horse breed comparative analysis between X and Y studs by evaluating the horses' exteriors, body measurement indices, temperaments, and keeping conditions. Research was carried out with a total of 12 horses, each stud farm consisting of 6 Žemaitukai breed horses (n = 6 horses/stud farm), which were selected by sex (4 mares and 2 stallions were selected from each stud) and age (the average horse age was 10 years old). The horses were assessed on their exterior according to a certain feature, 24 body parts measurements and their indices, temperament according to positive and negative traits, and keeping circumstances. The exterior of the horses showed that statistically significantly higher measurements were obtained for Žemaitukai horses bred in the Y stud farm: a significantly longer average forearm length by 1.8 cm, an almost 10 cm wider pelvis, and 5.4 cm longer foreleg length from wrist joint to hoof, compared to horses bred in the X stud farm ($P < 0.05$). Horse body size indices (foreleg length from heel to hoof; large body format; small body format) revealed that the X stud farm had 5.5, 2.8, and 6.6 cm lower indices than the Y stud farm ($P < 0.05$). Compared to the exterior of the horses according to the calibre type, the horses of the Y stud farm scored 0.8 points more than the X horses ($P < 0.05$). However, the horses from the X stud farm had a higher ossification of the fore cannon and the hind cannon ($P < 0.05$). After assessing the temperament of Žemaitukai horses, it was discovered that the horses from the X stud farm possessed a greater number of positive character qualities. These horses displayed features of peace, comfort, and energy for the most of the time. The Y stable had generally peaceful horses, but after evaluating the negative attributes, more of them were discovered on the X stud farm, primarily impatience and challenging management. The horses were more difficult to manage in the Y stud farm, even though the total number of negative features was lower. As an outcome, both studs possessed more positive than negative temperament traits. Examining the conditions of keeping Žemaitukai horses in X and Y stud farms revealed that horses are kept in different types of stables: the X stud farm had a pen system (n = 1 horse/pen), and the Y stud farm had a fencing system. However, the housing systems of both stud farmsmet the requirements. Overall, while the research provided relatively comparable findings, only a few significant differences in the exterior, body measurements, and indices of X and Y stud farm horses were detected. Despite varied horse-keeping practises, there were not enough significant differences in the traits studied, and no clear trend was revealed.

Keywords: Žemaitukai breed, horse, exterior, temperament, body measurement indices.

References:

[1] Wejer J, Lewczuk D. Effect of the Age on the Evaluation of Horse Conformation and Movement. Annals of animal science 2016; 16(3):863-870.

[2] Poyato-Bonilla J, Sánchez-Guerrero MJ, Cervantes I, Gutiérrez JP, Valera M. Genetic parameters for canalization analysis of morphological traits in the Pura Raza Español horse. Journal of animal breeding and genetics (1986) 2021; 138(4):482-490.

ANALYSIS OF CHANGES IN RADIOLOGICAL IMAGE MEASUREMENTS OF TARSAL JOINT OF YOUNG HORSES DURING PERFORMANCE TESTS

Roma Buczkowska[1], Mateusz Hecold[1], Dorota Lewczuk[2]

[1]Faculty of Veterinary Medicine, Warsaw University of Life Sciences, Nowoursynowska str. 159,02-776 Warsaw, Poland,
[2]Department of Biotechnology and Nutrigenomics, PAS, Jastrzębiec, ul. Postępu 36A, 05-552 Magdalenka, Poland

Corresponding author: d.lewczuk@igbzpan.pl

The study aimed to assess the effect of training on skeleton dimensions to understand changes connected with the horse OCD status of horses. Muscle and bone tissue respond to early training to varying degrees [1], and the lack of exercise delays the development of the musculoskeletal tissue of horses [2]. Young warmblood horses trained under controlled conditions on performance riding tests were investigated (19 stallions and 50 mares). Horses were 1249 days old (SD, 114.6). The average height at withers was 164.5 cm (SD, 3.02) with a circumference of the chest 189.8 cm (SD, 4.04) and 20.77 (SD, 0.78) for the fore limb (cannon bone). The average quality of their conformation was 78.15 (SD, 1.13) on a scale of 0–100 points. Lateral x-ray images of each hock joint were performed twice at the beginning and the end of the training. To characterize the changes in the dimensions of the hock joint area, 14 measurements of the hock joint were made (VetRay Vision 4.4.7 Vet Xp / 2000 software). The influence of investigated factors on the joint measurements was analyzed using Proc Mixed from the SAS program for all 191 observations. The statistical model included a random effect of the horse (69), fixed effects of training center (Bogusławice, Biały Bór), sex (stallion, mare), limb (left, right), training (investigations before and after training) and regression on age in days. The post-hock test for LSMs was used to evaluate differences between individual classes of the effects. The associations between measured parameters of hock bones structures were achieved using partial correlation analysis. The MANOVA statement in the GLM procedure of the SAS program corrected calculations for factors influencing the results (training center, sex, limb, training, age). Partial correlations between investigated measurements are presented for all *P*values levels. The training effect has the most significant influence on changes in the bone dimensions of young horses. It was statistically significant for all parameters on the level of $P < 0.001$. Gender turned out to be another important factor being significant for 11 of 14 measured parameters. The age effect was significant for two measured parameters. The right-left limb dimensions were different for five parameters, and the training center for one. The parameters characterizing the calcaneus were the most strongly correlated with each other and the other parameters. Obtained significant correlations are mainly between 0.28 to 0.5. Based on conducted research, it can be stated that training has a significant influence on the horse hock joint dimensions, so it should be taken into account by radiographic evaluation of the horse's health status.

Keywords: horse, tarsal joint, dimensions, training.

References:

[1] Firth E. C., et al. Mild exercise early in life produces changes in bone size and strength but not density in proximal phalangeal, third metacarpal and third carpal bones of foals. The Veterinary Journal 2011, 190, 383–389.
[2] Van Weeren P. R., Barneveld A., The influence of exercise during the first months of life on the development of the equine musculoskeletal system with special attention to osteochondrosis: Experimental set-up and scope of the study. Equine Vet J Suppl 1999, 31, 4-8.

THE POSSIBILITY TO USE BOTH HEALTHY AND RECOVERED COWS AS OOCYTE DONORS FOR THE OPU PROCEDURE

Renalda Juodžentytė[1], Vytuolis Žilaitis[2], Giedrius Palubinskas[1]

[1]Veterinary Center for Continuing Education and Counseling, LSMU, Tilžės str. 18, Kaunas, Lithuanian
[2]Large Animals clinic, Veterinary academy, LSMU, Tilžės str. 18, Kaunas, Lithuanian.

Corresponding author: renalda.juodzentyte@lsmu.lt

The main goal of successful animal husbandry is to quickly raise healthy, good offspring of a genetic breed. This can be achieved by applying innovative advanced technologies. Superovulation and embryo transplantation are one of the reproductive technologies capable of increasing animal productivity [1]. Nowadays, embryo transplantation technology is considered a key technique required to achieve good results in various artificial insemination technologies, especially *in vitro* fertilisation and animal cloning [2]. However, with innovative methods of improving reproduction, the success rate in farm practice is relatively low. There are many limitations associated with the internal and external factors that influence the success of these methods. Such factors include the choice of oocyte production methods, donor variety, and reproductive status. The influence of ovarian dysfunction on oocytes production and fertilization is increasingly being investigated. Finding ways to successfully obtain oocytes from donors without compromising their ovarian function and without prolonging recovery time is an increasingly widespread procedure around the world, ovum-pick-up (OPU), in which oocytes are obtained from secondary follicles [3]. The aim of this study was to investigate the influence of the reproductive status of cows on the success of the OPU procedure and embryo yield. For oocytes aspiration the donor cows (n = 20) were selected of the reproductive status (healthy group – cows without functional reproductive disorders, recovered group – cows after treatment of functional reproductive disorders, i.e., cysts, hypofunction). For this study, the Holstein cows (n = 20) in the 1st to –4th lactation, 60–100 days after calving with an average annual milk yield of 7.000–7.500 kg were selected. Oocyte aspiration was performed using the OPU procedure after hormonal stimulation. After oocytes aspiration, they were classified according to the expansion of the cumulus cells into classes. Quality grading (A, B, C, D) of the oocytes was performed on the basis of cumulus cell development and homogeneity of cytoplasm according to Chaubal et al. (2006) [4]. A total of 81 COCs were aspirated from ovaries. Only normal COCs (Class A and Class B) were used for maturation and fertilization. *In vitro* matured COCs were fertilized with frozen-thawed unsorted sperm. After fertilization, the embryonic cleavage was evaluated within 48 hours (cleavage rate). In the group of healthy cows, 31.91% of Class A oocytes were aspirated and 25.53% of Class B oocytes, of which 45.45% of Class A matured, and 27.27% of Class B matured. In the group of treated cows, 32.35% Class A oocytes and 23.32% Class B oocytes were aspirated of these, and 37.5% of Class A and 25% of Class B matured. In the assessment of oocyte fertilisation in the groups, we found that in the group of healthy cows, Class A oocytes were fertilised by 9.09% more ($P < 0.05$) in the group of healthy cows. In conclusion, this study shows that the OPU procedure can be performed on both healthy cows and cows with reproductive dysfunction, but the OPU procedure should only be performed on healthy cows to achieve higher embryo yields through hormonal stimulation.

Keywords: fertilization, *in vitro*, OPU, oocytes.

References:

[1] Faizah HMS, Richard F, Meena P, Stanley KL, Amriana H, Alhassany A, Yadav SB, Marie L, Crouch B, and Saipul BAR. Multiple Ovulation Embryo Transfer (MOET) In Dairy Cattle in Gatton. Malaysian journal of veterinary research 2018;9(2):109-116.
[2] Kennady V, Manimegalai J, Ranjeet V, and Chaudhiry V. Embryo transfer technology in animals: An overview. Journal of Entomology and Zoology Studies 2018;6(5):2215-2218.
[3] Watanabe YF, Henryli de Souza A, Mingoti RD, Ferreira RM, Emiliana Batista OS, Dayan A, Watanabe O, Meirelles FV, Nogueira MFG, Sterman FJB, Baruselli PS. Number of oocytes retrieved per donor during OPU and its relationship with in vitro embryo production and field fertility following embryo transfer. Anim Reprod 2017; 14:635- 644.
[4] Chaubal SA, Molina JA, Ohlrichs CL, Ferre LB, Faber DC, et al. Comparison of different transvaginal ovum pick-up protocols to optimise oocyte retrieval and embryo production over a 10-week period in cows. Theriogenology 2006; 65:1631-1648.