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Coccidiosis cases in cattle in Turkey

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ABSTRACT

Coccidiosis is a protozoan disease of the Eimeriidae family, mostly caused by *Eimeria* species, sometimes *Isospora* species, seen in all domestic and wild animals, especially in young animals, which can result in hemorrhagic diarrhea, depression, weakening, weight loss, and sometimes death. *Eimeria bovis*, *E. zuernii*, *E. auburnensis*, *E. ellipsoidalis*, and *E. alabamensis* cause clinical coccidiosis by showing pathogenic properties. The disease is more important for young people. In its diagnosis, the age of the animal, the hygienic condition of the environment and clinical signs are evaluated. Clinical findings and stool consistency in calves and calves are also important in diagnosis. In the treatment of coccidiosis is based on the principles of killing the causative agent or preventing its development, eliminating fluid loss, and treating secondary infections. The treatment of coccidiosis in calves naturally infected with *E. zuernii* and *E. bovis*, oral administration of 15mg/kg dose of toltrazuril is reported to be very effective and reduces economic losses by positively affecting the growth performance of animals. In studies conducted in our country, 11 *Eimeria* and 1 *Isospora* species have been found in cattle. In studies on bovine coccidiosis in Turkey, the prevalence of *Eimeria* species in calves and calves was found to be 16-93.3%. In line with the results obtained, it has been observed that coccidiosis continues to be a problem even in cattle farms with relatively good care and feeding conditions in Turkey. It was concluded that routine checks should be made in the enterprises, necessary precautions should be taken and hygiene rules should be followed.

Keywords: coccidiosis, *Eimeria*, cattle, Turkey.

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Introduction

Coccidiosis is a protozoan disease of the Eimeriidae family, mostly caused by *Eimeria* species, sometimes *Isospora* species, seen in all domestic and wild animals, especially in young animals, which can result in hemorrhagic diarrhea, depression, weakening, weight loss, and sometimes death (Jolley and Bardsley, 2006).

It is very common all over the world and in our country and causes economic losses by causing deaths. *Eimeria* species progress with morbidity manifested by increased feed conversion rates, reduced yield, loss of reproductive performance, continuous oocyst scattering, and increased susceptibility to secondary bacterial infections (Uslu and Ceylan, 2020).

Although coccidiosis in cattle is seen in animals of all ages, it is clinically more important in 0-6 and 6-12 months calves (Levine, 1985).

With this study, it was aimed to comprehensively examine the studies evaluating coccidiosis cases in cattle and coccidiosis disease in Turkey.

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Spread of disease and epidemiology

Coccidiosis in cattle is seen in animals of all ages. It is clinically important in calves less than 6 months old and calves 6-12 months old. In our country, 11 *Eimeria* and 1 *Isospora* species were found to be causative agents, and in studies conducted in the world, 17 *Eimeria* and 2 *Isospora* species were found to be bovine coccidiosis agents (Güven et al., 2010). While these species do not cause clinical coccidiosis in elderly animals, they cause developmental delay and mortality by causing acute bloody diarrhea in calves near the carriers. *E. bovis, E. zuernii, E. auburnensis, E. ellipsoidalis* and *E. alabamensis* cause clinical coccidiosis by showing pathogenic properties. The prevalence of infection varies according to climatic factors, diet, rearing, and cleanliness of barns (Köse, 2011; Karaer et al., 2012).

Studies to determine the spread of bovine coccidiosis in Turkey were examined.

Eimeria species were detected in 140 (93.3%) of 150 cattle feces collected from several provinces of Turkey and 11 different *Eimeria* species were reported (Sayın, 1970).

In the research conducted in the Ankara region, the distribution was determined as 16%, and 8 different *Eimeria* species were identified, and it was reported that the most common species was *E. zuernii* (Mimioğlu et al., 1956).

In a study conducted in the Bursa region, the distribution was determined as 49.3%, 10 different *Eimeria* species were determined. *E. bovis* (28.5%) was the predominant species, followed by *E. auburnensis* (17.2%), *E. ellipsoidalis* (14.7%), *E. zuernii* (12.4%), *E. canadensis* (6.2%), *E. cylindrica* (3.7%), *E. subspherica* (1.9%), *E. alabamensis* (1.6%), *E. brasiliensis* (1.2%), and *E. bukidnonensis* (0.5%) (Güleğen and Okursoy, 2000).

Erzurum *Eimeria* species were found at a rate of 25.9% in the region of the calves in dairy farms and 9 different *Eimeria* species were identified. Nine coccidia species, namely E. ellipsoidalis (51.0%), *E. auburnensis* (38.8%), *E. bovis* (32.7%), *E. zuernii* (22.5%), E. *subspherica* (16.3%), E. canadensis (6.1%), E. alabamensis (4.1%), *E. brasiliensis* (4.1%), and *E. bukidnonensis* (4.1%) were identified in infected calves (Aktaş et al., 2008).

In a study conducted in the Van region, they found 10 different *Eimeria* species and determined the distribution rate as 52.9% They were *E. bovis* (27.27%), *E. zuernii* (17.55%), *E. auburnensis* (9.09%), *E. cylindrica* (9.09%), *E. brasiliensis* (8.26%), *E. subspherica* (7.43%), *E. alabamensis* (7.43%), *E. bukidnonensis* (6.61%), *E. canadensis* (4.13%), *E. ellipsoidalis* (3.30%) (Gül et al., 2000).

In another study conducted in the Van region, *Eimeria* sp. The distribution of oocysts was determined as 22.5% (Gül et al., 2008). In a study conducted on calves, 86.4% prevalence and 10 different *Eimeria* species were determined in the Van region, Identified *Eimeria* species as follows were; *E. bovis* (38.4%), *E. zuernii* (35.2%), *E. auburne*nsis (30.4%), *E. cylindrica* (26.4%), *E. subspherica* (24%), *E. canadensis* (20%), *E. alabamensis* (19.2%), *E. ellipsoidalis* (16.8%), *E. bukidnonensis* (12%), and *E. brasiliensis* (11.2%) (Değer et al., 2001).

In a study in which the spread of coccidiosis in calves was determined, the prevalence in the Kars region was determined as 90.8% and the most common species was *E. bovis* (Arslan, 1997).

Eimeria oocysts were reported in 523 (68.1%) of 768 cattle feces in a study conducted in the Thrace region (Arslan and Tüzer, 1998).

In a study conducted in the Hakkari region, *Eimeria* species were found in 82 (89%) of 92 stool samples from calves (Göz and Aydın, 2005).

In other studies with coccidiosis in cattle, it was reported that the distribution was 50.6% in Kırşehir, 20.04% in Afyon, and 51.4% in Elazığ region (Güven et al., 2010; Çiçek et al., 2007; Dumanlı et al., 1993).

Biology of disease

Eimeria species that cause coccidiosis are single-hosted and spend part of their life cycle in the host and the other part in nature. The life cycle of *Eimeria* species occurs in three stages: merogony, gametogony, and sporogony. The merogony and gametogonia phases occur in the host's digestive system, while the sporogony phase occurs in nature (Denizhan and Kozat, 2022). *Eimeria* oocysts excreted with feces become infective by sporulating in an average of 1-2 weeks in accordance with environmental conditions. Sporulated oocysts containing sporozoites have infective properties for 1-2 years when conditions are appropriate. When sporulated oocysts in contaminated barns and pastures are taken with food, oocysts are broken down by bile, acid, enzyme, carbon dioxide effect and peristaltic movements in the digestive system of the animal and sporozoites come out. After that, the merogony phase begins. In the biology of *E. bovis* and *E. zuernii*, merogony occurs in 2 periods (Hatırnaz, 2015). The resulting sporozoites proliferate asexually in the epithelial cells of the large intestine and in the last part of the small intestine to form merozoites and complete the first merogony period with the

disintegration of the epithelial cells. The released merozoites enter the new epithelial cells and multiply again in the form of merogony. This second stage of merogony is the stage in which the epithelial layer is destroyed, hemorrhages develop most severely and symptoms appear. After the merogony stage, merozoites reproduce sexually, differentiate and form microgametes and, macrogametes and leave the epithelial cells. Macrogametes and microgametes unite extracellularly to form oocysts covered with a durable membrane. The biological cycle of oocysts excreted with feces is completed in 11 to 22 days (Tufan and Çam, 2008).

Pathogenesis and clinical symptoms

Coccidiosis usually has a clinical course in very few animals in a barn. However, in some cases, the incidence of disease (morbidity) can reach 100%. Generally, the disease is not seen in enterprises where care and feeding are appropriate (enzootic stability). In these enterprises, the amount of oocysts is sufficient to create immunity, and thus, limited oocyst removal does not cause an epidemic of disease-causing nature (premunition). Since *Eimeria* species have different growth sites specific to the species, different lesions and appearances may occur during the course of the disease. Although *Eimeria* species are located in the epithelial tissue of the small intestine, large intestine lesions are noted in *E. bovis* and *E. zuernii*, which are located in deeper tissues. Resorption disorders occur in large intestine changes. Water loss with diarrheal stools, resorption disorder due to the inhibition of Na and Cl ions in plasma concentration develops, and dehydration is observed. Thus, the cause of death at the beginning of the disease is due to loss of plasma proteins and minerals, but later to anemia due to erythrocyte loss. In addition, epithelial disorders, mucosal thickening, and catarrhal inflammation of the small intestine are formed. Immunity occurs in survivors and in subclinical infections according to each *Eimeria* species. There is no cross-immunity between species. Antibodies detected in contaminated animal serum are not protective, T lymphocytes are responsible for immunity (Karaer et al, 2012; Denizhan and Kozat, 2022).

The disease is seen as acute, per acute and, subclinical. While clinical signs such as diarrhea, weight loss, and dehydration are observed in calves with acute coccidiosis, the disease progresses more sub clinically in older cattle (Göz and Aydın, 2015; Aslan et al., 2015). Acute coccidiosis first manifests itself with foul-smelling, serous dark green diarrhea. The anus of the animal is contaminated with feces. In the second period of acute coccidiosis, the patient's feed consumption decreases, there is a rapid weakening and he cannot get up in his bed. Other symptoms that can be seen are a body temperature of 40-41°C, an increased need for water, decreased appetite, dry skin, and erect hair. Since excessive bleeding is seen in the intestines at the last stage of acute coccidiosis, the stool is bloody and rectal examinations show that the rectal mucosa is thickened, heavy, hyperemic, and edematous (Denizhan and Kozat, 2022).

Immunity

Immunity in coccidiosis is species-specific. The elderly show immunity due to the infections they have had. Immunity is not seen in young people. The disease is more important for young people. In the first infections, the number of oocysts excreted with feces is high. In subsequent infections, the number of oocysts decreases as a result of the immune system. Because of the immune response, sporozoites and merozoites are prevented from entering the cell. Immunity continues as long as the coccidia agent is present in the body. For this reason, continuous reinfections are required for the continuation of immunity (Chappell, 2001).

The role of T cells in the immune response against coccidiosis is very important. Because cellular immunity plays an important role in the immune response mechanism. First-generation meronts show strong antigenic stimuli. Serum antibodies (IgM, IgG1, IgG2) against 1st generation merozoite antigens are formed in animals infected with *E. bovis*. In particular, secretory IgA contributes to protective immunity. Humoral antibodies play a smaller role in protective immunity (Arslan and Sarı, 2010).

CD4+ (helper T lymphocytes) in initial infections with *Eimeria* species, and later infections; CD8+ (cytotoxic T lymphocytes) T lymphocytes are more important in protective immunity, and especially Th1 type cells play a role in cellular immunity. The effects of T lymphocytes are mediated by cytokines. These cytokines are interleukin-2 (IL-2) and interferon-gamma (IFN γ) secreted by lymphocytes activated by parasite antigens. Th1 cells activate cytotoxic CD8+ T and NK (natural killer) cells by secreting IL-2, and macrophages to produce nitric oxide through IFN γ. Macrophages activated with cytokines cause the destruction of sporozoites in the cell or show their effect by preventing the formation of meronts (Karaer et al., 2012).

Diagnosis

In its diagnosis, the age of the animal, the hygienic condition of the environment, and clinical signs are

evaluated. Clinical findings and stool consistency in calves and calves are also important in diagnosis. The stool is scored according to its consistency. Accordingly, stool; It is classified as normal (zero-0), soft (one-1), diarrhea (two-2), diarrhea and tissue residue (three-3), diarrhea, bloody and tissue residue (four-4). The stool is examined using the flotation method using various concentrated solutions ($ZnSO_4$, $ZnCl_2$, sugar water, $NaNO_3$, NaCl). With the McMaster technique, the number of oocysts in gram stool is determined. In addition, oocysts are sporulated in 2.5% potassium dichromate solution ($K_2Cr_2O_7$) for species identification (Aydenizöz et al., 1999).

Species identification can be made easily in bovine coccidiosis by taking into account the morphological features of non-sporulated and sporulated oocysts by stool examination. The shape and size of the oocyst is very important. Oocyst sizes range from 8 to 54 mm. The smallest oocysts are *E. subspherica* (8-14x8-13 mm); the largest ones belong to *E. bukidnonensis* (33-54x24-41 mm). The presence of oocyst in the stool does not mean coccidiosis. The presence of oocysts belonging to pathogenic species and clinical bloody diarrhea increases the possibility of coccidiosis. The absence of oocysts in the stool does not mean that there is no coccidiosis. Because especially in *E. zuernii* and *E. bovis* infections, pathological disorders occur before oocysts are excreted. By performing a necropsy, macroscopic lesions in the intestines are examined to support the diagnosis. In terms of meronts and gamonts, which are the developmental stages of Eimeria, histopathological preparations are prepared and examined microscopically. However, a necropsy for a definitive diagnosis of bovine coccidiosis is not economical and is not recommended. Only dead animals, if any, are necropsied. Clinically bloody diarrhea, oocyst counts in gram stool over 5.000-10.000, and detection of oocysts belonging to pathogenic species indicate clinical coccidiosis cases. In addition, species-specific molecular diagnostic methods (Polymerase Chain Reaction) have been developed. Coccidia oocysts begin to appear in calves' feces from 14 days of age. Oocyst density usually increases when they are one month old. Animals with clinically soft stools or bloody diarrhea have higher oocyst counts than those with normal stools. When making the differential diagnosis of coccidiosis in cattle, infections such as Clostridial enteritis, Salmonella spp., Escherichia coli, Bovine Viral Diarrhea, Rotavirus, *Campylobacter* spp., Coronavirus, giardiasis, cryptosporidiosis and intestinal helminthosis should also be considered, especially with diarrhea. Since clinical symptoms are formed before oocyst expulsion, repeated stool examinations should be performed by flotation method (Karaer et al., 2012).

Treatment of the disease

The treatment of coccidiosis is based on the principles of killing the causative agent or preventing its development, eliminating fluid loss, and treating secondary infections (Arslan and Sarı, 2010; Arslan 2001). In establishments with high density and large numbers of animals, medication should always be administered with food or by adding it to water (Chartier and Paraud, 2012). In such applications, the aim is a method of protection against coccidiosis before animals are infected and damaged. Not all types of coccidiosis in an animal show the same sensitivity to any drug. For this reason, it is important to determine the species for the treatment of the disease to be successful. When the risk factors are known and eliminated in the control of the disease, the disease can be prevented without using coccidiostatic drugs (Denizhan and Kozat, 2022).

Treatment of coccidiosis in calves requires drug treatment for all animals living in groups. The reason for this is that when the symptoms of the disease begin to appear, the deterioration of the intestinal epithelium in cattle is considered too late for treatment. It is very important in supportive treatments together with the treatment for the causative factors. Drugs used against coccidiosis are generally used with water, milk, and feed (Arslan and Sarı, 2010).

Since the clinical symptoms begin to appear in the last stage of the infection, it is necessary to pay attention to this situation in the treatment. 17-18 days of infection in *E. zuernii* and *E. bovis* coccidiosis. The first clinical symptoms are seen on the first day of life, therefore, the sooner treatment is started, the more successful the results. If several animals in the herd are sick, all animals should be treated together. Anticoccidial drugs and sulfonamide group antibiotics are used in the treatment of coccidiosis (Denizhan and Kozat, 2022).

While sulfonamides are used in combination with trimethoprim in clinical coccidiosis cases, sulfadimidine is used in advanced cases and emergencies. Toltrazuril is an effective and primarily used drug in the treatment of clinical coccidiosis in cattle. In the treatment of coccidiosis in calves naturally infected with *E. zuernii* and *E. bovis*, oral administration of 15mg/kg dose of toltrazuril is reported to be very effective and reduces economic losses by positively affecting the growth performance of animals. Since anticoccidial drugs generally affect meronts, this situation should be considered in treatment (Veronesi et al., 2011).

Table 1. Anticoccidial drugs (Denizhan and Kozat, 2022)

Active ingredient	Purpose of usage, dosage, etc.
Sulfaquinoxalin	15 mg/kg, 4 days, peros
Sulfamethazin	50-100 mg/kg, 4 days, peros
Sulfaguadin	100 mg/kg, 3 days, peros
Sulfathiazol	150 mg/kg, 3-6 days, peros
Sulfadimidin	50-100 mg/kg, peros
Sulfadimethoxine	55 mg/kg(first day), 27.5 mg/kg 4 days, peros
Toltrazurile	10 mg/kg, 2 times a day, 2 days, peros
Furazolidone	15-30 mg/kg, 3-7 days, peros
Amproliume	10 mg/kg, 5 days, peros

Disease prevention and control

Protection and control from coccidiosis is possible with some measures to be taken in barns and pastures. Ensuring the necessary hygienic conditions in the barn; In pasture, it is provided by using the pasture alternately with other animal species (horse, sheep, or cattle). Among the most important factors to be considered in the prevention of coccidiosis are herd management, care-feeding, and hygiene conditions. Young and old animals should be reared in separate compartments, especially calves should be separated from their mothers within 24 hours after birth and kept in separate compartments. The number of animals should not exceed the barn's capacity. This situation should also be considered in terms of the number of animals in the paddocks in semi-open and closed enterprises. Especially in dairy farms, in farms where the number of calves is high, animals should not be kept crowded in paddocks in the front of the barns (Göz et al., 2006).

Contamination of feed and drinkers with feces should be prevented in the barns, the walls should be made of solid concrete, there should be no cracks and crevices, the litter should be cleaned every morning and evening before feeding, animals should not be allowed to eat fodder such as hay and grass from the ground. The temperature of the barn should not exceed 18oC and it should be ventilated to prevent the environment from being humid. Care should be taken when the calves are left on the pasture during the post -weaning period and calves should not be left on the pasture in bulk. Calves should also be checked for gastrointestinal nematode, bacterial, and viral infections. Chemoprophylaxis and immunoprophylaxis are also important in the control and protection of coccidiosis in cattle. Anticoccidial drugs are used in chemoprophylaxis for prophylactic purposes. Anticoccidial drugs for prophylactic purposes should be used for calves up to 6 months of age especially during their laying in pasture, at the time of transfer from single to bulk compartments, and during weaning. Immunoprophylactic methods such as vaccination are practically not applied in bovine coccidiosis. Despite the prevention and control methods, the eradication of coccidiosis and the protection of animals are quite difficult. However, clinical cases are prevented and economic losses are reduced. Unsporulated oocysts lose their vitality in 4 days with less than 25% relative humidity and sunlight. Most of the oocysts lose their infective properties in an average of 60 days at -8 ^{o}C and all within 24 hours at -30 ^{o}C. High temperature (48 ^{o}C) causes shrinkage of oocysts and these types of oocysts cannot become infective because they cannot pass through the sporogonic growth stage. 5% phenol, 1% ammonia, 1.25% sodium hypochlorite, 25% formaldehyde, and 0.5% creosol are used in farm disinfection, which is lethal on oocysts (Karaer et al., 2012).

Conclusion

In studies conducted in our country, 11 *Eimeria* and 1 *Isospora* species have been found in cattle. In studies on bovine coccidiosis in Turkey, the prevalence of *Eimeria* species in calves and calves was found to be 16-93.3%.

In line with the results obtained, it has been observed that coccidiosis continues to be a problem even in cattle farms with relatively good care and feeding conditions in Turkey. It was concluded that routine checks should be made in the enterprises, necessary precautions should be taken and hygiene rules should be followed.

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Protective effects of vitamin C on hormonal level and testicular histopathology of rabbit bucks with metronidazole-induced toxicity

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ABSTRACT

Objective: This study evaluates the protective effects of Vitamin C on hormonal level and testicular histopathology in rabbit bucks with metronidazole-induced toxicity. Methods: Twenty adult rabbit bucks which were weighed and divided into four groups with five in each group were used for the study. Group I is the control, group II was given metronidazole 400 mg/kg/day for 30 days, group III was co-administered 400 mg/kg/day of metronidazole and 200 mg/kg/day of Vitamin C for 30 days and group IV was given 200 mg/kg/day Vitamin C for 30 days. At the end of the experiment, the rabbit bucks were weighed, and blood samples were collected from the marginal ear vein into a plain bottle and serum extracted through centrifugation for hormonal assay. FSH, LH and Testosterone assay were carried out using Enzyme Linked Immunosorbent Assay (ELISA) kits according to manufacturers' instruction. One testis from each rabbit was removed for testicular histology. Results: The study found out that there was no significant difference in the body weights of the rabbit bucks before and after the experiment, metronidazole significantly ($p < 0.05$) affects hormonal concentration in the bucks and there was significant improvement following vitamin C administration. The study also found out that metronidazole caused testicular degeneration which was reversed by Vitamin C administration. Conclusion: Vitamin C has protective effect against metronidazole-induced toxicity and its use in therapeutic application in prolong use of metronidazole is recommended.

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Introduction

Metronidazole (MTZ) is a pharmaceutical agent with antiprotozoal and antibacterial properties, which finds widespread application in the field of veterinary medicine. The antibiotic has been utilised in human and veterinary medicine for the therapeutic management of trichomoniasis, giardiasis, amebiasis, and anaerobic bacterial infections (Bergan, 1985). However, the prolonged utilisation of the drug has been documented to induce adverse effects on reproductive functions in rabbits (Foote, 2002). According to Castellini (2008), reproductive toxicity has been associated with many negative impacts on the reproductive system, such as diminished fertility rates, compromised semen quality, reduced hormone concentration, and other detrimental effects. According to a study conducted by Rhayf et al. (2014), it has been documented that metronidazole can induce reproductive toxicity in male rabbits. According to Rhayf et al. (2014), the administration of metronidazole resulted in a notable reduction in the weight of the testes, epididymis, and seminal vesicles. Additionally, it led to a decline in sperm count, motility, viability and reduced testosterone concentration. In a study conducted by Oyedeji et al. (2015), notable alterations were observed in the viability of sperm and a slight degradation of the germinal epithelia of the testes in Wistar rats.

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According to Roy et al. (2014), Vitamin C (Vit C) exhibits strong antioxidant properties and has demonstrated a protective influence against genotoxicity caused by metronidazole in mice. Vitamin C has been found to have a protective effect on spermatogenesis and is essential for maintaining semen integrity and fertility in both humans and animals. Additionally, it has been observed to elevate blood testosterone levels (Fernandes et al., 2011; Sonmez et al., 2005). According to a study conducted by Benabbou et al. (2017), the administration of Vitamin C has been found to have beneficial effects in mitigating the adverse effects of different drugs and substances on the reproductive system. Furthermore, it has been demonstrated in a study conducted by Sadeghzadeh et al. (2018) that vitamin C has the ability to mitigate the detrimental impacts of dexamethasone on sperm motility, testosterone levels, and spermatogenesis indexes in mice. According to a study conducted by Akorede et al. (2020), the administration of vitamin C had a protective effect against the modification of many parameters associated with oxidative alterations, sex hormones, sperm characteristics, relative pituitary and testicular weight, as well as histological changes.

Previous studies have suggested that vitamin C supplementation is beneficial in reducing the toxic effects of various drugs and chemicals on the reproductive system (Sadeghzadeh et al., 2018, Akorede et al., 2020, Hajjar et al., 2020). However, the potential of vitamin C in reducing the reproductive toxicity induced by metronidazole has not been thoroughly investigated, hence, the need for this study to evaluate the protective effect of Vitamin C on the hormonal level and testicular histopathology of male rabbits exposed to metronidazole.

Materials and Methods

Ethical approval

Ethical approval was sought from University of Ilorin Research Ethics Committee with approval No.: UREC/ FVM/PG20/68VO002 before commencement of the research and ethical guidelines for the use of animal was strictly followed.

Experimental animals and treatment

A total of twenty mature and apparently healthy, domestic rabbit bucks were used for this study. The rabbits had an average weight ranging from 1.6 to 1.8 kg. They were housed at the rabbit unit of the Faculty of Agriculture Research and Demonstration Farm of Federal University Wukari. The rabbit bucks were given unrestricted access to both feed and water. Prior to the start of the experiment, they were allowed to acclimatized for two weeks. The twenty rabbit bucks were divided into four groups with five bucks in each group. Group I was control, group II rabbit bucks were given oral administration of metronidazole (400 mg/kg BW/day) for 30 days, group III were co-administered 400 mg/kg BW/day of metronidazole and 200mg/kg BW/day oral dose of vitamin C for 30 days and group IV were given 200mg/kg BW/day dose of vitamin C for 30 days. Vitamin C tablet (Em-Vit-C® from Emzor Pharmaceuticals Nig Ltd) and metronidazole tablets (Metrone® 200 from Fidson Healthcare Plc) were purchased from New World Pharmacy in Wukari, Taraba State, Nigeria for the experiment. The drugs were first made into a solution by dissolving them in distilled water using 1ml of distilled water for each tablet before use. At the end of the treatment period, the rabbit bucks were weighed, and 3 mL of blood was obtained using a 5 mL syringe and a 23 G needle from the marginal ear vein of each rabbit. The blood was collected into a plain sample container. The blood samples were centrifuged at 3000 rpm for 5 minutes in order to obtain sera samples, which were subsequently used for hormonal level analysis. The rabbits were subjected to a humane slaughter, following which the testes were extracted using a scalpel blade and subsequently preserved in a solution of 10% formalin for testicular histology.

Evaluation of sex hormones concentration

Serum FSH, LH and testosterone were measured using Enzyme Linked Immunosorbent Assay (ELISA) kits obtained from Elabscience® (Texas USA) according to the manufacturer's instructions provided with the kits.

Evaluation of histological changes

The histological examinations of the testes of the rabbits were conducted using the methodology of Shyr et al. (2002). Each subject's testicular tissue was routinely prepared for paraffin embedding after being preserved in 10% formalin for 48 hours. Hematoxylin and eosin (H and E) staining was applied to the embedded tissues after they were serially sectioned using a Rotary Microtome at a 4µm thickness. The tissues were then treated in an alcohol-xylene series. The prepared slides were examined under the microscope at a magnification of x 400.

Statistical analysis

GraphPad Prism Version 5.03 for Windows developed by Graphpad Software in San Diego, California, USA was used to analyze the data. The concentration of sex hormones data was presented as mean ± SEM. They were tested using Tukey's post-hoc multiple comparison test after undergoing a one-way analysis of variance (ANOVA). Every value at $p < 0.05$ was deemed significant.

Results

Effects of Vitamin C on body weight of rabbit bucks exposed to metronidazole toxicity: Table 1 presents the mean (± SEM) values of body weight of rabbit bucks before commencement of experiment and after the experiment. The mean body weight did not differ significantly ($p>0.05$) among the groups before and after the experiment. The body weight for the control, MTZ, MTZ + Vit C and Vit C groups were 1.72, 1.62, 1.66 and 1.69kg respectively after the experiment.

E**ffects on sex hormonal concentration**

Effect of treatment on follicle stimulating hormone (FSH) concentration: The result in Table 2 shows the mean follicle stimulating hormone (FSH) concentrations in the different treatment groups. FSH concentration was significantly ($p < 0.05$) higher in the control group when compare to MTZ and MTZ + Vit C groups. Also, FSH concentration was significantly lower in MTZ group when compare with Control, MTZ + Vit C, and Vit C groups. FSH concentration was significantly ($p < 0.05$) lower in MTZ + Vit C when compare with Vit C group. The result indicates that MTZ caused a significant ($p < 0.05$) decrease in the FSH concentration as compared to the control group. There was significant improvement in the MTZ + Vit C group.

Effect of treatment on luteinizing hormone (LH) concentration: Table 2 also shows the mean Luteinizing Hormone (LH) concentrations in the different treatment groups. The LH concentration was significantly ($p < 0.05$) higher in the control group when compare to MTZ and MTZ + Vit C groups. The mean LH concentration in the MTZ group was significantly ($p < 0.05$) lower when compare to that of Control, MTZ + Vit C, and Vit C groups. Mean LH concentration in MTZ + Vit C was significantly ($p < 0.05$) lower when compare with Vit C group. The result indicates that MTZ caused a significant ($p < 0.05$) decrease in the LH concentration as compared to the control group. There was significant improvement in the MTZ + Vit C group.

Effect of treatment on testosterone concentration: Table 2 also shows the mean testosterone concentrations in the different treatment groups. The mean testosterone was significantly ($p < 0.05$) higher in the control group when compare to the MTZ and MTZ + Vit C groups. The mean testosterone concentration in the MTZ group was significantly ($p < 0.05$) lower when compare to that of control, MTZ + Vit C, and Vit C groups. Testosterone concentration in MTZ + Vit C group was significantly ($p < 0.05$) lower when compare with Vit C group. The result indicates that MTZ caused a significant ($p < 0.05$) decrease in the testosterone concentration as compared to the control group. There was significant improvement in the MTZ + Vit C group.

Effects of treatment on testicular histology: From the result in Figure 1, the photomicrograph showed a structure of the testis tissue, and the germinal epithelium was observed to be intact and normal in the control group (Plate A). In the MTZ group (Plate B), there were irregular and degenerated seminiferous tubules and a less compact arrangement of spermatogenic cells was observed in the structure of the testis. This could be due to the effect of the metronidazole on the testes. Meanwhile, the MTZ + Vit C group (Plate C) presented restoration of the germinal cells of the seminiferous tubules. Moreover, the Vit C group (Plate D) showed regular seminiferous tubules similar to the control group. Vitamin C was seen to have improved the histological changes observed in the MTZ group

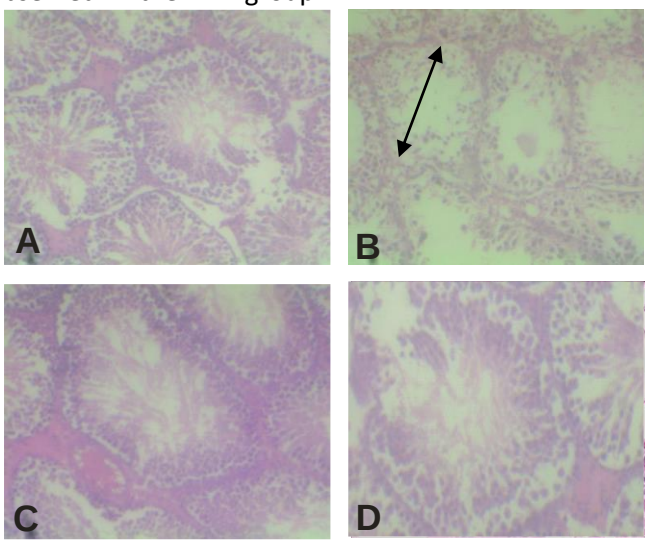

Figure 1: Photomicrograph of the testis of rabbit bucks exposed to Metronidazole and Vitamin C
A: Control, B: MTZ, C: MTZ + Vit C, D: Vit C (H&E x400). A: No visible histological changes. B: Degenerative changes in the seminiferous tubules (Black arrow), less populated with spermatogenic cells, C: Restoration of seminiferous tubules histoarchitecture, D: No visible histological changes.

Table 1. Effects of Vitamin C on body weight of rabbit bucks exposed to metronidazole toxicity

	Control	MTZ	MTZ + Vit C	Vit C
Before Experiment	1.69 ± 0.04	1.61 ± 0.03	1.65 ± 0.06	1.67 ± 0.04
After Experiment	1.72 ± 0.04	1.62 ± 0.04	1.66 ± 0.05	1.69 ± 0.09

($p >0.05$)

Table 2. Mean ± SEM of serum FSH, LH and testosterone concentrations in different treatment groups

	Control	MTZ	MTZ + Vit C	Vit C
FSH (mIU/mL)	0.836 ± 0.01[a]	0.296 ± 0.04[b]	0.689 ± 0.03[c]	0.852 ± 0.03
LH (mIU/mL)	0.833 ± 0.02[a]	0.331 ± 0.03[b]	0.675 ± 0.03[c]	0.879 ± 0.03
Testosterone (ng/mL)	2.859 ± 0.02[a]	1.674 ± 0.05[b]	2.470 ± 0.05[c]	2.918 ± 0.04

MTZ = Metronidazole. MTZ + Vit C = Metronidazole + Vitamin C. Vit C = Vitamin C. a = ($p < 0.05$) higher when compare with MTZ and MTZ + Vit C groups. b = ($p < 0.05$) lower when compare with Control, MTZ + Vit C and Vit C groups. c = ($p < 0.05$) lower when compare with Vit C group.

Discussion

The study found out that there was no significant difference in the body weight of rabbit bucks administered metronidazole and vitamin C before and after the experiment (Table I). This could be due to the absence of androgenic properties in the metronidazole since it has been reported that androgen possess anabolic activities (Johnson & Everitt, 1998). It could also be due to absence of anorectic and lipolytic properties in this drug (Carvajal et al., 2009). This finding is similar to that of Oyedeji et al. (2015) and Kumari & Singh (2015) who found in their separate researches no significant changes in the body weight of rabbit bucks exposed to metronidazole.

In comparison to the Control, MTZ + Vit C and Vit C treated groups, the FSH and LH concentrations were significantly ($p < 0.05$) lower in the MTZ treated group. This finding is in line with the findings of Davood et al. (2007), who observed a substantial decrease in rats treated with 400 mg/kg of metronidazole as compared to the non-treated control group. Grover et al. (2001) similarly observed a comparable decrease in FSH and LH concentrations after metronidazole treatment. The inhibition of gonadotropic releasing hormone (GnRH) secreted by the hypothalamus by metronidazole may be the cause of the decreased serum concentrations of FSH and LH in the metronidazole-treated group. GnRH aids in releasing gonadotropins (FSH and LH) from the anterior pituitary.

The development of accessory reproductive organs and the process of spermatogenesis are androgen dependent. The quantity and functional state of mature Leydig cells will decline as androgen production declines (Osuntokun et al., 2017). In males, FSH controls seminiferous tubules. Because it maintains Sertoli cell activity, which in turn maintains many aspects of sperm cell maturation, follicle-stimulating hormone (FSH) is also critical to sperm production (Egba et al., 2014). Diminished gonadal function (hypogonadism) may be the outcome of decreased LH or FSH production. Males with the disorder usually show up as lacking a normal number of sperm Egba et al., 2014). Spermatogenesis is directly stimulated by FSH when it binds to receptors in sertoli cells (O'Donnel et al., 1994). According to O'Donnel et al. (1994); Singh et al. (1995), LH stimulates Leydig cells to secrete more testosterone. This testosterone may then act on the Sertoli and peritubular cells of the seminiferous tubules, indirectly stimulating spermatogenesis.

Serum FSH and LH concentrations were found to have significantly improved with supplementation with vitamin C. Sadeghzadeh et al. (2019) have reported that vitamin C can enhance the levels of FSH and LH after dexamethasone administration-induced oxidative damage. By reducing the damage caused by oxidative stress, vitamin C can increase the concentration of glutathione (GSH) and the activity of testicular antioxidant enzymes like catalase and superoxide dismutase, which are vital for sperm survival (Olorunshola et al., 2011, Ekaluo et al., 2013).

This study also showed a decrease in the testosterone serum concentration in the MTZ treated group in comparison to the Control, and MTZ + Vit C and Vit C groups. The results of earlier research (Davood et al., 2007, Samah, 2012, Oyedeji et al., 2015, and Kumari & Singh 2015) are consistent with the finding. Testosterone levels were significantly reduced by metronidazole. This could mean that the drug prevents the mechanism that interferes with the Leydig cells' ability to synthesise testosterone. The inhibitory effect of metronidazole on pituitary gonadotropin (FSH and LH) production may be the cause of the decreased serum testosterone levels in the metronidazole group. Similar to this, direct damage to Leydig cells—possibly as a result of oxidative injury—may account for the decrease in testosterone concentration observed in the metronidazole-treated group (Oliva & Miraglia, 2009). Additionally, it's possible that metronidazole causes a decrease in testosterone concentration because it causes the liver's aromatase enzyme to be produced. The enzyme which converts testosterone to estradiol thereby reducing testostosterone concentration (Vijay et al., 2009).

The testosterone concentration improvement seen in the MTZ + Vit C group may indicate that vitamin C helps to mitigate testicular changes caused by metronidazole. This is because vitamin C possesses

antioxidant properties that have been shown to protect biological tissues from reactive oxygen species. Because vitamin C affects the hypothalamic-pituitary-testicular axis, which raises blood testosterone levels, it may also be related to the improvement in hormone concentration in the MTZ + Vit C (Ashamu et al., 2010). Vitamin C or E deficiency causes oxidative stress in the testes, which interferes with testosterone production and spermatogenesis (Rekha et al., 2009).

When compared to the control and vitamin C groups, histological analysis of the testes of rabbit bucks treated with metronidazole showed increased interstitial space and degradation in the spermatogenic cells lining the seminiferous tubule. The current study confirms the observations of Samah (2012), who reported that metronidazole altered the rat seminiferous tubules. The metronidazole crosses the blood-testis barrier and reaches the germ cells in the seminiferous tubules may be the source of this cell injury and histological changes (Dixon & Lee, 1977). There is evidence that metronidazole damaged DNA strands, causing cell necrosis and death (Samah, 2012). Saad et al. (2018) observed a similar histological effect on the seminiferous tubules. Additionally, Kumari & Singh (2015) found that after administering metronidazole, laboratory mice had altered testicular histology, impaired spermatogenesis, and had smaller seminiferous tubules. The erosion of germinal epithelia has been reported by Oyedeji et al. (2015), which is consistent with the results of our investigation. Given that spermatogenesis is reportedly triggered by testosterone, which is produced by Leydig cells and acts on Sertoli cells and peritubular cells, the low population of germinal epithelium may be the result of insufficient testosterone (Sharpe, 1987).

Additionally, the study revealed that the histoarchitecture of the testes of rabbit bucks in the MTZ + Vit C group was gradually restored. The antioxidant properties of vitamin C might be the cause of this. This aligns with the research conducted by Sadeghzadeh et al. (2018), which demonstrated that Vitamin C can reverse the negative histological changes, such as apoptosis in Leydig cells, degeneration of seminiferous tubules, and a decrease in spermatocyte and spermatid counts, in the testes of mice exposed to dexamethasone. Compared to the MTZ and MTZ + Vit C treated groups, the seminiferous tubule and germ cells in the Vit C group were significantly normal.

Conclusion

This study investigated the protective effects of vitamin C on hormonal levels and testicular 264 histopathology in rabbit bucks that were subjected to metronidazole-induced toxicity. The study found out that metronidazole caused significant deleterious effects on the hormonal concentration of rabbit bucks exposed to the drug for 30 days. The drug also caused degenerative effects on the histoarchitecture of the testes of rabbit bucks. The research has provided valuable findings regarding the therapeutic advantages of vitamin C in alleviating the adverse effects of metronidazole on the reproductive system of rabbit bucks. Vitamin C was fond to be a useful protective agent against the toxic impacts of metronidazole on the hormonal level and testicular histopathology of male rabbits. The study's findings on the protective properties of vitamin C have significant clinical significance in management of reproductive toxicity caused by prolong administration of metronidazole. The findings of the study could potentially guide further detailed research in clinical settings.

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Conflict of Interest

The authors have no conflict of interest.

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Association of the FTO gene with obesity and cancer in dogs

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Review Article

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ABSTRACT

Nowadays, obesity is one of the most serious problems that significantly affect health in both human and animal populations. Fat mass and obesity-associated gene (FTO), increases the risk of obesity and other metabolic diseases such as cancer, with taking part in many complex molecular pathways. On the other hand, environmental and genetic factors cause changes in FTO gene variants and expression levels, which result in phenotypic differences. Advanced knowledge on the genetic basis of human FTO gene and its association with cancer and obesity, has paved the way for the investigation of FTO gene in animals as well. In this review, we summarized current state of knowledge about the FTO gene, which is considered as an important marker of obesity in humans, as well as obesity, cancer and the association of FTO polymorphisms with these diseases in dogs by considering humans with other animal species. Understanding the molecular background of the FTO gene in dogs will be leading to the development of individual treatment methods and prediction of possible phenotypic effects in other species.

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Introduction

Obesity is defined by the World Health Organization (WHO) as an excessive or abnormal increase in fat mass that can lead to diseases such as cancer, cardiovascular disease, or diabetes mellitus (Phillips, 2013). The morbidity of this disease increases year by year in many countries and threatens human health (Ng et al., 2013). Especially in the past 20 years, increased sedentary lifestyle and excessive consumption of high-calorie foods have caused an increment in the prevalence of overweight and obesity in people approximately 3 times. Thus, obesity has become a major problem in both developed and developing countries (Finucane et al., 2011; Haidar and Cosman, 2011). The fact that studies have reported that more than 1.9 billion adults were overweight in 2016, of whom 650 million were obese, and this number is expected to increase to 1.12 billion by 2030, clearly highlights the seriousness of the obesity problem. (WHO, 2023).

The inordinate accumulation of adipose tissue leading to obesity is a growing problem in both animal populations and humans (Switonski and Mankowska, 2013). According to the 2009-2010 National Pet Owners Survey conducted by Pet Products Manufacturers Association there are 77.4 million purebred and crossbred dogs in USA with 39% of households have at least 1 dog and 24% of them have 2 dogs. Considering this the increase in body weight of pets, especially animals such as cats and dogs that share the same environment with humans, it is usual to be positively correlated with the owner's Body Mass Index (BMI) and to encounter the problem of obesity for these animals (Nijland et al., 2010; Davis and Ostrander, 2014). In addition, after it was reported that 22% of dogs visiting veterinary clinics were overweight and 1% were obese in 1991, the number of overweight dogs increased to 34-59% and obese dogs to 5-10% in about 20 years, this proves that obesity is a year by year growing problem in dogs as well as in humans (Kronfeld et al., 1991; McGreevy et al., 2005; Colliard et al., 2006; Lund et al., 2006; Courcier et al., 2010).

In this review, it is aimed to explain obesity and cancer diseases in dogs, which are one of the pets that share the common environment with humans, and to explain the molecular basis of the FTO gene in dogs, which can cause these diseases.

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Obesity in dogs

Dogs are considered overweight when their body weight exceeds 15% of the optimal weight, and they are considered obese when it exceeds 30% (German, 2006). The reproductive and health status of dogs is significantly affected in the presence of obesity. Furthermore, obesity also leads to the triggering of some diseases such as fatty liver, metabolic syndrome, heart diseases, bone disorders, and cancer, or an increased risk of developing these diseases (Switonski and Mankowska, 2013).

In addition to environmental factors such as nutrition, gonadal status, and inadequate physical activity, individual genetic factors such as age, breed, and gender also play a role in the emergence of the obesity problem (Robertson, 2003; Courcier et al., 2010; Hess and Bruning, 2014).

Between 30% and 70% of common obesity is inherited (Huđek et al., 2017). Gene-dependent obesity can be monogenic, syndromic, oligogenic, or polygenic. However, obesity is mostly a polygenic condition. The mechanism underlying polygenic obesity is quite complex and is based on the intricate interactions between the gene itself and the gene-environment relationship (Huvenne et al., 2016). Studies have shown that while there is no significant gender-obesity relationship in mostly unneutered dogs, spayed animals, especially females, tend to be more prone to obesity. Additionally, it has been noted that the risk of obesity increases with age (Robertson, 2003; Lund et al., 2006; Courcier et al., 2010).

The difference between breeds is one of the most significant genetic factors influencing obesity prevalence in dogs. Depending on age, body composition, which is the ratio of fat body mass (FBM) to lean body mass (LBM), varies between breeds (Speakman et al., 2003). In a study by Speakman et al. (2003) comparing metabolism and body composition in three different dog breeds based on age, it was noted that FBM and LBM were not age-related in Labrador and Papillon breeds, while FBM increased with age in Great Dane breed. The same study also indicated that resting metabolic rate (RMR) decreased with age in all three dog breeds examined. Another study suggested that the varying predisposition of obesity between breeds could be attributed to changes that occur during the selection process in genes related to fat metabolism associated with body composition and fat mass (Jeusette et al., 2010). Population studies have reported that Beagles, Cocker Spaniels, Dachshunds, Rottweilers, Shetland Sheepdogs, Dalmatians, and Retrievers are prone to obesity, supporting the hypothesis proposed by Jeusette et al. (Kronfeld et al., 1991; Colliard et al., 2006; Lund et al., 2006). This situation indicates the interbreed predisposition differences demonstrate that in some breeds the gene pool contains DNA polymorphisms that may be responsible for obesity (Switonksi and Mankowska, 2013).

Cancer disease in dogs and its relationship with obesity

Cancer is an extremely serious disease in dogs, affecting approximately 4 million dogs annually and being the most common cause of death in dogs of all ages, particularly those over the age of 10 (David and Ostrander, 2014; Gardner et al., 2016). While 50% of older dogs are diagnosed with cancer, one-fourth of these cases result in death (Bronson, 1982; Vail and MacEwen, 2000; Adams et al., 2010; Dobson, 2013; ACF, 2023).

Registrations regarding cancer in dogs are predominantly retrospective due to the inherent nature of the disease, and they are often limited to a defined patient population (Bronson, 1982; Dobson et al., 2002). In a retrospective necropsy study conducted by Bronson (1982) on 2,002 dogs, it was reported that 45% of dogs aged 10 and above, and 23% of dogs of all ages, died due to cancer and this disease was one of the leading causes of death in dogs. Data from the Genoa Animal Tumor Registry suggests an estimated cancer incidence in dogs ranging from 99.3 to 272.1 cases per 100,000 dogs (Merlo et al., 2008).

There are many differences among breeds regarding predisposition to specific types of cancer. For instance, breeds like Boxers, Golden Retrievers, French Bulldogs, Boston, and Rat Terriers are severely prone to the brain and central nervous system cancers, while this risk is low in Cocker Spaniels and Doberman Pinchers. Additionally, meningiomas are more common in dolichocephalic (long-nosed) breeds, while brachycephalic (flat-nosed) breeds are more prone to gliomas (Song et al., 2013). As multiple dog breeds are at risk for the same type of cancer, these breeds are likely to share an underlying genetic predisposition (Davis and Ostrander, 2014).

Obesity has been reported to increase the risk of at least 13 different types of cancer, including esophageal adenocarcinoma, colon cancer, endometrial cancer, kidney cancer, hematopoietic cancers, and postmenopausal cancers in humans (Calle and Kaaks, 2004; Goodwin and Stambolic, 2015). According to a study conducted in the USA, in 2003, 14% of men and 20% of women who died from cancer, particularly colon, rectum, esophagus, kidney, pancreas, gallbladder, ovary, endometrium, liver, prostate, and hematological malignancies, were overweight or obese (Calle et al., 2003).

There are several mechanisms that can explain the relationship between obesity and increased cancer risk. Obesity is associated with the compensatory increase of hyperinsulinemia in the production of insulin resistance and growth factors. This first stimulates mitogenesis and consequently carcinogenesis, multiple signaling pathways, oxidative stress, and inflammatory processes (Sarfstein et al., 2013). In an epidemiological study linking obesity and cancer, it was reported that the cancer risk in obese women is 50% higher than in women with normal weight (Calle and Kaaks, 2004).

History of the FTO gene

The FTO gene, identified through Genome-Wide Association Studies (GWAS), is considered the most powerful predictor of polygenic obesity today. It can influence the development of obesity by regulating fatty acid transport and fat metabolism and is the first obesity-related gene to be described. (Loos and Bouchard, 2008; Loos and Yeo, 2014). This gene was first cloned through exon capture analysis in mice with the 'fused toes (Ft)' mutation in 1999, and it was identified as one of the six genes deleted in these mice (Peters et al., 1999). Since homozygous mice with the Ft mutation displayed severe developmental anomalies resulting in embryonic death and heterozygous mice exhibited syndactyly and thymic hyperplasia, scientists initially considered that this gene might be associated with programmed cell death (Van Der Hoeven et al., 1994; Fischer et al., 2008). It was stated that the observed phenotype in mice occurred as a result of a 1.6 Mb deletion in chromosome 6, and three of the six deleted genes belonged to the IrxB (Irx3, Irx5, Irx6,) cluster of Iroquois family genes, while the other three had no known function, i.e. dysfunctional. Thus, the first of the genes were named Ft1 (later Fts), the second was named Phantom (Ftm) due to technical difficulties in its characterization, and the third was named Fatso (later FTO) because of the fact that mice with Ft mutation did not have any signs of obesity both metabolically and phenotypically, only because of the size of the gene (Fischer et al., 2008; Larder et al., 2011). However, in 2007, the FTO gene was identified as an obesity susceptibility gene through GWAS, and single nucleotide polymorphisms (SNPs) located in intron 1 were reported to be strongly associated with BMI, body fat ratio, waist circumference, hip circumference, and energy intake (Dina et al., 2007; Frayling et al., 2007; Scuteri et al., 2007). As a result, the gene in question was named a fat mass and obesity-associated (FTO) gene (Lan et al., 2020).

According to current genomic research, the FTO gene is found only in vertebrates and a few types of marine algae, along with highly conserved nucleotide and amino acid sequences, but not in invertebrates, fungi, and green plants (Robbens et al., 2008). Furthermore, the FTO protein is thought to have a similar function among all vertebrates as it is conserved with 85% sequence similarity between humans, mice, sheep, cattle, and horses (Fredriksson et al., 2008).

Association of FTO gene with obesity

The association between the FTO gene and obesity was first reported with GWAS, detecting that there are approximately 500,000 autosomal SNPs in a population of individuals with type 2 diabetes (T2D) in the UK (Frayling et al., 2007). In this study, an SNP (rs9939609) that has a strong relationship with both T2D and increased BMI was identified in the 1st intron of the gene in question was identified, and it was considered that this SNP caused a predisposition to diabetes by affecting the body weight. The authors noted that individuals homozygous for the risk allele (A) of this SNP were approximately 3 kg heavier than those homozygous for the non-risk allele (T), and these individuals have a 1.7 times higher risk of obesity. The association of FTO gene variants with obesity and other diseases is shown in Table 1 and Table 2.

After the publication of the initial study, the association of the FTO gene with obesity was primarily confirmed in European populations, and other SNPs such as rs9930506 (Scuteri et al., 2007), rs1121980 (Dina et al., 2007), and rs8050136 (Stratigopoulos et al., 2008) were identified.

Later, studies expanded to include populations from Africa, the Americas (such as Mexican-Mestizo), Afro-Americans, and Asia (such as Han Chinese and indigenous Oceanian), and the association of FTO variants with other metabolic diseases, particularly with obesity and T2D, across other ethnicities was evaluated (Ohashi, 2007; Li et al., 2008; Villalobos-Comparán et al., 2008; Hassanein et al., 2010; Li et al., 2013). Accordingly, in the study conducted by Hassanein et al. (2010) investigating the FTO locus in an African population, the strongest association with obesity was not observed with the SNPs rs9939609 or rs8050136, which are frequently examined in European populations, but rather with two less characterized SNPs, rs3751812 and rs9941349. Furthermore, in a study investigating the risk of Polycystic Ovary Syndrome (PCOS) associated with the rs9939609 variant of the FTO gene, Chinese women, as PCOS (741) and control group (704), were evaluated. The results of the study in which the samples were divided into 2 groups, obese PCOS patients and non-

Table 1. Association of FTO gene intronic variants with obesity and other diseases in humans (Reviewed from Hernández-Caballero and Sierra-Ramírez, 2015, Köksal, 2019, Kucher, 2020)

Locus	Location	Diseases	Description
rs9939609	Intron 1	Obesity	It causes an increase in BMI.
		T2DM	It causes T2DM development unrelated to BMI.
		PCOS	BMI unrelated studies have been reported to have a stronger effect.
		Endometrium Cancer	AA genotype increases the risk of endometrial cancer.
		Pancreas Cancer	There is a risk of pancreatic cancer in the A allele (BMI unrelated) and the AT genotype (BMI associated).
		Colorectal Cancer/ Adenoma	Associated with BMI.
		Lung Cancer	The AA genotype reduces the risk of lung cancer. (BMI related)
		Kidney Cancer	The AA genotype increases the risk of kidney cancer.
		Prostate Cancer	The A allele causes high-grade prostate cancer. (BMI related)
		Cardiovascular Disease	The A allele increases the risk of coronary heart disease, heart attack, and atrial fibrillation. (BMI related)
rs17817449	Intron 1	Obesity	It causes an increase in BMI.
		Colorectal Cancer/ Adenoma	G allele reduces the risk of colorectal adenoma. (BMI related)
		Breast Cancer	There is a risk of ER+ breast cancer. (BMI unrelated)
rs3751812	Intron 1	Obesity	The T allele causes an increase in BMI.
rs1421085	Intron 1	Obesity	It causes an increase in BMI.
		T2DM	Associated with BMI.
		PCOS	BMI unrelated studies have been reported to have a stronger effect.
rs9930506	Intron 1	Obesity	The A allele causes an increase in BMI.
rs8050136	Intron 1	Obesity	It causes an increase in BMI.
		T2DM	It causes T2DM development unrelated to BMI.
		Pancreas Cancer	There is a risk of pancreatic cancer in the AC genotype. (BMI related) There is a risk of pancreatic cancer in T2DM patients who are carriers of the A allele.
		Prostate Cancer	Polymorphisms reduce the risk of prostate cancer.
		Colorectal Cancer/ Adenoma	The A allele reduces the risk of colorectal adenoma. (BMI related)
rs7202116	Intron 1	Obesity	It causes an increase in BMI.
rs8043757	Intron 1	Obesity	The T allele causes obesity.
rs9928094	Intron 1	Obesity	A/G alleles cause extreme obesity.
rs9941349	Intron 1	Obesity	The T allele causes extreme obesity.
rs1558902	Intron 1	Obesity	A/T alleles cause obesity.
rs1121980	Intron 1	Obesity	A/T alleles cause extreme obesity.
		Breast Cancer	There is a risk of breast cancer in T/C alleles. (BMI unrelated)

Table 2. Association of non-obesity-related intronic variants of the FTO gene with other diseases in humans (Reviewed from Hernández-Caballero and Sierra-Ramírez, 2015, Köksal, 2019, Kucher, 2020)

rs7185735	Intron 1	T2DM	A/G alleles cause T2DM. (BMI related)
rs9936385	Intron 1	T2DM	The C allele causes T2D. (BMI related)
rs12933928	Intron 1	Melanoma	It increases the risk of melanoma.
rs6499640	Intron 1	Endometrial Cancer	There is a risk of endometrial cancer in the A allele. (BMI unrelated)
rs1477196	Intron 1	Breast Cancer	There is a risk of breast cancer in the GG genotype (BMI-related)
rs62048402	Intron 1	Breast Cancer	There is a risk of breast cancer in the A allele.
rs56094641	Intron 1	Nephropathy	The G allele causes nephropathy in patients with T2DM.
rs7187250	Intron 1	Cardiovascular Disease	It causes mineral density in the heel bone.
rs8044769	Intron 1	Osteoarthritis	The T allele causes osteoarthritis. (BMI related)
rs9930333	Intron 1	Osteoarthritis	G/T alleles cause osteoarthritis of the hip and knee. (BMI related)
rs9940128	Intron 1	Metabolic Syndrome	The A allele causes metabolic syndrome. (BMI related)
rs11642841	Intron 2	T2DM	The A allele causes T2DM.
rs11075995	Intron 2	Breast Cancer	There is a risk of ER- breast cancer. (BMI unrelated)
rs12932428	Intron 7	Melanoma	The risk of melanoma is increased.
rs12599672	Intron 7	Melanoma	The risk of melanoma is increased.
rs1125338	Intron 8	Melanoma	The risk of melanoma is increased.
rs16953002	Intron 8	Melanoma	It causes melanoma risk in the A allele (BMI unrelated)
rs56077980	Intron 8	Breast Cancer	There is a risk of breast cancer in the CT genotype.
rs7195994	Intron 8	Rheumatoid Arthritis	The A allele causes rheumatoid arthritis.
rs7187423	Intron 8	Rhinitis	It is associated with seasonal allergic rhinitis.

obese PCOS patients, revealed the relationship of the aforementioned variant with not only obesity but also non-obese cases in Chinese women (Li et al., 2013). Contrary to the initial studies that interpreted FTO's influence on obesity to potentially lead to metabolic diseases like T2D by affecting BMI (Frayling et al., 2007), this situation indicates that FTO might contribute to susceptibility to certain metabolic diseases independently of its effect on weight gain and that environmental or other genetic factors could lead to distinct relationships between the FTO gene and ethnic groups (Larder et al., 2011).

Function of the FTO gene

The physiological function of the FTO gene is not yet fully understood. Frayling et al. (2007) referred to the FTO gene as “a gene with an unknown function in an unknown pathway”. In sequence analysis, the FTO protein was predicted to have a double-stranded β-helix structure homologous to members of the non-heme Fe (II) and 2-oxoglutarate (2-OG) oxygenase superfamily (Clifton et al., 2006; Ozer and Bruick, 2007). Through advanced bioinformatics analyses, Gerken et al. (2007) revealed that the FTO gene encodes Fe(II)/2-OG dependent demethylase, which is the ninth protein found in mammals from the alpha-ketoglutarate dependent dioxygenase (AlkB) family, which is Escherichia coli DNA repair enzyme. Due to its homologous sequence with proteins from the AlkB family, this protein is also referred to as ALKBH9. Based on this, it has been hypothesized that FTO plays a significant role in DNA repair and post-translational modifications. Additional experiments have determined that FTO signals the cellular presence of oxygen, is functionally involved in fatty acid metabolism and energy metabolism, and has a role in the catalysis of nucleic acid demethylation (Han et al., 2010). In a study

2010). In a study conducted a few years later by Jia et al. (2012), it was stated that N6-methyladenosine (m6A) in nuclear RNA is the primary substrate of FTO. Therefore, FTO was initially described as the first RNA demethylase, opening the door for further research into both RNA epigenetic modifications and the functions of FTO proteins in the subsequent years (Wei et al., 2018).

While the FTO gene can influence the proliferation and differentiation of adipocytes, the FTO protein can regulate the growth and development of adipocytes as a transcriptional co-activator (Qiong, 2010; Ronkainen et al., 2015; Chen et al., 2017). It also plays a regulatory role in biological processes such as the regulation of animal β-cell function and oxidative stress (Bravard et al., 2011; Russell and Morgan, 2011). On the other hand, as the FTO gene regulates adipocyte differentiation and causes obesity, mutations in this gene may also affect growth characteristics in animals (Wang et al., 2021). For instance, in a study focused on Italian Duroc pigs, it has been indicated that the g.276T> G polymorphism in the pig FTO gene is closely associated with adipose tissue characteristics, marbleization, backfat thickness, and intramuscular fat content (Fontanesi et al., 2009). Another study investigating the relationship between FTO gene variants and growth and carcass characteristics in cattle indicated that the FTO gene had a significant impact on growth and carcass characteristics in Simmental and Brown cattle in the Slovenian population. In a statistical analysis conducted between these traits and 34 SNPs in the FTO gene, it was reported that there is a significant correlation between FTO variants and the percentage of lean muscle mass (Jevsinek Skok et al., 2016). Moreover, in a study focusing on the effects of insertion/deletion (InDel) polymorphisms in the FTO gene on fat tail size and growth traits in the Tong sheep, a breed famous for its fat-tailed characteristics in China, as a result of fat tail diameter and body measurements, a total of 10 InDel loci were identified in 75 sheep, 4 (InDel 4,5,7,8) of which were significantly correlated with tail fat accumulation, and 8 (InDel 1,2,3,4,5,7,8,10) were significantly associated with certain growth characteristics (Wang et al., 2021). In addition, there are studies reporting that genetic variations in the FTO gene can also affect growth characteristics in chickens and rabbits (Jia et al., 2012; Zhang et al., 2013).

Data from numerous human studies have suggested an association between various FTO risk alleles and increased energy intake. In the conducted studies, it has been reported that cases with at least one risk-free allele showed an increase in food consumption, more frequent eating, impaired satiety, and loss of eating control compared to individuals with homozygous risk-free allele (Speakman et al., 2008; Haupt et al., 2009; Tanofsky-Kraff et al., 2009; Wardle et al., 2009). In detailed analyses on this subject, the observed increase in energy intake among risk allele carriers is attributed to an increased tendency towards high-fat, energy-dense foods, rather than an increase in the quantity of consumed food (Cecil et al., 2008; Timpson et al., 2008). Furthermore, the role of the FTO gene in regulating energy balance and energy expenditure has been confirmed through experiments conducted on mice and rats (Fischer et al., 2009; Fredriksson et al., 2008). For example, a study conducted by Fischer and colleagues (2009) demonstrated that the absence of the FTO gene in knock-out mice leads to a drastic reduction in both fat tissue and lean body mass, resulting in postnatal growth retardation. Considering the studies, it is understood that the energy balance of the FTO gene is more effective on the "input" side (Larder et al., 2011). In a study examining the FTO gene locus for milk fat characteristics in Holstein dairy cattle, it has been reported that the FTO region regulates not only milk fat yield but also the total energy content of milk (Zielke et al., 2013).

Animal models

Animal models are crucial for understanding the role and effects of FTO in different anatomical regions (Larder et al., 2011). This gene is strongly expressed in the hypothalamus, the region of the brain that controls appetite behavior (Gerken et al., 2007; Stratigopoulos et al., 2008). In mice, the expression of the FTO gene in the hypothalamus increases after feeding with a high-fat diet (Tung et al., 2010). Due to the different amounts of transcripts obtained in the regions of the brain that control food intake, depending on food intake and deprivation, it has been suggested that the role of FTO in regulating body weight depends on the activity of FTO protein in these regions of the brain (Fredriksson et al., 2008). In studies on pigs and sheep, it has been reported that FTO expression is significantly higher in the cortex, hippocampus and hypothalamus regions of the brain (Madsen et al., 2009; Sebert et al., 2010). Other studies on pigs have proven that FTO is associated with intramuscular fat and average daily gain (Fan et al., 2009; Fontanesi et al., 2009). Furthermore, it has been reported that the FTO gene is widely expressed in several tissues and organs involved in the control of energy metabolism and cardiovascular function in animals, including adipose tissue, liver, pancreas,

kidney, brain, skeletal muscle, and heart (Stratigopoulos et al., 2008; Wåhlén et al., 2008; Madsen et al., 2009).

Mouse experiments are used to understand the anatomical role of the FTO gene as well as its metabolic effects. For example, in the study by Church et al. (2009) on mouse model, were studied the metabolic effects and expression level of the human FTO gene, for this, were used mice with a dominant missense mutation (I367F), which causes a reduced body weight and fat mass phenotype. As a result, it was mentioned that physical activity and food intake did not change in mutant mice, but the metabolic rate was high. Also, in mutant mice, fat and carbohydrate metabolism was increased and mutant mice fed with a high-fat diet had lower fat mass than the wild-type. According these results, it is stated that mice are a model for the human phenotype and may provide evidence that the FTO gene may be one of the underlying causes of obesity.

Besides mouse experiments, dogs are also important biomedical models (Lequarré et al., 2011). Knowledge of powerful molecular tools such as sequence of the canine genome, marker genome maps, haplotype distribution or SNPs, facilitate the identification of monogenic hereditary diseases associated with quantitative traits or gene mutations responsible for polymorphisms in humans as much as dogs (Breen, 2008). In particular, dog obesity attracts attention as a model, because many hereditary diseases seen in humans have similarities in different dog breeds and also differences that may arise from the gene-environment interactions are minimised due to their presence in the same living place with humans (Tsai et al., 2007; Ricci and Bevilaqua, 2012).

FTO gene in dogs

The canine FTO gene (Gene ID: 478125) is a protein-coding gene located on chromosome 2 with a length of 416.31 kb (428.861 nt) (NCBI, 2023). In total, there are 4.767 variants in all regions including SNPs and InDel, and the majority of these variants (4.428) are localizated in introns. There are a total of 9 protein-coding FTO transcripts (from 428 to 620 aminoacids) ranging in length from 4797 to 1485 bp (Ensembl, 2023). This gene, enables metal ion binding and activates mRNA N6-methyladenosine dioxygenase, oxidative DNA demethylase and transferase activities in biological process. Also, it is involved in DNA dealkylation, RNA repair, oxidative single-stranded DNA demethylation, oxidative single-stranded RNA methylation and regulation of multicellular organism growth (GO Annotations, 2023).

The alpha-ketoglutarate-dependent dioxygenase protein, which is encoded by the FTO gene and uses Fe (II) as a cofactor, is localized in the nucleus, nuclear speckle and cytoplasm of the cell, performs a number of molecular functions and takes part in biochemical processes (Wei et al., 2018).

Roles of canine FTO protein

The canine FTO protein acts as an RNA demethylase, mediating the oxidative demethylation of different types of RNA such as messenger RNA (mRNA), transfer RNA (tRNA) and small nuclear RNA (snRNA) and as a regulator of fat mass, adipogenesis and energy homeostasis (Gerken et al., 2007; Jia et al., 2008; Mauer et al., 2017). Also, it shows demethylase activity against m6A RNA, which is the most common modification of mRNA, especially in higher eukaryotes. This demethylation mediated by FTO, regulates expression levels of certain target genes by affecting the expression and stability of mRNA (Mauer et al., 2017). Among the genes targeted by the FTO gene in humans are Ghrelin, SRSF2, CCNA2 and CDK2, which can cause obesity; ASB2, RARA, ADAM19, EPHA3, KLF4, CDKN2A, BRCA2, TP53I11, BNIP3, MZF1, USP7, PKM2, MERTK, BCL-2, PD-1, CXCR4, and SOX10, which can be associated with various types of cancer (Reviewed from Lan et al., 2020 Table 1). Furthermore, FTO protein is also demethylates m6A in U6 small nuclear RNA (U6 snRNA) and it mediates demethylation of the N6,2'-O-dimethyladenosine cap (m6A(m)), which is located in the 5' cap, by demethylating m6A at the second transcribed position of mRNA and U6 snRNA (Mauer et al., 2017). This process ensures mRNA stabilization by increasing the sensitivity to uncapping. Also, FTO functions as a tRNA demethylase by removing N1-methyladenine (m1A) from tRNAs. In mRNA, FTO regulates dopaminergic activity in the midbrain through its ability to demethylate m6A (Hess et al., 2013). In addition, it is able to repair alkylated DNA and RNA by oxidative demethylation. FTO executes this by demethylating single-stranded RNA containing 3-methyluracil (3-meU) and single-stranded DNA containing 3-methylthymine (3-meT), as well as its low demethylase activity towards single-stranded DNA containing 1-methyladenine or 3-methylcytosine (3-meC) (Jia et al., 2008). However, the mentioned ability of repairing DNA and RNA is not definitively established in vivo (Gerken et al., 2007; Jia et al., 2008). It has no detected activity against double-stranded DNA as well (Jia et al., 2008).

The FTO protein causes the development of obesity by affecting the m6A levels of hormones related to nutrition (Lan et al., 2020). Furthermore, it contributes to the regulation of body size and fat accumulation by playing a role in the regulation of

adipogenesis, fat mass and body weight (Fischer et al., 2009; Church et al., 2009; Church et al., 2010; Hess et al., 2013; McMurray et al., 2013). Thus, it regulates both thermogenesis and the differentiation of brown and white fat cells (Church et al., 2009; Fischer et al., 2009).

Association of the canine FTO gene with obesity and cancer

Studies on the canine FTO gene are quite limited. Grzes et al. (2011) studied on the FTO and insulin-induced gene 2 (INSIG2), which are two candidate genes associated with fat accumulation in four species of Canidae family including dog, red fox, Arctic fox, and raccoon dog. In this study, a comparative genomic analysis was perfomed on the mentioned genes and as a result, a total of 29 SNPs were identified with 13 of them located in the FTO gene. Two of the analyzed 29 SNPs were determined to be missense in dogs and these SNPs were further investigated in 14 different dog breeds. As a result of the investigations, the presence of the variant identified as missense mutation in exon 1 (23C>T, Thr>Met) was reported in 5 dogs representing different breeds, including Labrador and Golden Retriever. In addition, 2 synonymous mutations, one in the FTO gene and the other one in the INSIG2 gene, were used for association studies in red foxes. In conclusion, it has been indicated that both genes are potential candidates for growth and adipose tissue development in canids. On the other hand, Grzemski et al. (2019) investigated the association of the FTO and Iroquois homeobox protein 3 (IRX3) gene with obesity in Labrador dogs. For this purpose, polymorphisms were investigated in 32 Labradors and also 165 Labrador dogs were used to examine the orthologous regions of these genes with humans. However, none of the identified 12.217 polymorphisms were reported to be significant in lean and obese dogs. The study concluded that FTO and IRX3 genes are not indicators of obesity in Labrador dogs.

Although there is no other published study on the canine FTO gene, it has been stated that dogs can be used as valuable models in genetic studies particularly related with cancer which is one of the most important diseases that obesity increases the risk of it, since they often experience spontaneous diseases and can develop many types of cancer observed in humans. Also, considering that domestic dogs are divided into more than 175 breeds and these breeds show similar phenotypic characteristics, it was mentioned that the breed barrier may enhance the utility of the dog model, especially in genetic studies that consider breed-based cancer susceptibility expressed by few genes (Switonski and Mankowska, 2013; Davis and Ostrander, 2014).

FTO Gene and cancer relationship

FTO proteins play a role in adipogenesis as well as in tumorigenesis with their m6A-dependent demethylase activity. In this way, FTO is highly expressed in many cancer tissues by acting as an oncogene and participating in the regulation of the malignant phenotype of cancer cells (Lan et al., 2020). In the study by Cui et al. (2017), it was reported that high levels of m6A promote the growth, self-renewal of glioblastoma stem cells (GSCs) and tumor development in these cells. Furthermore, several studies have reported that SNPs in the FTO gene are associated with breast cancer risk (Kaklamani et al., 2011; Zhang et al., 2014; Hernández-Caballero and Sierra-Ramírez, 2015).

Following the first publications showing the association between variants of FTO gene and obesity, researchers have started questioning and exploring the existence of the relationship between these variants and cancer risk in obese people from various ethnic groups (Hernández-Caballero and Sierra-Ramírez, 2015). In the study conducted by Brennan et al. (2009) on 7,000 people from Central and Eastern Europe, the rs9939609 variant of the FTO gene was investigated, however, it was reported that the A allele was associated with a low risk of lung cancer, while the risk of kidney cancer showed minimal increase. Subsequently, Lewis et al. (2010) suggested in their study that the A allele of the same variant is a protective factor against prostate cancer, reducing the possibility of low-grade cancer but potentially increasing the possibility of high-grade cancer. In 2011, an association between obesity and pancreatic cancer have been suggested and in two case-control studies of the non-Hispanic white population and the European population, the A allele of the rs8050136 variant was shown to be significantly associated with pancreatic cancer (Pierce et al., 2011; Tang et al., 2011). Besides the rs8050136A variant, Tang et al. (2011) were also studied on rs9939609A. In both variants a decreased risk of pancreatic cancer was observed in people with normal BMI, and an increased risk in people with high BMI (Tang et al., 2011). In the following years, the association of rs9939609 variant with pancreatic cancer risk was investigated in the Japanese population and it was reported that the TT genotype had a 1.5 times higher risk of pancreatic cancer than the TA genotype (Lin et al., 2013).

In relation to endometrial cancer and breast cancer, Delahanty et al. (2011) examined the relationship between obesity and endometrial cancer

risk in Chinese women and they revealed a strong association between the FTO SNPs they studied and endometrial cancer, but the association was not related to BMI. In another study conducted in the same year, it was reported that there is a significant association between the rs1477196 variant and breast cancer, predominantly in individuals of Caucasian descent (Kaklamani et al., 2011). In addition, in a study which the FTO SNPs rs1121980 and rs9939609 were analyzed in Brazilian population, it was reported that the risk of developing breast cancer was 4,9 times higher when the rs17782313 variant of melanocortin receptor-4 (MCR4) and these FTO SNPs were found in combination (da Cunha et al., 2013). In the study conducted by the GenoMEL consortium (2013), it was stated that SNPs located in an intron of the FTO gene unrelated to obesity, such as intron 8, may be associated with the risk of developing cancer. Later, in 2015, the study by Tan et al., examining the expression of the FTO gene in the breast tissue of Chinese women stated that FTO was overexpressed in cancerous breast tissue compared to healthy breast tissue.

Conclusions

The results obtained from all the mentioned studies on association of the FTO gene with obesity and cancer suggest that the relationship between cancer development, being overweight and having some FTO polymorphisms is based on ethnic origins as well as some genetic and environmental factors. Hence, it is clear that there is a multifactorial relationship between FTO variants and cancer disease rather than a simple association. In addition, the size and quality of the working group raise concerns about the reliability of certain results in these studies. For example, in a study that examines gender comparison, the ethnic backgrounds of individuals may hide the differences between genders. The character of the tumor in cancer diseases is another factor that influences the relationship between the polymorphic variants of FTO. Especially in studies focusing on breast cancer, the nature of tumors in the working group, whether they are spontaneous, hereditary or estrogen receptor status (ER+/-) should be considered (Hernández-Caballero and Sierra-Ramírez, 2015). Furthermore, in studies, the interaction of individual genes with the FTO gene or protein, the existence of an effective FTO pathway and the impact of FTO expression on other genes should be taken into consideration (Popović et., al., 2023).

Although excessive fat accumulation primarily promotes tumor growth rather than initiating cancer, it is essential to understand the underlying factors of obesity development. Even though the polymorphisms in intron 1 have been reported to be associated with obesity in studies, further investigations are needed to reveal their relationship with cancer. In addition, the reported association of polymorphisms in intron 8, which are not related to obesity, with cancer suggested that FTO polymorphisms may cause cancer with a mechanism other than obesity and opened a new field of research (Hernández-Caballero and Sierra-Ramírez, 2015). When the obtained results are evaluated, FTO gene and its intronic polymorphisms are likely to be associated with cancer as well as obesity.

In contrast to studies on human FTO gene, researches on the FTO gene in animals are quite limited. Since the identification of the FTO gene as an obesity gene, studies have mainly focused on farm animals such as pigs, cattle, sheep, and chickens. Regarding to pet animals, dogs have been studied in only two studies while cats have never been studied. The reason for this is probably the fact that the number of farm animals is high because they are usually keep in herds and use in breeding, also, their genetic origins can be followed easily thanks to selective breeding programs and certain traits such as tail fat, milk fat, back fat and intramuscular fat content are highly important in terms of the quality of food obtained from these animals. Furthermore, there are no studies conducted on the FTO gene in animals regarding diseases such as cancer and cardiovascular diseases, which are associated with an increased risk due to obesity. On the other hand, mice and chickens have been used as models in studies of the human FTO gene. Understanding the molecular basis of the FTO gene in dogs will provide an opportunity to develop individualized treatment strategies for diseases related to this gene. Also, considering that especially Beagle breed dogs are predicted to show phenotypes similar to the phenotypic characteristics caused by variations in the human FTO gene in terms of BMI and metabolic traits (Akey et al., 2010), the knowledge about the phenotypic effects of variations in dogs will pave the way for investigating the genetic origin of variations in both dogs and humans.

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Stethoscopes as vectors of staphylococci at a veterinary teaching hospital

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ABSTRACT

The issue of nosocomial infections, or healthcare-associated infections (HAIs), remains a significant concern in healthcare settings worldwide. In recent times, there has been growing attention towards medical devices, notably stethoscopes, as potential vectors for pathogen transmission. This study aimed to evaluate the prevalence of Staphylococcal contamination on stethoscopes used by students and staff at Istanbul University-Cerrahpasa Faculty of Veterinary Medicine's animal hospital. Furthermore, it gathered information about stethoscope usage habits, cleaning practices, handwashing routines, participants' knowledge about nosocomial infections, and their interest in learning more about these infections and stethoscope hygiene. The analysis of 50 stethoscope samples revealed that 27 (54%) were contaminated with one or more Staphylococci. The isolated 30 *Staphylococcus* spp. included *Staphylococcus epidermidis* (n=17; 56.7%), *Staphylococcus hominis* (n=10; 33.3%), *Staphylococcus pasteuri* (n=1; 3.3%), *Staphylococcus capitis* (n=1; 3.3%), and *Staphylococcus schleiferi* (n=1; 3.3%). Notably, the absence of the highly pathogenic *Staphylococcus aureus* in all samples provides some reassurance. However, the presence of various *Staphylococcus* spp. raises concerns due to their pathogenic potential. These findings align with previous research on stethoscope contamination, emphasizing the persistent problem of bacterial colonization on these crucial medical devices. Despite variations in bacterial prevalence among studies, *Staphylococcus* spp. consistently emerge as common contaminants, emphasizing the need for comprehensive stethoscope hygiene protocols in veterinary healthcare settings. To the best of our knowledge, this is the first report describing the vector potential of stethoscopes in a veterinary setting within Turkey. The study suggests the necessity for further research, taking a proactive approach to tackle the challenges of nosocomial infections. This would enable the development of strategies to ensure a safer healthcare environment for patients and healthcare providers.

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Introduction

Nosocomial infections, also known as healthcare-associated infections (HAIs), continue to be a significant concern in healthcare settings worldwide. While close contact between humans and their animal companions can lead to the exchange of bacterial strains (Kaspar et al., 2018; Rossi et al., 2020), similarly close contact in a hospital environment can lead to infections, increased morbidity, mortality, and healthcare costs (Cohn & Middleton, 2010). While the role of hands and surfaces in the transmission of HAIs is well-established (Al-Beeshi et al., 2021; Nivedhitha et al., 2021; Rojas et al., 2017; Suleyman et al., 2018), the potential role of medical devices, such as stethoscopes, in the dissemination of pathogens has gained attention in recent years. Their contamination serves as a potential source for transmitting pathogens among individuals in the veterinary healthcare setting (Fujita et al., 2013; Leite et al., 2023; Souza et al., 2022).

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Stethoscopes are indispensable tools for veterinary healthcare providers, facilitating accurate diagnosis and patient assessment. However, their frequent and close contact with patients, coupled with inadequate cleaning and disinfection practices, can contribute to microbial contamination. Approximately four decades ago, the emergence of nosocomial infections highlighted the possibility of stethoscopes serving as potential vehicles for transmission (Gerken et al., 1972; Mangi & Andriole, 1972).

Ever since then, numerous researchers have showcased the ability of stethoscopes to harbour pathogens (Fujita et al., 2013; Heldeweg et al., 2022; Marinella, 1997; Núñez et al., 2000). In a study conducted by Gerken et al., an examination was carried out on the cultures of 100 stethoscopes utilized in a teaching hospital. The findings revealed that approximately 21% of these stethoscopes harboured Staphylococci (Gerken et al., 1972). In the year 1996, Smith et al. conducted a study where they analysed 200 stethoscopes obtained from hospitals and outpatient clinics. The results indicated that a staggering 80% of the stethoscopes were found to be contaminated with bacteria. The predominant type of bacteria detected was *Staphylococcus* spp. (Smith, 1996). In a study by Gupta et al., a total of 50 stethoscopes were evaluated, and it was found that all of them were culture positive for bacterial growth. The predominant bacteria isolated from these cultures was coagulase negative *Staphylococcus* (CoNS), accounting for approximately 77% of the isolates (Gupta et al., 2014).

Although more study has been conducted in this area at human medicine, stethoscope studies that include veterinary hospitals have also shown the same results (Leite et al., 2023; Souza et al., 2022). These collective discoveries have sparked worry that stethoscopes might serve as a potential origin for hospital-acquired infections. To address this concern, it is essential to assess the extent of microbial contamination on stethoscopes and explore the associated hygiene practices among healthcare professionals.

The main aim of this research was to evaluate the prevalence of Staphylococcal bacterial species on stethoscopes used by students and staff at Istanbul University-Cerrahpasa Faculty of Veterinary Medicine. Additionally, the study anonymously collected data on stethoscope usage patterns, cleaning practices, handwashing routines, participants' knowledge of nosocomial infections, and their interest in learning more about these infections and proper stethoscope hygiene.

Materials and Methods

This study was performed from April 2022 to July 2022 at Istanbul University-Cerrahpasa Faculty of Veterinary Medicine. Samples from stethoscope diaphragms of randomly chosen students and staff of the faculty were obtained at the University's animal hospital and analysed at the laboratory of the Microbiology Department. In this study, 50 stethoscope samples were collected from 33 participants, as 8 of the participants owned more than one stethoscope, also 9 of the stethoscopes were the belonging of the facility for the usage of teaching staff and students. During sampling, information was collected about the participants' demographic information, professional roles, gender, and stethoscope usage habits, like the frequency of stethoscope usage, cleaning frequency, materials used for cleaning, and hand washing practices after examinations (Table 1-2). Additionally, their knowledge about nosocomial infections and interest in learning more about nosocomial infections and stethoscope hygiene were recorded (Table 3).

The diaphragms of stethoscopes were swabbed using sterile transport swabs moistened with phosphate-buffered saline solution (PBS). Following sampling, the swabs were immediately transported to the laboratory and inoculated onto mannitol salt agar and blood agar plates. The inoculated plates were incubated at 37°C for 24-48 hours. The identification of isolates showing staphylococcal morphology continued using standard microbiological identification techniques and classical biochemical tests (Table 4), which are routinely employed in the laboratory to confirm the identity of the *Staphylococcus* spp.

Results

The study comprised 33 participants who were randomly selected during their shift at the teaching hospital of Istanbul University-Cerrahpasa Faculty of Veterinary Medicine, with 14 undergraduate students (42.4%), 12 PhD students (36.4%), and 7 assistants/ instructors (21.2%). Of the participants, 16 were female (48.5%) and 17 were male (51.5%) (Table 1).

Table 1. Participant demographics

	n	%
Role		
Undergraduate student	14	42.4
PhD student	12	36.4
Assistant/Instructor	7	21.2
Gender		
Male	17	51.5
Female	16	48.5

In this article, Table 2 presents the data on stethoscope usage frequency, cleaning practices, and handwashing routines after examination among the participants. The frequency of stethoscope usage varied among participants, with 14 individuals (43%) reporting daily use, 11 (33%) reporting usage a few times a week, 3 (9%) reporting usage once a week, 3 (9%) reporting rare usage, and 2 (6%) reporting never using a stethoscope. Regarding stethoscope cleaning practices, only one participant (3%) reported always cleaning the stethoscope after use, while 4 participants (12%) cleaned it often, 11 participants (34%) cleaned it sometimes, 9 participants (27%) cleaned it rarely, and 8 participants (24%) never cleaned it. The most commonly used material for stethoscope cleaning was alcohol (n=15, 45.5%), followed by clean fabric (n=5, 15.2%), while 5 participants (15.2%) used other materials, and 8 participants (24.2%) reported not using any material for cleaning. Regarding hand washing after examinations, 20 participants (60.6%) reported always washing their hands, 6 participants (18.2%) reported often, 2 participants (6.1%) reported sometimes, and 5 participants (15.2%) reported rarely washing their hands. No participant reported never washing their hands.

Table 2. Participants' general hygiene, stethoscope usage and cleaning practice

Usage frequency	**n**	**%**
Daily	14	42.4
Few times a week	11	33.3
Once a week	3	9.1
Rare	3	9.1
Never	2	6.1
Cleaning frequency		
Always	1	3.0
Often	4	12.1
Sometimes	11	33.3
Rarely	9	27.3
Never	8	24.3
Stethoscope cleaning material		
Alcohol	15	45.4
Clean fabric	5	15.2
Other	5	15.2
Nothing	8	24.2
Hand washing after examination		
Always	20	60.6
Often	6	18.1
Sometimes	2	6.1
Rarely	5	15.2

Additionally, Table 3 showcases participants' knowledge about nosocomial infections and their interest in learning more about nosocomial infections and stethoscope hygiene. In terms of knowledge about nosocomial infections, 12 participants (36.4%) were aware of nosocomial infections and took necessary precautions, 15 participants (45.5%) were aware but did not always take precautions, 5 participants (15.2%) had heard about nosocomial infections but did not possess much knowledge, and only one participant (3%) had never heard about nosocomial infections. Regarding participants; interest in learning more about nosocomial infections and stethoscope hygiene, 23 participants (69.7%) expressed a desire to acquire further knowledge, 4 participants (12.1%) showed no interest, and 6 participants (18.2%) were hesitant.

Out of the 50 stethoscopes, 30 Staphylococcal species were isolated from 27 (54%) stethoscope swabs, 11 samples (22%) showed no bacterial growth, while the remaining samples (24%) showed single- or mixed cultures other than *Staphylococcus* spp. The isolates obtained from the stethoscopes revealed a diverse array of *Staphylococcus* spp. Among the isolates, 17 were *Staphylococcus epidermidis* (56.67%), 10 were *Staphylococcus* hominis (33.33%), 1 was *Staphylococcus pasteuri* (3.33%), 1 was *Staphylococcus capitis* (3.33%), and 1 was *Staphylococcus schleiferi* (3.33%). Notably, S. aureus, a significant pathogenic species, was not detected in any of the samples.

Table 3. Knowledge and interest in HAIs and stethoscope hygiene

Knowledge	n	%
Aware and take precautions	12	36.3
Aware but not always take precautions	15	45.4
Do not have much knowledge of the topic	5	15.1
Never heard about nosocomial infections	1	3.0
Interest in further learning		
Yes	23	69.7
No	4	12.1
Hesitant	6	18.2

Table 4. Methods and biochemical tests for identification of Staphylococci from bacterial isolations

Basic Characteristics	**Fermentation of**	**Enzymatic Reactions**
Catalase	Arabinose	Arginine dihydrolase
Coagulase	Cellobiose	Lysine decarboxylase
Gelatine hydrolysis	DNAse	Ornithine decarboxylase
Haemolysis	Fructose	
Nitrate reduction	Lactose	
Oxidase	Maltose	
ONPG	Mannitol	
Pigment	Mannose	
Shape	Raffinose	
Urease	Ribose	
6.5% NaCl	Sucrose	
	Trehalose	
	Xylose	

Limitations: Single-Centre Study: The study was conducted at a single veterinary teaching hospital in Istanbul, Turkey. This may limit the generalizability of the findings to other veterinary healthcare settings in different regions.

Limited Bacterial Identification: The decision to narrow the scope of bacterial identification to *Staphylococcus* spp. was made to specifically assess their prevalence and potential implications in the veterinary environment. However, future research endeavors may explore a broader range of bacterial species to gain comprehensive insights into stethoscope contamination and its impact on veterinary healthcare.

Antibiotic Resistance Testing: The assessment of antibiotic resistance profiles of the isolated *Staphylococcus* spp. was beyond the scope of this study. Future investigations should focus on exploring the resistance patterns and evaluating the potential risk posed by these bacteria in the context of HAIs, thereby contributing to a more comprehensive understanding of their clinical implications and informing effective infection control measures.

Discussion

The presence of bacteria on the diaphragms of veterinary stethoscopes is a predictable discovery that aligns with previous culture surveys conducted on stethoscopes used by physicians.

Upon comparing multiple studies on stethoscope contamination, several consistent findings emerge regarding the bacterial species identified and *Staphylococcus* spp. were commonly isolated. Our study found single or multiple *Staphylococcus* species in 27 (54%) out of 50 stethoscopes, including S. epidermidis, S. hominis, S. pasteuri, S. capitis, and S. schleiferi. Other studies also reported *Staphylococcus* spp. as the predominant bacteria. Gupta et al. found that all 50 stethoscope samples were culture positive, with CoNS being the most commonly isolated bacteria (77%) (Gupta et al., 2014). Similarly, Leontsini et al. found that pathogenic or potential pathogenic CoNS were isolated in 87.6% of the stethoscopes sampled, with S. epidermidis being the most predominant species (39.4%), followed by S. hominis (19.7%) (Leontsini et al., 2013). This founding is also similar to ours.

Unlike most studies that primarily investigate stethoscopes used by human physicians, our research with Souza et al., Leite et al., and Fujita et al.'s study specifically examined stethoscopes employed in veterinary settings (Fujita et al., 2013; Leite et al., 2023; Souza et al., 2022). In the study by Souza, bacterial growth was observed in 73.33% of stethoscopes before disinfection, and *Staphylococcus* spp. accounted for 35.3% of the isolates (Souza et al., 2022).

The study by Fujita et al. reported similar findings to ours, demonstrating the presence of diverse bacteria on the stethoscope diaphragms. The recovered organisms included normal skin flora, agents of opportunistic infections, and potential pathogens. Among the isolated bacteria, S. epidermidis was present in 15% of the samples, while the resistant strain of S. epidermidis constituted 8%. S. pseudintermedius and S. hominis represented 12% and 4%, respectively, while S. simulans and S. warneri each accounted for 4% of the total isolates (Fujita et al., 2013).

S. aureus, a significant pathogenic species, was notably absent in our and Fujita's study. Leite et al. investigated the occurrence of antimicrobial -resistant S. aureus in a Brazilian veterinary hospital environment by sampling humans, animals, surfaces, mobile phones and stethoscopes. In the study, out of 110 bacterial isolates, only 10 were identified as S. aureus, and these were found in human nasal cavities rather than on stethoscopes (Leite et al., 2023). The consistent findings were also observed in Leontsini et al.'s study, which investigated physicians' stethoscopes and reported the absence of S. aureus isolation from both the diaphragms and earpieces (Leontsini et al., 2013).

Anticipatedly, the isolation of S. aureus in animals is infrequent since, according to various studies, pets are more prone to being colonized by other types of *Staphylococcus*, such *as S. epidermidis*, *S. felis, S. intermedius, S. pseudintermedius, S. schleiferi,* and *S. simulans* (Bagcigil et al., 2012; Bierowiec et al., 2019; González-Domínguez et al., 2020). This finding is supported by other studies that display the fact that S. aureus organisms are seldom found on inanimate objects in the community due to their limited ability to survive on dry surfaces for more than 24 hours (Domon et al., 2016).

These distinctions are important because it provides valuable insights into the bacterial contamination associated with veterinary practices. By exploring the microbial presence on veterinary stethoscope diaphragms, these studies shed light on the potential risks and hygiene considerations in animal healthcare environments.

To the best of our knowledge, while numerous studies have been conducted in the country's multiple healthcare environments concerning human health (Eriş, F. N., et al., 2000; Kilic et al., 2011; Oguzkaya-Artan et al., 2016), and environmental sample from areas at veterinary education facilities have been examined (Bagcigil et al., 2012), this report is the first to describe the vector potential of stethoscopes in a veterinary environment in Turkey.

It is important to note that the prevalence of specific bacterial species may vary across studies due to factors such as study population, sample size, geographical location, and healthcare settings (Lee et al., 2018; Sivri et al., 2016). However, the consistent presence of Staphylococcus spp., particularly CoNS, suggests their potential role as common contaminants on stethoscopes. Also, a significant proportion of this study's participants reported irregular stethoscope cleaning practices, with only a small percentage consistently cleaning the instrument after use. Studies that investigated the frequency of stethoscope cleaning among students found controversial results. A survey conducted among 51 French students revealed that 82% of the participants reported cleaning their stethoscopes either regularly or occasionally (Duroy & Le Coutour, 2010). A Serbian study showed that a substantial number of students in the study had cleaned their stethoscopes (Gazibara et al., 2015). Similarly to our study, a survey amongst medical and nursing students in Jordan showed that only 8% cleaned their stethoscopes between patients, 38% did not clean their stethoscopes at all, 67% did not know how to effectively clean their stethoscopes (Bataineh et al., 2022).

The controversy in findings may be influenced by various factors, including cultural norms, educational settings, and individual attitudes towards hygiene. Additionally, variations in sample sizes and demographics of the surveyed populations could impact the results. These contrasting findings emphasize the need for further research to gain a comprehensive understanding of stethoscope hygiene practices among students worldwide. Policymakers and educators should consider implementing standardized guidelines and training programs to promote proper stethoscope disinfection.

Conclusion

This study aimed to assess the prevalence of Staphylococcal contamination on stethoscopes used at a veterinary teaching hospital. The results revealed Staphylococcal contamination in 54% of the examined stethoscopes, with various Staphylococcus spp., including S. epidermidis, S. hominis, S. pasteuri, S. capitis, and S. schleiferi.

While the absence of highly pathogenic S. aureus is reassuring, the presence of diverse Staphylococcus spp. warrants attention due to their potential pathogenicity and risk of opportunistic infections. This highlights the persistent challenge of bacterial colonization on stethoscopes in veterinary healthcare settings.

Through an extensive analysis of stethoscope usage habits and hygiene practices among the participants, a concerning pattern of inadequate cleaning protocols emerged. A mere fraction of participants adhered to regular cleaning after usage, with the majority employing suboptimal or infrequent cleaning practices. This lack of stringent hygiene measures accentuates the potential risk of cross-contamination and pathogen dissemination among patients and healthcare practitioners. Also, considering the interest shown by participants in learning more about nosocomial infections and stethoscope hygiene, educational interventions or training programs should be considered to raise awareness and improve hygiene practices.

This study significantly contributes to the limited literature on stethoscope contamination in veterinary healthcare, especially in Turkey. It is the first report examining stethoscope contamination in a veterinary environment in this region, providing valuable insights into microbial contamination associated with animal healthcare practices.

Future research should involve larger and diverse participant populations from various veterinary healthcare settings to ensure comprehensive representation. Additionally, in-depth studies exploring potential antibiotic resistance profiles of the isolated species are crucial to understanding their virulence and infection risk.

Through such research, the healthcare community in Turkey can proactively address the challenges of nosocomial infections and enhance patient safety in veterinary healthcare settings.

Conflict of interest

The authors declared that there is no conflict of interest.

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Investigating the relationship between heart rate and respiratory changes and the human-animal bond: Insights from an external telemetry system

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ABSTRACT

Heart rate changes and respiratory activities are vital physiological phenomena that provide valuable insights into the physiological and psychological states of family dogs. The bond between humans and their pet dogs necessitates a deeper understanding of this relationship. Therefore, the objective of this study was to investigate the human-animal bond by examining heart rate (HR), heart rate variability (HRV), respiratory rate (breathe per minute, BPM), and tidal volume (TV) using an external telemetry system. A total of ten dogs were selected as participants, and their cardio-respiratory responses were evaluated in an unfamiliar environment. The baseline data for the study was established during the first stage of the Strange Situation Test (SST), known as "dog with owner." The analysis focused on changes in HR, HRV, BPM, and TV throughout the different stages of the SST. Interestingly, the results demonstrated that changes in HR did not consistently correspond to changes in HRV across all stages. During the initial encounter with the stranger (episode b, stranger entering), there were notable percentage changes in HR, HRV, and TV, despite an overall increase in BPM, although not significant. In the third stage (stranger alone with the dog), both HR and HRV parameters, as well as TV, displayed increased percentage changes, whereas BPM exhibited a decrease. Furthermore, when the dog interacted with the stranger for the second time (episode f), HR and BPM increased, while HRV and TV decreased. This pattern suggests a shift towards a more active and alert state in response to the renewed social interaction. In contrast, when the dog was left alone (episode e), HR and BPM decreased, while HRV and TV increased. Overall, these findings provide evidence that changes in heart rate and respiratory parameters reflect the emotional stress experienced by family dogs in various social contexts. Moreover, the utilization of the external telemetry system in this study offers a promising model for investigating the effects of pharmacological interventions, behavioral interventions, and animal-assisted therapy in animals. By gaining a deeper understanding of the human-animal bond, we can further enhance the well-being and quality of life for both dogs and their human companions.

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Introduction

Behavior is a fundamental component in establishing and sustaining both internal and external balance, which encompasses various physiological mechanisms. The stress response, a complex interplay of physiological and behavioral reactions orchestrated by the central nervous system, is triggered in response to various stimuli. Stress behavior represents a non-specific adaptive reaction of the organism, exerting a profound impact on the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. In order to maintain homeostasis, the organism must adapt to changes in the social and physical environment through coordinated behavioral, physiological, and metabolic responses. These responses work in tandem to restore homeostasis, involving the activation of thermoregulatory

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processes, cardiovascular system adjustments, respiratory system modulation, and behavioral adaptations (Koolhaas et al., 1993).

Heart rate (HR) and heart rate variability (HRV) are essential measures that capture the fluctuations in the time interval between successive normal heartbeats, reflecting the activity of the autonomic nervous system. HRV serves as a practical, non-invasive, and effective method for assessing the balance of the autonomic nervous system. Notably, HRV has gained increasing popularity as a physiological phenomenon for evaluating both normal and pathological conditions (Rasmussen et al., 2012; Pirintr et al., 2012; Bogucki and Noszczyk-Nowak, 2015). Its widespread use stems from its ability to provide valuable insights into the dynamic interplay between sympathetic and parasympathetic influences on the cardiovascular system, offering a window into the physiological and pathological states of individuals.

HRV can be assessed through various intervals, and one of the most widely used techniques for analyzing HRV parameters is spectral analysis in the frequency domain. Key HRV parameters include NN (the time interval between two consecutive normal beats), SDNN (standard deviation of NN intervals), and rMSSD (root mean square of successive NN interval differences), as comprehensively reviewed by Bilman (2011). While some studies have reported no significant differences in HRV parameters among dogs (Bogucki and Noszczyk-Nowak, 2015), others have observed lower HRV values, such as NN or rMSSD, in the context of certain diseases (Piccirillo et al., 2009; Rasmussen et al., 2012).

Moreover, HRV analysis can be conducted using both long-term and short-term electrocardiogram (ECG) recordings. Measure the accurate HRV is very important excluding abnormal NN intervals that causes by ectopic pulses, artifacts or noises etc. Its possibility with short-term ECG recording under standart conditions with minimal artifact was reported (Kleiger et al., 2005). It was also determined that the abnormal NN intervals may be assessed by functional changes of body such as physical activity, hormones, reflexes and cardiac rhythm’s changes related to physiological or pathological changes (Kleiger et ak., 2005; Stein and Reddy, 2005). However, in order to obtain a comprehensive assessment of circadian rhythm, researchers have explored the use of long-term measurements, such as 24-hour Holter monitoring (Rasmussen et al., 2012; Gacsi et al., 2013). Conversely, some studies have suggested that short-term recordings may be more advantageous compared to 24 -hour monitoring (Handlin et al., 2011; Voss et al., 2013; Bogucki and Noszczyk-Nowak, 2015; Baisan et al., 2020). For instance, Handlin et al. (2011) employed a polar system to analyze heart rate, collecting data every 15 seconds in dogs. Their findings indicated that dogs exhibited normal behavior in unfamiliar environments when accompanied by their owners. Similarly, in a recent study by Baisan et al. (2020), short -term HRV recordings of 5 minutes were conducted, revealing the potential utility of this approach for assessing HRV in small dogs.

External telemetry is a sophisticated system used for real-time monitoring of various physiological parameters, including body temperature, heart rate, blood pressure, electrocardiographic parameters, respiratory rate and depth, spatial positioning of the body, and locomotor activity. This system implements electrodes placed on the skin to record and transmit the collected data to a computer, where it is analyzed using specialized software provided with the telemetric system (Napoleoni et al., 2010; Roche et al., 2011; Bailey et al., 2012). The utilization of external telemetry has greatly facilitated the investigation of changes in HR, HRV, respiratory rate (breath per minute; BPM), and tidal volume (TV) in response to environmental stimulation, making it a prominent tool in physiological research. The advantages of external telemetry systems extend beyond their non-invasive nature and extended data collection capabilities, as they also offer great potential for investigating the physiological responses of animals in pharmacological, behavioral, and animal-assisted therapy studies.

During the course of development, it is crucial for individuals to share their physiological and psychological states. Psychologist Mary Ainsworth devised the Ainsworth's Strange Situation Test (SST) to observe the attachment relationship between a child and caregiver. The procedure focuses on meeting a child's social needs provided by the caregiver (Ainsworth et al., 1978). Modified versions of this test have been employed for assessing attachment in both canines and humans. These adaptations are based on the behaviors exhibited by dogs towards their owner and/or an unfamiliar person. The interaction between pets and their owners has been likened to the attachment observed between children and parents. These modified versions involve physical and behavioral tests established by researchers studying human-animal bonding and interaction (Prato-Previde et al., 2003; Palestrini et al., 2005; Gacsi et al., 2013). These studies have noted that separation from the owner, akin to a child's separation from their parent, leads to physiological changes such as alterations in cardiac or respiratory parameters. Gacsi et al. (2013) reported an increase in heart rate during separation

processes, cardiovascular system adjustments, respiratory system modulation, and behavioral adaptations (Koolhaas et al., 1993).

In this present study, our aim was to employ an experimental approach to examine the impact of the Strange Situation Test using an external telemetry system in family dogs. To achieve this, we used an external telemetry system to capture and analyze relevant data related to HR, HRV, BPM, and TV. By utilizing this approach, we aimed to gain a comprehensive understanding of the dogs' physiological and emotional states. Furthermore, this methodology enabled us to assess the impact of the human-animal bond on these physiological parameters, providing valuable insights into the intricate nature of the relationship between humans and their canine companions.

Material and Methods

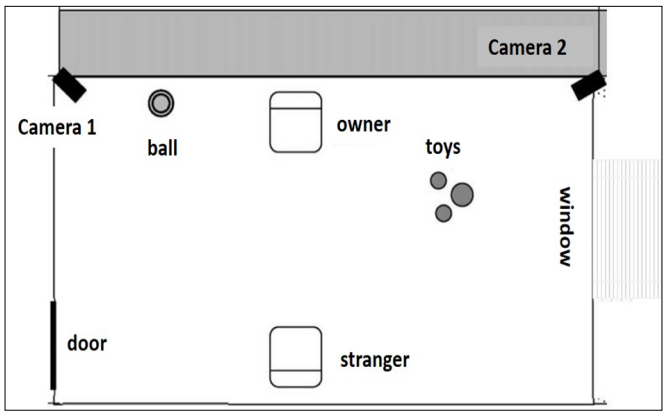
Animals

The study was carried out with the permission of the Bursa Uludag University Experimentation Local Ethics Committee (approval no. 2014.3/1). The present study aimed to include a sample of ten dogs (four males, six females) that were voluntarily enrolled with the consent of their respective pet owners. A thorough assessment of the health status, including vaccination records, was conducted prior to the commencement of the study to ensure the inclusion of healthy dogs. The sample consisted of dogs of various breeds (Golden retriever, French bulldog), genders (female and male), and ages (3 months – 1 year old), all of which were primarily kept as pets in family settings.

Experimental set up

The research was conducted within the Human-Animal Interaction Laboratory, an experimental facility located at the Department of Ethology in the Veterinary Faculty (see Picture 1). The laboratory space was specifically designed for this study and consisted of an unfurnished room with parquet flooring. The room maintained a controlled temperature ranging between 22 and 24°C, while artificial lighting was provided by a ceiling-mounted fluorescent lamp. To ensure hygiene and minimize the influence of confounding odors, the room was thoroughly cleaned after each experimental session.

For the purpose of behavior recording, two cameras were positioned within the experimental setting. One camera was positioned above the door, providing an overhead view of the room, while the second camera was placed on the opposite side of the room, capturing a different perspective. During the experimental procedure, the room was minimally



Picture 1. The experimental rool (Human-animal interaction Laboratory, located at the Department of Ethology in the Veterinary Faculty)

furnished, containing only two chairs and a selection of pet toys such as ropes, tennis balls, and large balls. This simplified environment was specifically arranged to facilitate the implementation of the Strange Situation Test (SST) and to allow for unobstructed observation and analysis of the dogs' behaviors.

Pet attachment scale & owner personality

At the onset of the research, the Pet Attachment Scale was administered to the dog owners to assess the level of attachment and emotional bond between the owners and their dogs (Hamano, 2002). It is important to note that owner personality plays a significant role in shaping this bond and influencing the overall well-being of the animals. The utilization of the Pet Attachment Scale provides valuable insights into the nature of the human-animal relationship and has been employed in various settings such as military, hospital, companion, and home environments. Previous studies, including those conducted by Holcomb et al. (1985) and Johnson et al. (1992), have explored the dimensions of pet attachment and the dynamics of affectionate relationships between humans and animals. The questionnaire used in this study was adapted from Dr. Hamano's pet attachment scale (Hamano, 2002) and modified to align with the specific research objectives and context.

Strange situation test (SST)

The Ainsworth's SST was originally devised by psychologist Mary Ainsworth as a means of assessing the attachment relationship between an infant and their caregiver. This standardized procedure aims to observe and evaluate the social and emotional needs of the child as they are met by their caregiver (Ainsworth et al., 1978).

Modified versions of the SST have been adapted and utilized in both canine and human research to assess attachment relationships (Palestrini et al., 2005; Vas et al., 2005; Mongillo et al., 2013). These modified

versions focus on observing the behavior of dogs towards their owner and/or an unfamiliar person. In our recent study, we employed a modified method based on the model proposed by Vas et al. (2005).

Preparation of external telemetry recording

Prior to the study, a preparation procedure was conducted on the dogs. Specifically, the thorax area of each dog was shaved in three circular patches with a diameter of 5 cm. Adhesive patches were then utilized to secure multiple derivative electrodes in place. In order to measure the physiological parameters of the dogs, they were fitted with DSI external telemetry equipment (DSI, USA), which included electrodes for monitoring heart rate and activity, as well as respiratory belts. To ensure proper placement and stability of the electrodes, a jacket was utilized in accordance with the guidelines provided by the equipment manufacturer. Following the attachment procedure, dogs and their owners were given approximately 5 minutes of free time to familiarize themselves with the equipment and the experimental room. If a dog did not acclimate to the equipment or the room during this time, we excluded that pet from the study

Procedure

The study consisted of two phases. In the first phase, the pet attachment scale was administered to assess the bond between the dog and its owner. This scale was completed through a survey conducted with the owner, and the responses were evaluated statistically. In the second phase, the human-animal bond between the dog and its owner was assessed using the Ainsworth's SST. During this test, the dog's behavior was observed in three different conditions: with the owner, with a foreign person, and when left alone. The entire test consisted of seven episodes, each lasting three minutes that modified by researches (Rehn et al., 2013; Gerencsér et al., 2013; Bogucki and Noszczyk-Nowak, 2015). The behaviors exhibited by the dogs during these episodes were recorded using cameras for later analysis.

Episodes

Episode 1 (a; 3 min): The owner sat quietly and no interaction with the dog. The dog was free to explore the room. Episode 2 (b; 3 min, 1nd reunion episode): The stranger who was always played by the same woman and who had never met the dog before, entered the room and sat quietly. She initiated a conversation with the owner for 30 second. And then during third minute she approached the dog and attempts to engage the dog by throwing a bal lor offering a toy. At the end of this episode the owner left the room unobtrusively. Episode 3 (c; 3 min, 1st separation episode): The stranger interacts and plays with the dog if the dog allows. After 60 seconds, the stranger only interacts with the dog if it permits. Episode 4 (d; 3 min, 2nd reunion episode): The owner entered the room while the stranger promptly exits. Throughout the episode, the owner behaves according to the dog's preferences. At the end of the episode, the owner gives the "stay" command and leaves the room. Episode 5 (e; 3 min, 2nd separation episode): The dog is left alone for three minutes in the room. Episode 6 (f; 3 min): The stranger enters the room, sits, and waits for the dog to initiate interaction. She then followed the same protocol as in episode 3. Episode 7 (g; 3 min): The owner calls the dog from outside the door without entering. Meanwhile, the stranger quietly leaves the room. The owner and the dog have unrestricted time together throughout the episode.

External telemetry measurements

The HR, HRV, TV, and BPM were measured using the external telemetry system developed by DSI (Data Sciences International, Inc, USA). To assess HRV, first of all, the NN from sinoatrial nodes were evaluated for NN intervales from system software (Ponemah, version 5.20-SP10, DSI). To calculate the SDNN, the standart deviation of all NN during record were analyzed, and for rMSDD it was calculated the root mean square of successive NN interval difference. Values for all stages were evaluated as percentage changes by averaging the frequency numbers obtained in the previous stage. The system was applied to the animals through a jacket, and the collected data were analyzed using specialized software (Ponemah, version 5.20-SP10, DSI).

Data analysis

The dogs remained in the room during 21 minutes ECG recording. The HRV parameters were determined from all recording time electrocardiogram (Bogucki and Noszczyk-Nowak, 2015). The analysis of HRV was preceded by manual screening of preselected ECG segments, to identify the representing a pure sinus rhythm and lacking any artifacts. The mean lenght of the NN intervals and the number of QRS complexes were determined for each of analyzed segments. The following time-domain parameters were calculated: mean of all normal NN intervals; standart deviation of all NN intervals (SDNN), and the square root of the mean of the sum of the squares of differences between adjacent NN intervals (rMSSD). The total number of differences were evaluated as percentage differences between the stages.

Statistical analysis

Statistical analyses were performed using SPSS (Version 20.0; Chicago, IL, USA). Data were examined

for normality distribution (Shapiro-wilk test). If normally distributed, a one-way ANOVA test was applied, and the differences between groups were analyzed using Tukey's post hoc test. Differences were considered significant at $p < 0.05$, and the means and standard errors were calculated. Nonparametric tests were also used as the data did not provide normal assumptions. Therefore, the differences between the groups were analyzed using the Kruskal–Wallis and Mann–Whitney U tests. Once again, differences were considered significant at $p < 0.05$, and the median values (minimum–maximum) were calculated.

Results

The analysis of HR, BPM, TV and HRV (NN, SDNN, rMSSD) expressed as percentages differences between episodes in Table 1.

Percentage differences of heart rate (HR)

A percentage decrease of HR (-0,05) was determined when the animal encountered the stranger for the first time while owner was with it (episode b, dog with owner and stranger). Besides that, there was an increase in the percentage differences of episodes b-c (separation episode; in which dog with stranger alone) compared to the percentage differences of episodes a-b in which dog with owner and stranger (p:0,001; a-b:-0,05 vs b-c: 0,01). Although not significant, there was an increasing tendency in percentage differences of episodes c-d (reunion episode; dog with owner again) compared to percentage differences of episodes b-c in which dog with stranger alone (p>0.05; b-c:0.01 vs c-d:0.05). However, it was found that there was a significant decrease in the percentage difference of episodes d-e which dog was alone for the first time (episode e) compared to the percentage difference of episodes c-d which animal with owner again in reunion episode (p<0.0001; c-d: 0.05 vs d-e: -0.06). In addition, there was no statistical differences found between the percentage differences in episodes d-e and e-f (p>0.05; d-e:0.06 vs e-f:-0.07), in which the animal remained alone and afterwards encountered the stranger again. In the last episode of trail, when the owner encountered the room again, the percentage differences between episodes f-g, there was a slight increase in HR (p>0.05; e-f: -0.07 vs f-g: -0.02) compared to the percentage differences between episodes e-f in which dog with stranger alone.

Percentage differences of respiratory rate (breath per minute; BPM)

The percentage differences of episode b was decreased (-0.02). Also, a slight decrease was found in the percentage differences of episodes b-c in which dog with stranger alone compared to the percentage differences of episodes a-b in which dog with owner and stranger (p>0.05; a-b:-0, 02 vs b-c: -0.16). However, there was a significant increase in the percentage differences of episodes c-d in which dog with owner again compared than the percentage differences of episodes b-c in which dog with stranger

Table 1. Percentages changes of HR, NN, SDNN, rMSDD, BPM and TV between episodes of the study groups (n:10)

	Percentages of episodes					
Parameters	Differences between the first and second episode (a-b)	Differences between the second and third episode (b-c)	Differences between the third and forth episode (c-d)	Differences between the forth and fifth episode (d-e)	Differences between the fifth and sixth episode (e-f)	Differences between the sixth and seventh episode (f-g)
Heart rate (HR)	-0.05 (-0.17 - 0.06)	0.01[a] (-0.12 - 0.17)	0.05 (-0.08 - 0.26)	-0.06[c] (-0.22 - 0.08)	-0.07 (-0.15 - 0.13)	-0.02 (-0.13 - 0.11)
Normal to normal intervals (NN)	0.05 (-0.05-0.15)	0.11 (-0.14-0.21)	0.002 (0.00-0.00)	0.06[c] (-0.05-0.26)	0.04 (-0.10-0.20)	-0.06 (-0.17-0.12)
Standart deviation of all NN during record (SDNN)	0.03 (-0.03-0.07)	0.06 (-0.07-0.10)	0.05 (0.04-0.06)	0.03 (-0.03-0.12)	0.02 (-0.05-0.09)	-0.03 (-0.09-0.06)
Root mean square of successive NN interval difference (rMSDD)	-0.06 (-0.88-2.80)	0.32 (-0.46-1.56)	0.01 (0.00-0.03)	1.02[c] (0.21-2.81)	-0.29 (-0.77- -0.05)	-0.04[e] (-0.53-3.22)
Respiratory rate; Breath per minute (BPM)	-0.02 (-0.41-0.73)	-0.16 (-0.48-0.75)	0.29[b] (-0.19-0.76)	-0.26[c] (-0.54-0.17)	0.04[d] (0.02-0.08)	0.12[e] (-0.18-1.04)
Tidal volume (TV)	-0.05 (-0.64-1.72)	0.11 (-0.43-2.05)	-0.15[b] (-0.56-0.87)	-0.32[c] (-0.32-1.78)	-0.22[d] (-0.57-0.48)	-0.12 (-0.61-1.18)

p<0.05 ;

a; Differences between the second and third episode (b-c) versus Differences between the first and the second episode (a-b)

b; Differences between the third and forth episode (c-d) versus Differences between the second and third episode (b-c)

c; Differences between the forth and fifth episode (d-e) versus Differences between the third and forth episode (c-d)

d; Differences between the fifth and sixth episode (e-f) versus Differences between the forth and fifth episode (d-e)

e; Differences between the sixth and seventh episode (f-g) versus Differences between the fifth and sixth episode (e-f)

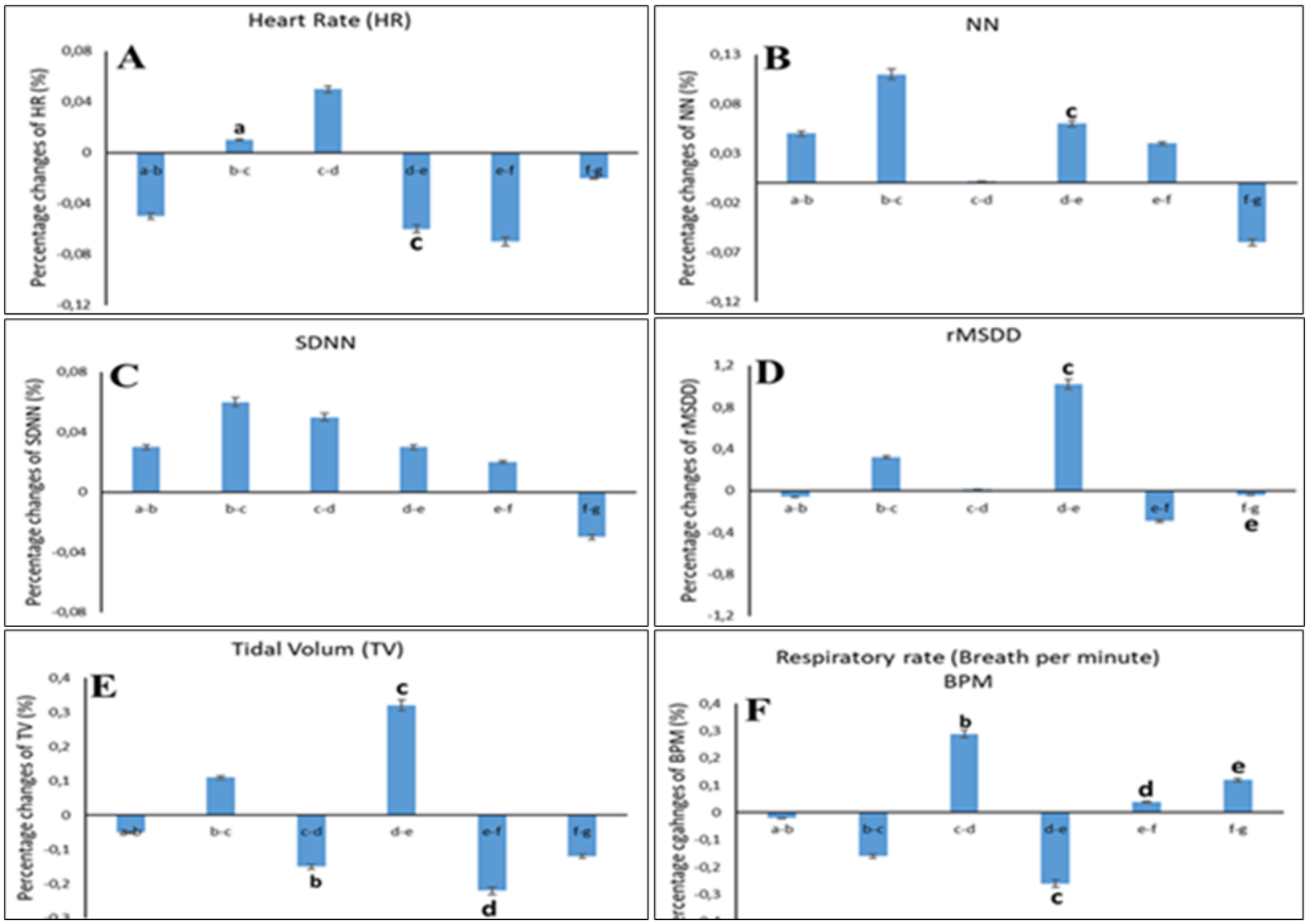


Figure 1. The percentage changes of heart rate, heart rate variabilities (NN, SDNN, rMSDD) and respiratory parameters (BPM, TV) between episodes of the study groups (n:10). A: Percentage changes of heart eart rate (HR); B: Percentage changes of NN interval; C: Percentage of SDNN intervals; D: Percentage changes of rMSDD; E: Percentage of tidal volum (TV); F: Percentage of respiartory rate (breath per minute, BPM).

P<0,05;
a; Differences between the second and third episode (b-c) versus Differences between the first and the second episode (a-b)
b; Differences between the third and forth episode (c-d) versus Differences between the second and third episode (b-c)
c; Differences between the forth and fifth episode (d-e) versus Differences between the third and forth episode (c-d)
d; Differences between the fifth and sixth episode (e-f) versus Differences between the forth and fifth episode (d-e)
e; Differences between the sixth and seventh episode (f-g) versus Differences between the fifth and sixth episode (e-f)

alone (p<0.0001; b-c: -0.16 vs c-d:0.29). A significant decrease was found in the percentage differences of episodes d-e in which dog was alone comparison to the percentage differences of episodes c-d in which dog with owner again (p<0.0001; c-d:0.29 vs d-e:-0.26). A significant increase was determined in the percentage differences of episodes e-f which dog encountered stranger again comparison to the percentage differences of episode d-e which dog was alone in the room (p<0.0001; d-e:-0.26 vs e-f:0.04). However, in the last period the percentage differences of episodes f-g which dog with owner again increased compared to the percentage differences of episodes e-f which dog with stranger again (p:0.031; e-f:0.04 vs f-g:0.12).

Percentage differences of tidal volume (TV)

A percentage decrease (-0,05) was found in episode b which the dog encountered the stranger for the first time. Although not significantly, the percentage differences of episodes b-c which dog was alone increased compared to the percentage differences of episodes a-b which dog was with owner and stranger (p>0.05; a-b:-0, 05 vs b-c: 0.11). In addition, the percentage differences of episode c-d which dog encountered owner again decreased as to the percentage differences of episodes b-c which dog was with stranger alone (p:0.006; b-c: 0.11 vs c-d:-0.15). However, there was a significant increase in the percentage differences in episode d-e which dog was alone compared to the percentage differences in episode c-d which dog was with owner again (p<0.0001; c-d:-0.15 vs d-e:0.32). A statistical decrease was determined in the percentage differences in episode e-f which dog was stranger again comparison to the percentage differences in episode d-e which dog was alone (p<0.0001; d-e:0.32 vs e-f:-0.22). In the last episode, the percentage differences of episodes f-g

which dog with owner again increased than to the percentage differences in episodes e-f which dog with stranger although not significantly (p>0.05; e-f:-0.22 vs f-g:-0.12).

Percentage differences of heart rate variability parameters (HRV; NN, SDNN, rMSSD)

The percentage differences of HRV parameters were examined when the dog encountered stranger for the first time (a-b), an increase was determined in NN (0.05) and SDNN (0.03), while a decreasing trend was observed in rMSSD (-0.06). Also, increases were observed in the percentage differences in episode b-c which dog with stranger alone compared to the percentage differences in episode a-b while dog with owner and stranger, although not significantly (p>0.05; NN; a-b:0.05 vs b-c:0.11 / SDNN; a-b:0.03 vs b-c:0.06 / rMSDD; a-b:-0.06 vs b-c:0.32). Although not statistically significant, HRV parameters were decreased in the percentage differences of episodes c-d which dog with owner again compared to the percentage differences in episodes b-c which dog with stranger alone (p>0.05; NN; b-c:0.11 vs c-d:0.002 / SDNN; b-c: 0.06 vs c-d:0.05 / rMSDD; b-c:0.32 vs c-d:0.01). However, the percentage differences in episode d-e when dog alone, the NN (p:0.002; c-d:0.002 vs d-e:0.06) and rMSSD (p<0.0001; c-d:0.01 vs d-e: 1.02) were increased significantly while SDNN (p>0.05; c-d:0.05 vs d-e:0.03) was slightly decreased compared to the percentage differences in episode c-d which dog with owner again. The decreases in the percentage differences of episodes e-f which dog with stranger again were determined comparison to the percentage differences in episodes d-e which dog was alone (NN: p>0,05; d-e:0,06 vs e-f:0,04 / SDNN: p>0,05; d-e:0,03 vs e-f:0,02 / rMSSD: p<0,0001; d-e:1,02 vs e-f:-0,29). In addition, the last episode in which dog with owner again NN (p>0.05; e-f:0.04 vs f-g: -0.06) and SDNN (p>0.05; e-f:0.02 vs f-g: -0.03) were decreased while rMSSD (p:0.019; e-f:-0.29 vs f-g: -0.04) was increased as to the percentage differences in episodes e-f which dog with stranger again.

Discussions

Individuals often choose to have pets based on factors such as lifestyle management or environmental influence, rather than considering their own personal needs and preferences. This decision can lead to challenges and difficulties in establishing compatibility and effective communication between humans and animals. The objective of this study was to examine the physiological and behavioral impacts of human-animal interaction in family dogs. Specifically, we aimed to assess the changes in HR, HRV, BPM, and TV through the use of an external telemetry system. The primary objective of this research was to examine the percentage changes in HR, HRV, BPM, and TV during the strange situation testing procedures. Our findings revealed that brief separations from owners and interactions with a stranger could potentially induce notable alterations in these physiological parameters in family dogs.

To the best of our knowledge, there is limited research available on the assessment of short-term HRV in normal and healthy dogs, specifically within a 3-minute duration. In the present study, we focused a systematic approach to record HRV parameters at 3-minute intervals over a span of 21 minutes. The objective was to closely examine the impact of interactions with a stranger on family dogs. Throughout the various phases of the study, we observed consistent patterns in the HRV parameters, specifically NN, rMSSD, and SDNN, indicating both decreases and increases. These fluctuations were observed consistently across all experimental conditions. During Episode b, which marked the initial encounter with a stranger, we noted a decrease in the percentage changes of HR, rMSDD and TV, while NN, SDNN and BPM exhibited an increase. The comparison of HR between the percentage differences of episodes b-c in which dog with stranger alone and episodes a-b in which dog with owner and stranger showed that a significant increase (p:0,0001; a-b:-0,05 vs b-c:0,01). Also, HRV parameters (NN, SDNN and rMSDD) and TV increased in the percentage differences of episodes b-c compared to the percentage differences of episodes a-b, although non significantly. However, BPM values decreased in the percentage differences of episodes b-c than the percentage differences of episodes a-b, not significantly. Consistent with our findings, Gacsi et al. (2013) conducted a study using a holter system to assess heart rate responses in dogs encountering a stranger and their owner. They reported an increase on HR during the period of stranger interaction, supporting the notion that the presence of an unfamiliar person can elicit an arousal response in dogs. Furthermore, Handlin et al. (2011) conducted a study that focused on heart rate changes in dogs in unfamiliar surroundings with their owners. They utilized a polar system to monitor heart rate every 15 seconds. The results of their study indicated that dogs exhibited normal behavior in such unfamiliar environments when accompanied by their owners. In contrast to our study, a study conducted by Palestrini et al. (2005) reported a decrease in HR when dogs were exposed to a friendly stranger. This finding suggests that the presence of a familiar and trusted individual, such as the owner, can have a calming effect on dogs and mitigate potential stress responses

associated with novel situations. Therefore, the context of the interaction, including the presence of the owner, appears to play a significant role in modulating heart rate changes and the overall behavior of dogs in unfamiliar surroundings.

Dogs share a strong bond with their owners, yet their behavioral patterns can vary when they are separated from them. In the recent study, the effects of the stranger on HR at the reunion episode (c-d; which dog with owner again) had a slight increase in mean of percentage differences versus 1st separation episode (b-c; which dog and stranger alone; p>0.05; b-c:0.01 vs c-d:0.05). However, all HRV parameters had decreasing tendencies in percentage differences of episodes c-d when dog encountered owner again compared to percentage differences of episode b-c (p>0.05; NN; b-c:0.11 vs c-d:0.002 / SDNN; b-c: 0.06 vs c-d:0.05 / rMSDD; b-c:0.32 vs c-d:0.01). However, respiratory rate (BPM) increased (p<0.0001; b-c: -0.16 vs c-d:0.29), while TV decreased (p:0.006; b-c: 0.11 vs c -d:-0.15) in percentage differences of episodes c-d compared to percentage differences of episodes b-c, significantly. Similarly our results, Zupan et al. (2016) conducted a study on dogs, and reported that the rMSSD, a measure of HRV, exhibited different patterns depending on the dogs' emotional state. Specifically, when the dogs were in a positive state, such as during interaction with a familiar person or in the presence of food, the rMSSD decreased. On the other hand, when the dogs were in a resting state, the rMSSD increased. The authors suggested that this observed pattern may be attributed to parasympathetic deactivation in response to positive emotions elicited during social interactions or when being petted. Thus adds to our understanding of the complex relationship between emotions and physiological responses in dogs. According to our results, during positive states such as episode d and the percentage differences of episodes c -d (when the owner entered the room and the stranger left, and the owner engaged in petting), we observed an increase in HR and BPM, accompanied by a decrease in HRV measured by rMSSD, as well as a decrease in TV. It was observed that, the reaction to separation from the attachment figure like as owner may has an impact role on dog emotional stress.

Emotional states have been reported depend on motor and respiratory activity as well as behavioral changes. Fallani et al. (2006) found that guide dogs display controlled emotional responses during attachment testing procedures with their owners, indicating the influence of the dog-owner bond on their behavior during separation. Furthermore, our study investigated the physiological responses of dogs when they were left alone or in the absence of the owner. The differences between episodes d-e compared to episodes c-d, responses of remaining dog alone, we observed decreases in HR (p<0.0001; c-d: 0.05 vs d-e: -0.06), SDNN (p>0.05; c-d:0.05 vs d-e:0.03) and BPM (p<0.0001; c-d:0.29 vs d-e:-0.26), indicating a potential activation of the autonomic nervous system and decreasing breathing activity. In addition, NN (p:0.002; c-d:0.002 vs d-e:0.06), rMSSD (p<0.0001; c-d:0.01 vs d-e: 1.02) and TV (p<0.0001; c-d:-0.15 vs d-e: 0.32) showed increases significantly. These findings are in line with the study conducted by Fallani et al. (2007) showed that cardiac activation increased during episodes involving meeting strangers but decreased during isolation episodes. On the contrary, the study by Palestrini et al. (2005), which reported an increase in HR when dogs were left alone in both first and other episodes of loneliness. This discrepancy may be attributed to the specific conditions and experimental designs of each study, highlighting the complexity of canine physiological responses in different social situations. Also, the similarity suggests a consistent physiological response to solitude across different contexts.

In the recent study, the decreases in HR (p>0.05; d-e:0.06 vs e-f:-0.07), and HRV parameters (NN: p>0,05; d-e:0,06 vs e-f:0,04 / SDNN: p>0,05; d-e:0,03 vs e-f:0,02 / rMSSD: p<0,0001; d-e:1,02 vs e-f:-0,29) and BPM (p<0.0001; d-e:-0.26 vs e-f:0.04) while significant increase in TV (p<0.0001; d-e:-0.32 vs e-f:-0.22) was observed in the percentage differences of episode e-f which dog with stranger again compared to percentage differences of episode d-e which dog remained alone. Contrary our results, Palestrini et al. (2005) observed that in the second instance of meeting a stranger, HR increased. This suggests that the dogs' HR response may vary depending on the specific context and familiarity of the stranger. In addition to our findings, previous studies have reported increased HR and SDNN in dogs during the process of being petted by unfamiliar individuals (Kuhne et al., 2014). These researchers suggested that such interactions may create a social conflict situation for dogs when they are being petted by unfamiliar humans. Furthermore, it has been demonstrated that emotional stress can be induced by the presence of strangers and an unfamiliar environment, which may lead to cardiac acceleration as a cardiovascular response to minor motor activity. All of these factors can be regarded as potential sources of stress.

It has been reported that for dogs, psychological changes, especially separation from the owner and subsequently being reunited, are associated with increased physical activity and changes in some physiological parameters (Gacsi et al., 2013). The study

determined that the presence of the owner had a significant impact on heart rate (HR) and heart rate variability (HRV). In the present study, in the last episode, when the owner encountered the room again, the percentage differences between episodes f-g, there were slight increases in HR ($p>0.05$; e-f: -0.07 vs f -g: -0.02) and TV ($p>0.05$; e-f:-0.22 vs f-g:-0.12) while significant increases were observed in rMSSD (p:0.019; e-f:-0.29 vs f-g: -0.04) and BPM (p:0.031; e-f:0.04 vs f-g:0.12) compared to the percentage differences between episodes e-f in which dog with stranger alone. In addition, NN ($p>0.05$; e-f:0.04 vs f-g: -0.06) and SDNN ($p>0.05$; e-f:0.02 vs f-g: -0.03) were decreased in the percentage differences of episodes f-g as to the percentage differences of episodes e-f which dog with stranger again. These findings suggest that positive interactions with the owner can influence the physiological parameters of dogs, leading to changes in their cardiovascular and respiratory responses.

There is a negative correlation between HR and HRV generally due to physiological and mathematical reasons (Sacha, 2013; Billman, 2013). Also, it was suggested that to overcome this negative correlation, increase of parasympathetic activity is necessary which is elevated against a heightened HR. This change of patterns is a result of concurrent activity of the sympathetic and the parasympathetic branches of the autonomic nervous system. In the recent study, HR and HRV parameters were demonstrated increases in the percentage differences of stages b-c in which dog alone with stranger than episodes a-b in which dog with owner and stranger at first. Dogs showed high HR and HRV even if they with their owner when stranger encountered and also with stranger alone. Following episodes, when dog with owner again, the percentage differences of episodes c-d (reunion episode) than episodes b-c (1st separation episode), the HR increased while the HRV parameters decreased. Also, HR increased while HRV decreased in percentage differences of episodes f-g (dog with owner, last episodes percentage) in comparison to episodes e-f in which dog with stranger again after remaining alone. This would suggest that stressing event for dogs was the stranger that a source of stress (Palesterini, 2005). Interestingly, both HR and HRV decreased in percentage differences of episodes e-f in which stranger encountered compared to episodes d-e in which dog remained alone. Stranger entrance was similar to that observed towards the owner. This may be due to encountered the same stranger again after remaining alone which may be a distressful situation for dogs. Changes in HR during specific episodes such as encountered stranger, remaining alone, reunion the owner, the emotional responses can be associated with cardiac acceleration.

In animals experiencing emotional states, there can be alterations in cardiac responses, such as HR, HRV, and respiratory parameters. Despite experiencing excitement or anger, the parasympathetic regulation of the nervous system can help maintain a balance with these physiological responses. Various studies have observed that dogs can exhibit diverse emotional responses when interacting with both familiar and unfamiliar individuals (Prato-Previde et al., 2003; Palestrini et al., 2005; Gacsi et al., 2013; Rehn et al., 2013). Moreover, it is important to note that the sample size and variations are crucial factors in elucidating these attachment behaviors. The present study, it was suggested that to investigate the relationship between physiological and behavioral responses of family dogs during the Strange Situation Test using an external telemetry system can be important for clarifying the human animal bond. The external telemetry system offers researchers a comprehensive tool for studying the effects of pharmacological interventions, investigating animal behavior and responses, as well as facilitating therapeutic applications in animal-assisted interventions. This innovative approach can enhance our understanding of physiological and behavioral processes in animals, ultimately leading to improved treatments and interventions in various contexts.

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Conflict of Interest

The authors declare that there are no conflicts of interest regarding the findings and conclusions presented in this study.

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The effect of egg shape index on pore number and hatching performance in Sussex hens

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ABSTRACT

This study was carried out to investigate the effect of egg shape index on pore number and hatching performance in Sussex chickens. The material of the study consisted of 63 eggs obtained from the Sussexbreed chicken flock of a private enterprise engaged in chicken rearing in Konya. Eggs were divided into two groups as below 75 (Group-1, 75<) and above (Group-2, ≥75) according to their shape index values. Some egg external quality characteristics, regional pore numbers as well as chick weight values of the experimental groups were examined Shape index, egg width, number of pores at the equator end up, number of pores at the pointed end up and chick weight were significant ($p<0.01$), but egg length and number of pores at the blunt end up were similar ($p>0.05$). As a result, the obtained data showed that the shape index had an effect on the external egg quality and chick weight along with the number of pores in the eggs in Sussex chickens.

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Introduction

Nowadays, there are more than 200 chicken breeds in the world, which are differentiated in terms of features such as color, shape, size and yield. Sussex chickens were developed in the first half of the 19th century as a subspecies of Dorking without five toes. They are an indispensable breed of European countries that demand white skin in their parent lines in broiler production, as they are white-skinned, well-developed and also can inherit these characteristics. It is still used as a broiler line in the production of white-skinned broiler chickens (Sarıca, 2014, Dere and Guler 2022).

Poultry species provide the continuity of their generation with eggs. In addition to albumin and egg yolk, which provide nutrients to the embryos during incubation, the egg shell, which protects its weight values against external environments and is responsible for gas exchange, constitutes the three main elements of an egg (Hincke et al., 2012, Nangsuay et al., 2015). In poultry species, genotype (Tůmová et al., 2009, Lewko et al., 2021; Krawczyk et al., 2023; Tadele et al., 2023), rearing conditions (Tůmová et al., 2011), nutrition (Pérez-Bonilla et al., 2011), age (Akyurek and Okur, 2009; Zita et al., 2013; Chung and Lee, 2014; Park and Sohn, 2018) and storage (Tilki and Saatci, 2004; Demirel and Kırıkçı, 2009; Günhan and Kırıkçı, 2017; Nasri et al., 2020; Çam et al., 2022) affect the external quality of eggs.

The quality of egg shells and egg quality is determined by both genetic and environmental factors related to genotype, rearing system, parent stock age, nutrition, health, and physiological state, cage and room temperature storage of eggs (Jones and Musgrove, 2005, Banaszewska et al., 2019, Eleroğlu, 2021, Wengerska et al., 2023). The pores on the eggs; Although their number varies depending on the species, the regional (pointed, equatorial, and blunt)

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localization on the egg differs numerically (Rokitka and Rahn, 1987). Alasahan et al., (2019) reported that the egg shell contains approximately 10.000 pores, depending on the species. These pores provide gas and water exchange to the development of the chick embryo during incubation (Eddin et al., 2019).

Pores are also effective not only in gas exchange during incubation, but also in water and weight loss of the stored egg (Ar et al., 1974; Peebles and Brake, 1987).Internal and external variables can have an impa ct on the growth and development of embryos (Tong et al., 2013; van der Wagt et al., 2020).

Hatched eggs had more pores than the late embryonic death and pip groups, according to Peebles and Brake (1985), who also noted that insufficient pores may be th e cause of embryonic deaths (Peebles and Brake, 1985).

To maintain a normal overall diffusive water loss, eco logical and taxonomic heterogeneity may modify some of the linkages expected for the 'average' egg (Amos and Rahn, 1985).

The development of the embryo is associated with egg quality characteristics and the number of pores. For this reason, it is stated by Narushin & Romanov (2002) that eggs with an average egg quality value (egg weight etc.) are more suitable for hatching. One of the important egg quality characteristics affecting hatching performance is the shape index (Narushin and Romanov, 2007).

Shell structure, which is a feature that affects egg quality and hatching performance, correlates with embryonic deaths in hatching. The aim of this study was to determine the relationship of shape index with pore number and embryonic mortality in Sussex chicken eggs.

Materials and Methods

Animal materials

The material of this study consisted of 63 eggs obtained from the Sussex breed chicken flock of a private enterprise engaged in chicken breeding in Konya.

Experimental design

The Sussex eggs obtained from the brood flocks were collected daily and stored at 14 oC temperature and 70% humidity conditions for seven days. These eggs were divided into two groups as 35 eggs below 75 (Group-1, 75<) and 28 eggs above (Group-2, ≥75) according to their shape index values. The shape index of the eggs was calculated with the formula (Egg Width / Egg Length) x 100.

The eggs in the two groups were incubated at 37.7°C and 60% humidity in the incubator, and 37.6°C and 75% humidity in the hatching machine. The live weights of the hatched chicks were determined by weighing them with a digital scale (KERNPFB 100) with a precision of 0.01 g.

Determining the number of hatching performance

Egg fertilization and embryonic death of chick hatching were determined according to the method reported by Hamburger and Hamilton (1990).

Fertility rate (%) = (number of fertile eggs / number of set eggs) x 100

Hatchability of fertile eggs rate (%) = (number of hatched chicks / number of fertile eggs) x 100

Hatchability rate (%) = (number of hatched chicks / number of set eggs) x 100 Determining the number of pores

Peebles and Brake (1985) method was used to determine the number of pores in eggs with and without hatching. Eggs that were broken during pore counting or that did not have a clear image in pores were not evaluated.

The solution prepared with 89% 0.5 g of methylene blue in 1 liter of 70% ethanol was stained by the method of Board and Halls (1973).

Pores were counted with a dissection microscope by marking areas of 0.25 cm^2 from the blunt, equatorial, and pointed ends of each egg.

Egg pore counts of three regions were stained according to the same method, and photographed under the microscope, and the pore counts obtained from the counts were recorded in the Microsoft Excel program.

Statistical analysis

In the study, Kolmogorov-Smirnov and Shapiro-Wilk tests were used to examine the normality of quantitative variables. Comparison of the means of two groups, where the assumption of normality was met, was made with the Independent t test. In examining the correlations between variables, Pearson correlation coefficient was used. IBM SPSS Statistics 29.0 package program was used in statistical analysis and $p<0.05$ value was considered statistically significant.

Results

Some egg external quality characteristics and regional pore number, and chick weights according to egg shape were presented in Table 1.

Shape index values of Group-1 and Group-2 were measured 73.99 and 77.17 respectively ($p<0.001$). The traits of eggs in poultry have important indications as to which eggs were chosen for hatching. Egg length and egg width values was calculated as 55.85-41.28 and 56.59-43.61, respectively (Table 1). In this study, the number of pores in the experimental groups was

Table 1. Some egg external quality characteristics and regional pore number, and chick weights according to egg shape index on Sussex eggs.

Variables		Group-1		Group-2		p values
		n	Mean ± SE	n	Mean ± SE	
Shape Index		35	73.99±0.44	28	77.17±0.70	0.000
Egg length		35	55.85±0.30	28	56.59±0.38	0.128
Egg width		35	41.28±0.11	28	43.61±0.24	0.000
Pore number	EEU	28	34.54±2.17	20	25.05±2.15	0.004
	BEU	28	29.32±2.16	20	27.30±2.43	0.541
	PEU	27	30.70±1.74	17	19.94±1.22	0.000
Chick weight		20	30.51±0.50	15	34.86±0.67	0.000

Group-1: SI<75, Group-2: SI>75. EEU: Equator end up, BEU: Blunt end up, PEU: Pointed end up.

calculated as 34.54-25.05, 29.32-27.30-30.70-19.94 per 0,25 cm^2 at the equator, blunt and pointed ends in group-1 and group-2, respectively. Shape index, egg width, chick weight ($p<0.01$), and pore number of EEU and PEU ($p<0.01$) were found significant. On the other hand, egg length and pore number of BEU were determined no significantly ($p>0.05$) (Table 1).

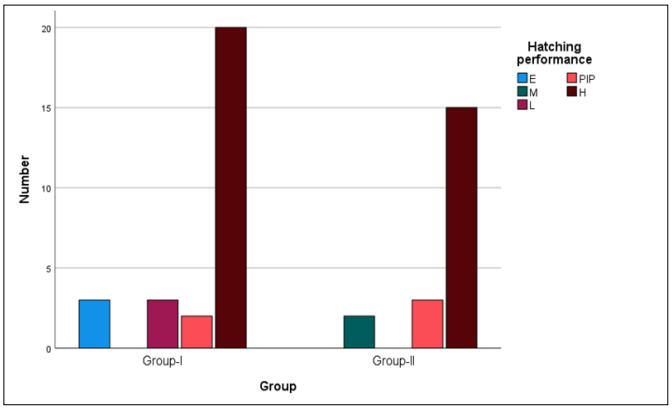

Figure 1. Hatching performance of different egg shape index in Sussex

Group-1: SI<75, Group-2: SI>75. E: Early embryonic mortality, M: Middle embryonic mortality, L: Late embryonic mortality, PIP: Pipped embryonic mortality, H: Hatching chicks.

Rate of fertility, hatchability and hatchability of fertile eggs in Sussex eggs were given in Table 2.

Table 2. Hatching performance of egg shape index groups in Sussex

	Fertility rate (%)	Hatchability of fertile eggs rate (%)	Hatchabiliy rate (%)
Group-I	80.00	71.43	57.14
Group-II	71.43	75.00	53.57
Pearson X^2	0.630	0.075	0.080
P value	0.427	0.784	0.777

Group-1: SI<75, Group-2: SI>75.

Fertility (%), hatchability of fertilized eggs (%), as well as hatchability of the shape index groups were found to be similar in Table 2.

Correlation of pore numbers in different regions of eggs were given in Table 3.

Table 3. Correlation of pore numbers in different regions of eggs

		Equator	Blunt	Point
Blunt	Pearson correlation	0.157	-	
	Sig. (2-tailed)	0.288	-	
	N	48		
Point	Pearson correlation	0.086	0.611**	-
	Sig. (2-tailed)	0.577	0.000	-
	N	44		

** Correlation is significant at the 0.01 level (2-tailed).

As seen in Table 3, it was determined that there was a significant correlation between the blunt and pointed ends of the egg ($p<0.01$).

Discussion

In this study, some egg quality characteristics, pore number and chick weight values belonging to different shape index groups were analyzed. Shape index, egg lenght, egg width, regional pore number, and chick weights were given in Table 1. The effect of egg shape index on chick weight was found to be significant. This finding is consistent with Kruenti et al. (2022), which reported that chick weight increases as egg size increases in Japanese quail.

The size of the egg yolk increases so that the old parent stock produce larger follicles. Accordingly, the size of the eggs increases with age. However, because the amount of calcium carbonate existing to form the

shell remains the same, shells were examined against the growing eggs and eggs with a number of pores with a number of pore were obtained than the eggs of parent stock (Stringhini et al., 2011). According to Ancel and Girard (1992), the number of porosity is decreased from the end to pointed. This is explained as a biological condition with larger pore concentration at the wide end of the egg (Araújo et al., 2017).The average number of pores (32.5) reported in Sussex-66 line chickens by Lewko et al. (2021), who reported that different genotypes and age differences affect the number of pores in eggs, was found to be similar with the values obtained in this study. In a study examining the pore numbers of geese at different parent flock ages, it was reported that the number of pores in the blunt and equatorial regions was higher in the first year, while the total number of eggs reached the highest level in the fourth year (Kucharska-Gaca et al., 2023).

As seen as Table 1, the effect of the shape index on the equator and pointed end was significant ($p<0.01$), but the number of pores at the blunt end was found to be similar. Araújo et al., (2017), who reported that eggshell porosity showed a correlation of 0.870 with the weight of the hatched chicks, reported that the effect of age, eggshell region, and age*eggshell region on the pore number was significant ($p<0.01$). In addition, in the same study, they calculated the number of pores of eggs obtained from 29, 35, and 59-week-old broiler chickens as 125, 112, and 95 per cm2 at the blunt, equatorial, and pointed ends, respectively.

As a result, researchers (Meir and Ar, 2008; Stringhini et al., 2011; Kucharska-Gaca et al., 2023) argue that although the species are different, the number of egg pores generally varies regionally and increases with breeding age. The findings of this study, in which the effect of shape index on the number of pores was determined to be significant, are compatible with the information reported by Stringhini et al. (2011), who reported that larger eggs have a higher number of pores.

Shape index consists of the role of egg shape in the direction of turning during incubation and the determination of embryo movements for nutrient utilization (Hristakieva et al., 2017). The effect of egg shape index on hatching result evaluation parameters was not found to be statistically significant ($p>0.05$). Ascı and Durmus (2015) reported that the shape index should be between 72-76% for optimum hatching and breeding eggs, and that the shape index is a factor affecting hatching performance. In this study, it was determined that shape index did not affect hatching performance (Table 2). This finding was found to be different from the researchers who argued that egg size affects hatching and fertility rates in Koekoek chickens Molapo and Motselisi (2020) and who reported that it affects some hatching performance in Japanese quails (Khurshid et al., 2004; Alasahan and Copur, 2016; Gutiérrez et al., 2021). This may be due to genotype or age factors (Keskin et al., 2022).

Conclusion

As fertilized eggs obtained from Sussex chickens were examined, it was determined that different shape index groups had a significant effect on egg width and chick weight. The higher chick weight in the group with higher egg width can be considered as a factor affecting the shape index and chick weight. While no difference could be found in the blunt end in the shape index groups according to the number of pores, this difference occurred at a significant level ($p <0.01$) in the equator and pointed end.

The shape index value calculated based on the width and length values of the eggs showed a close relationship with the hatching performance. In this study, a high number of pores were determined in large eggs in terms of shape index. Thousands of pores on the egg prevent fluid loss during storage and incubation.

As a result, the obtained data showed that the shape index had an effect on the external egg quality and chick weight along with the number of pores in the eggs in Sussex chickens.

Authors' contributions

All authors contributed to the study conception and design. First draft writing and data collection: [EA], [RO]; general control and interpretation: [KK]; Formal and statistical analysis: [HY]; Material preparation and methodology: [EA] and [RO]; the methodology: [EA] and [KK]. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval; This study was approved by the Animal Experiments Ethics Committee of Selçuk University Experimental Medicine Application and Research Center (SUVDAMEK) with reference number 2023/043 dated 27.04.2023.

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JIVS JOURNAL OF ISTANBUL VETERINARY SCIENCES

Impact of betaine on the performance and specific haemato-biochemical parameters in heat-stress exposed broiler chickens

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ABSTRACT

The present study revealed that there was a significant (P<0.05) effect of betaine on body weight, feed intake, and feed conversion ratio (FCR) of the broiler. Productive performance and blood cholesterol level of the broiler. A total of 150-day-old broiler chicks (Cobb 500) were placed into five dietary treatment groups: T0 (control diet), T1 (0.03% betaine in water), T2 (0.06% betaine in water), T3 (0.09% betaine in water), and T4 (0.12% betaine in water). Each group consisted of three replications containing 10 birds in each. Body weight gain (BWG), mortality rates, and meat yield characteristics were recorded. The collected data were analyzed by one-way ANOVA using SPSS version 25.00 software. Above, the total body weight was significantly highest in T2 (1758.3 ± 7.61 g), followed by T1 (1602.6 ± 10.16), T3 (1632.5±12.68 g), T4 (1606 ± 22.65 g), and T0 (1425.5 ± 10.14 g), respectively. The FCR was found to be lowest in T2 (1.36) and highest in T0 (1.49), whereas the FCR of T1, T3, and T4 were 1.43, 1.39, and 1.38, respectively. It was found that there was a significant difference (P<0.05) between the dietary groups for carcass weight, live weight, thigh weight, and breast weight. It was found that there was a significant difference among the treatment groups for cholesterol levels. During the experimental period, there was no mortality among the dietary groups. The T2 group generated a much larger net profit per broiler. Betaine supplementation in broilers is advantageous for growth performance, economic benefit, and lipid profile when used at 0.06% through drinking water, according to this study's findings. In the production of broilers, it may also be the best substitute as a growth promoter, stress reliever, and immune booster.

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Introduction

A high environmental temperature is one of the most important factors that causes heat stress among chickens and negatively affects poultry production (Meremikwu et al., 2013). Usually, the optimum temperature for growing broilers is 18 to 22 °C, and any temperature higher than that range could cause heat stress (Daghir, 2009). Heat stress normally happens in the summer season when there is a negative balance between the environmental temperature and body heat production. The management of heat stress (HS) is a subject of increasing concern for industry with increasing global

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temperatures and the incidence of sub-tropical and tropical broiler production (Henry et al., 2012). Broilers are susceptible to increased environmental heat loads for a variety of reasons, including that they lack sweat glands and feather coverage, and that increased selection for muscling means they are comparatively less resilient to heat than other production animals (Renaudeau et al., 2012). Broilers that are reared under hot conditions have a reduced growth rate, in part due to reduced feed intake. However, only half of the consequential reduction in growth rates is due to feed intake alone (Geraert et al., 1996), and skeletal muscle from thermally challenged broilers has reduced rates of protein synthesis and, to a lesser extent, proteolysis. Betaine is a naturally occurring substance found in a variety of plants and animals. It is the tri-methyl derivative of the amino acid glycine (Ratriyanto et al., 2009). A hydrocarbon made up of hydrogen and carbon is called betaine. It has three (CH3) methyl groups bound by the amino group of glycine amino acids to an amide group. Betaine is produced naturally and artificially from various plants and animals (Boch et al., 1994). Beet roots naturally contain higher concentrations of betaine (Wang et al., 2004). Betaine has a variety of purposes, one of which is to donate its labile methyl group, which is then employed in trans-methylation processes to create carnitine and creatine (Eklund et al., 2005; Kidd et al., 1997). When betaine reacts with homocysteine, it exerts a methionine-saving effect by donating a methyl group rather than methionine (Paniz et al., 2005). Osmolyte betaine supports cellular water equilibrium (Klasing et al., 2002). Supplementing with betaine in the diet likely lessens the need for other methylgroup donors like methionine and choline (Siljander-Rasi et al., 2003). Under heat-stressed conditions, betaine supplementation in feed enhances growth performance and feed intake (Hassan et al., 2005). Betaine has a beneficial effect since it makes chickens' bodies less hot to the touch (Klasing et al., 2002). Methionine is assumed to be spared from this function since betaine is a methyl-group donor, allowing methionine to be used more for growth and muscle development (Paniz et al., 2005). Heat-stressed birds have a dramatic drop in feed intake as a physiological reaction to reduce intrinsic heat production and preserve thermal homeostasis, which lowers feed efficiency, live weight gain, and survival rates (Geraert et al., 1996; Koh et al., 1999; Deaton et al., 1982; Faria Filho et al., 2007). The other detrimental impacts of heat stress that reduce the economic value of broiler carcasses are reduced breast-meat yield and increased carcass-fat deposition. In broiler production, a number of feed additives have been used to improve growth, feed efficiency, immune status, and antioxidant capacity (Abudabos et al., 2016). Various techniques are also practiced to reduce heat stress in poultry (Chand et al., 2016). Such methods include the use of electric fans, cooling pad systems, and the sprinkling of water through foggers (Khan et al., 2014). As most of these methods cannot be practiced due to high expenses, other strategies such as nutritional therapies, including the use of balancing nutrient contents and the addition of vitamin C, sodium bicarbonate, potassium carbonate, and aspirin in drinking water, can be followed. One of these nutritional strategies for reducing stress in the broiler is the use of betaine as a feed additive in the poultry diet (Zimmermann et al., 1996). Betaine, when examined as water or feed additives, has been shown to have many benefits to the poultry sector, involving enhanced carcass composition by altering the lipid metabolism (He et al., 2015), boosted intestinal morphology, enhanced antioxidant defenses, and decreased lipid peroxidation in the breast meat (M. Alirezaei et al., 2012). During heat stress, the body cells of birds are subjected to osmotic stress. In such instances, water is pulled out of the cell because of a higher concentration of salts or solutes outside the cells. This loss of water can cause the cells to shrink in volume, and if this water loss is not corrected, the cell will eventually die. Although poultry do not have a specific requirement for betaine, the osmolytic property of betaine could be beneficial to heat-stressed birds. The present study was conducted with the following objectives: 1. To investigate the effect of betaine on the productive performance of broilers. 2. determining the effect of betaine on the blood cholesterol level of broilers.

Materials and Methods

Experimental site

The experiment was carried out at the poultry farm of Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, to determine the impact of supplementing with betaine on the performance and meat yield traits of broilers (Cobb 500) during the summer season from April 13 to May 11, 2022.

Experimental birds

For the experiment, 150-day-old broiler chicks (Cobb 500) were collected from the Kazi Farm hatchery via local traders.

Layout of the experiment

The chicks will be randomly assigned to one of five nutritional treatment groups (T0, T1, T2, T3, T4), each of which will consist of three replications with ten birds each. The following are the treatments: T0 = control, T1 = control + 0.03% betaine per liter of water, T2 = control + 0.06% betaine per liter of drinking water, T3 = control + 0.09% betaine per liter of water, and T4 = control + 0.12% betaine per litre of drinking water.

Collection and preparation of betaine and feed

Betaine was purchased from the local market in Dinajpur. For the experimental trial, ready-feed was used. The feed used in the experiment was bought from a feed store in the town of Dinajpur.

Managemental practices

Housing, litter, and feed (CP Feed Co. Ltd.) Broiler pre-starter: 1–7 days; broiler starter: 8–15 days; broiler grower: 16–28 days; water, lighting, sanitization, and vaccination were all necessities provided. Adequate precautions were implemented throughout the study period. During the whole experimental period, room temperature was maintained at 35 ºC. This study evaluated the survival ability of broilers at high temperatures with a supplement of betaine.

Calculation

1. Total gain in weight (g) = final weight – initial weight. **2**. Dressing percentage = (dressed weight ÷ body weight) x 100. **3**. Total feed consumption (g) = total feed offered – total left-over. **4.** Feed efficiency = total feed consumed / total gain in weight. **5.** Mortality rate (%) = no. of dead chickens / total no. of birds as a group × 100.

Hematological analysis

Blood samples were collected after 4 weeks using a vacutainer tube through the wing vein puncture tubes (BD vacutainer SST Gel-5 ml). They were then permitted to coagulate at room temperature (25 degrees Celsius) for an hour. The serum was recovered from the blood sample after centrifuging it at 2000 rpm for 15 minutes. Separated, non-hemolyzed serum samples were stored in clean, dry Eppendorf tubes in the deep freezer (-20 °C) for later use. The serum cholesterol concentration was tested using a suitable commercial analytical kit manufactured by the German cholesterol agent company Randof (2016). The experiment was carried out using a Merck Microlab 300 biochemistry analyzer (India) in accordance with the protocol provided in the manufacturer's leaflet.

Statistical analysis

SPSS version 25 software, using the one-way ANOVA method, was used to examine the data of feed consumption, growth performance, carcass features, and hemato-biochemical data in accordance with the principles of Complete Randomized Design (CRD). Significance was assessed when ($P<0.05$) and all results were given as mean ± SEM. Using the Duncan test, the means of the treatment groups were compared.

Results and Discussion

Performance of broiler of experimental birds

Feed consumption, feed conversion ratio, live weight gain, bird mortality, hemato-biochemical properties, cost effectiveness, heat stress, and carcass features were studied in this experiment to observe how broilers responded to various dietary doses of betaine. The results are shown in several tables and explained under the following topic.

Body weight

In the T0, T1, T2, T3, and T4 groups, the initial body weight (g/broiler) was 38.6±1.33, 37.3±1.33, 40.0±0.00, 38.6±0.67, and 39.3±0.67, respectively. At 7th and 14th days old, the body weights of different treatment groups were not significantly varied. At the ages of 21st and 28th days, there was a significant ($P<0.05$) effect of betaine on body weight. The T2 (1758.3±7.61g) group had the highest body weight, which was followed by T1 (1602.6±10.16 g), T3 (1632.5±12.68 g), 1606±22.65 g, and T0 (1425.5±10.14 g), respectively (Table.1). The results of this experiment's body weight gain are consistent with those of Attia et al. (2009). Broilers fed on 0.006% betaine in water showed higher ($P<0.05$) live body weight and body weight gain, according to Bowmaker and Gous (1991) and Hassan et al. (2005). However, Schutte et al. and Rostagno (1997) disagree. According to their findings, broilers treated with betaine at levels greater than 0.08% in the water level displayed ($P<0.05$) reduced live body weight. The fact that betaine is an amino acid supply and works as a protein source may be the cause of the broilers' improved weight gain while utilising betaine. Betaine supplementation affects carcass and part weights because of its methyl-group donor feature, which would boost methionine, cystine, and glycine for

Table 1. Effect of betaine on body weight of broiler

Age in days	Dietary groups (%)					Significance
	T_0	T_1	T_2	T_3	T_4	
Initial body weight	38.67 ± 1.33	37.33 ± 1.33	40.00 ± 0.00	38.6 ± 0.67	39.3 ± 0.67	NS
7^{th} day	174.6 ± 1.76	176.6 ± 0.88	177.8 ± 0.73	174.8 ± 0.73	173.1 ± 1.01	NS
14^{th} day	276.1 ± 1.96	286.0 ± 3.46	287.1 ± 2.59	279.6 ± 5.92	284.3 ± 2.46	NS
21^{st} day	427.1 ± 1.74^{a}	492.8 ± 3.19^{b}	580.5 ± 2.60^{d}	513.5 ± 3.04^{c}	509.6 ± 16.90^{c}	*
28^{th} day	508.8 ± 3.35^{a}	609.8 ± 1.30^{c}	672.8 ± 1.69^{e}	625.8 ± 2.32^{d}	599.5 ± 1.61^{b}	*
Total body weight (1st-28^{th})	1425.5 ± 10.14^{a}	1602.6 ± 10.16^{b}	1758.3 ± 7.61^{d}	1632.5 ± 12.68^{c}	1606 ± 22.65^{b}	*

a, b, c means having different superscript in the same row differed significantly ($P<0.05$). * = 5% level of significance , NS= Non-significant

Table 2. Feed intakes (g) in different groups at different ages of birds

Age in days	Dietary groups (%)					Significance
	T_0	T_1	T_2	T_3	T_4	
7th day	189.5 ± 1.89	191.5 ± 2.02	184.8 ± 2.74	183.1 ± 1.45	181.7 ± 3.58	NS
14th day	373.1 ± 1.17	386.5 ± 0.87	383.3 ± 1.88	378.6 ± 5.78	378.6 ± 0.88	NS
21st day	684.8 ± 1.88^a	732.5 ± 1.32^b	853.5 ± 27.00^e	746.3 ± 2.03^c	764.1 ± 6.93^d	*
28th day	882.7 ± 3.26^a	985.8 ± 2.20^c	972.3 ± 1.30^b	965.2 ± 2.66^b	883.8 ± 2.32^a	*
Total feed intake	2130.2 ± 8.2^a	2296.3 ± 6.41^d	2394.0 ± 32.92^e	2273.4 ± 11.92^c	2208.4 ± 13.71^b	*

a, b, c means having different superscript in the same row differed significantly (P<0.05). *= 5% level of significance , NS= Non significant

protein synthesis and also help to decrease fat deposition in the carcass through various metabolic pathways (McDevitt et al., 2000; Partridge, 2002).

Feed intakes

For broilers fed at 21 and 28 days of age, the difference in feed consumption was significant (P<0.05). At different dietary levels, feed intake did, however, vary modestly between 7 and 28 days of age. The amount of food consumed at 21 and 28 days of age varied depending on the diet. At 21 and 28 days old, feeding behavior seemed significant P<0.05) (Table 2). At 21 and 28 days old, feed consumption increased (P<0.05) when betaine was added to the water. It was discovered that the treatment groups at 0.03%, 0.06%, 0.09%, and 0.12% consumed the most, while the group at 0% consumed the least. This conclusion is consistent with that of Bowmaker and Gous (1991), who discovered that feed consumption peaked (P<0.05) at 0.06% betaine inclusion levels in water at various broiler developmental stages. There was a considerable impact on the betaine level in water during the entire period of the ages' rest. The findings of the current study demonstrated that broilers' growth performance and immunological state were considerably impacted by high ambient temperatures, while these parameters were enhanced by betaine supplementation. When under heat stress, there is a decrease in feed intake, which may be because there is minimal energy needed to maintain heat (Freeman, 1988). According to Awad et al. (2014), giving betaine at a level of 0.06% in the water results in significantly increased feed intake when compared to the control group. Similar to this, Sakomura et al. (2013) showed that broilers treated with betaine consumed considerably more feed than the control group.

Feed conversion ratio (FCR)

At ages 21 and 28, broilers in various treatment groups had varying weekly feed conversion ratios (P<0.05). There was no difference in the betaine levels at 7 and 14 days of age, which could have been caused by the increase in betaine in feed conversion. The lowest FCR was recorded at a betaine level of 0.06%. At 21 and 28 days of age, it was (P<0.05) superior to betaine levels of 0.00%, 0.03%, 0.09%, and 0.12% (Table 3). Our findings demonstrated that adding betaine considerably increased FCR in water at a rate of 0.03–0.08%. With higher levels of betaine, the feed conversion ratio seemed to improve. The inclusion of betaine at a level of 0.06% increased live weight and FCR in broilers, according to many investigators.

Table 3. Feed conversion ratio (feed intake/wt gain) of different birds of different groups

Age in days	Dietary groups (%)					Significance
	T_0	T_1	T_2	T_3	T_4	
7th day	1.08 ± 0.02	1.08 ± 0.01	1.04 ± 0.02	1.05 ± 0.01	1.05 ± 0.02	NS
14th day	1.35 ± 0.01	1.35 ± 0.01	1.34 ± 0.02	1.35 ± 0.01	1.33 ± 0.01	NS
21st day	1.60 ± 0.01^c	1.49 ± 0.01^a	1.47 ± 0.05^a	1.46 ± 0.01^a	1.50 ± 0.04^b	*
28th day	1.73 ± 0.02^d	1.62 ± 0.00^c	1.45 ± 0.00^a	1.54 ± 0.01^b	1.47 ± 0.01^a	*
Final FCR (1st-28th)	1.49 ± 0.81^c	1.43 ± 0.63^b	1.36 ± 4.33^a	1.39 ± 0.94^a	1.38 ± 0.61^a	*

a,b,c means having different superscript in the same row differed significantly (P<0.05). *= 5% level of significance NS= Non significant

Table 4. Effect of feeding betaine to broilers on dressing parameters at different ages

Parameter (g)	Dietary groups (%)					Significance
	T_0	T_1	T_2	T_3	T_4	
Live weight	1425.5 ± 10.14^a	1602.6 ± 10.16^b	1758.3 ± 7.61^d	1632.5 ± 12.68^c	1606 ± 22.65^b	*
Carcass weight	782.6 ± 6.73^a	886.1 ± 3.40^b	984.5 ± 2.86^e	953.1 ± 2.70^d	899.8 ± 3.21^c	*
Breast weight	231.0 ± 3.22^a	345.0 ± 1.93^d	403.1 ± 2.36^e	324.8 ± 6.67^c	309.6 ± 3.05^b	*
Thigh weight	200.2 ± 3.54^a	232.4 ± 3.04^b	285.0 ± 1.70^e	254.2 ± 2.29^c	269.8 ± 1.66^d	*
Head Weight	31.0 ± 0.71^a	35.2 ± 0.66^a	41.0 ± 0.71^c	37.2 ± 0.58^b	34.2 ± 0.37^a	*
Shank weight	37.8 ± 0.86^a	43.6 ± 0.51^a	52.4 ± 0.93^b	48.0 ± 0.71^b	41.2 ± 0.58^a	*
Gizzard weight	40.8 ± 0.86^a	42.0 ± 0.71^a	51.4 ± 1.08^b	58.8 ± 0.86^c	41.2 ± 0.86^a	*
Liver weight	38.6 ± 1.03^a	56.8 ± 1.43^c	74.0 ± 0.89^e	46.0 ± 1.82^b	71.4 ± 1.03^d	*
Heart weight	16.1 ± 0.57^a	19.1 ± 0.21^a	22.2 ± 0.57^b	22.5 ± 1.45^b	21.3 ± 1.15^b	*
Spleen weight	7.2± 0.35^a	7.8 ± 0.31^a	12.9 ± 0.34^b	11.6 ± 0.42^b	11.6 ± 0.70^b	*
Intestine weights	97.0 ± 0.71^a	111.6 ± 2.71^c	105.8 ± 1.59^b	126.6 ± 1.89^d	94.0 ± 1.58^a	*

a, b, c means having different superscript in the same row differed significantly (P<0.05). *= 5% level of significance , NS= Non significant

According to our findings, adding betaine significantly improved FCR in water at a rate of 0.03–0.08%. The results of this study are consistent with those of Attia et al. (2009), who found that adding betaine at a rate of 0.08% in water could partially alleviate chronic heat stress in poultry when compared to the negative treatment. Similar to this, EL Husseiny et al. (2007) found that the addition of betaine at a level of 0.75 g/L in water significantly improved FCR in comparison to the control group.

Meat yield characteristics

Table 4 shows that the highest live weight (1758.3±7.61 g) in group T2 and the lowest live weight (1425.5±10.14)in groups T0 and T1 weight (1602.6±10.16),other weight (1632.5±12.68) T3, and group (1606±22.65)T3, respectively, are significant. Carcass weights were significant, with the highest (984.50± 2.86) found in the T2 dietary group and the lowest (782.6± 6.73) in the T0 dietary group (Table 4). For the percentage of breast meat, drumstick meat, head weight, shank weight, gizzard weight, liver weight, spleen weight, heart weight, and intestinal weight at various diets, significant (P<0.05) variances were found. Broiler meat, particularly the breast and drumstick, nearly rises linearly as betaine levels rise. The tabulated results show that betaine levels significantly influenced the quality of the meat. However, there were changes in breast meat, drumstick meat, abdomen fat, and skin that were significant (P<0.05). Our findings demonstrated that supplementing with betaine at a dose of 0.06% considerably (P<0.05) increased dressing percentage. The osmotic effects of betaine, which promote water retention, may be to blame for the increase in dressing percentage (Waldroup and Fritts, 2005). Our findings are consistent with those of EL Shinnawy (2015). They stated that feeding with betaine at a rate of 800 mg/L at 32 days of age increased the dressing percentage significantly. The current study's findings are consistent with those of Attia et al. (2009), who found that adding betaine to water at a rate of 0.08% could partially relieve chronic heat stress in chickens as compared to the unfavorable treatment. El-Husseiny et al. (2007) and Mahmoudnia, N., et al. (2012) also found that adding betaine to water at a concentration of 0.75 g/L significantly increased FCR compared to the control group.

Measurement of effect of dietary betaine on heat stressed broiler

Heat stress reduced the BWG and feed intake, whereas it increased the FCR. Dietary betaine supplementation tended to improve the BWG and feed intake of broilers under heat stress. The effect of dietary betaine on heat stress broilers was measured by improved production performance rates of panting per minute and wings outstretched and feathers erect. Heat stress can be understood by the rectal temperature, which was between 41 and 42°C (Table 5). The tabulated results

show that betaine levels significantly influenced the quality of the meat. Rectal temperature T0 is the highest, and T2 is the lowest. T3 heart rate is the lowest, while T0 is the highest. The improvement in production performance rate of panting/minute and wings outstretched and feathers erect were used to test the impact of dietary betaine on heat stressors in broilers (Mutibvu et al., 2017; Collins et al., 2012; and Ayo et al., 2011). Heat stress can be understood by the rectal temperature, which was between 41 and 42°C (Table. 5). The tabulated results show that betaine levels significantly influenced the quality of the meat.

Blood biochemical properties in broiler

Table. 6 shows the impact of betaine on the lipid profile of broilers. Between the therapy groups, there was a significant (P< 0.01) difference in total cholesterol levels, with T0 recording higher levels (138.1±0.91 mg/dl blood) and T3 recording lower levels (175.1±2.23 mg/dl blood). Triglycerides were also statistically significant (P<0.01), with T0 recording higher blood triglyceride levels of 72 ± 0.577 mg/dl and T4 recording lower levels of 42.6± 0.882 mg/dl. T3 recorded a greater value of 43.7± 0.312 mg/dl, and T0 recorded a lower value of 34.7± 0.371 mg/dl of blood for high-density lipoprotein (HDL), which was statistically significant (P<0.01). Additionally, low-density lipoprotein (LDL) was statistically significant (P<0.01), with T0 recording a higher value of 97.4 ± 0.56 and T3 recording a lower value of 50.5 ± 0.704 mg/dl blood. LDL levels were lower in the betaine-treated group than in the control group. The experimental group receiving betaine supplements had higher blood levels of HDL mg/dl. Betaine significantly reduced blood LDL levels. This may be because betaine acts as an antioxidant to stop the oxidation of LDL and cholesterol (Mathur et al., 1996), which slows the thermogenesis process. The level of betaine supplement was gradually reduced as it was increased in all treated groups. The results of Dalal et al. (2018) and Alzawqari, et al. (2016), who found that increasing levels of natural supplementation led to a reduction in serum cholesterol and achieved the best results with betaine supplementation, appear to be closely related to those of this study. Additionally, they noted that increasing levels of betaine supplementation led to higher and lower serum HDL (mg/dl) levels. The outcome appears to be consistent with the findings of Aljumaily et al. (2019), who discovered that natural organic supplements like betaine recorded lower triglyceride levels than control; Dalal et al. (2018), who discovered that betaine supplements had similar serum triglyceride levels to the control group (mg/dl), as well as similar LDL (mg/dl) levels.

Cost-effectiveness of broiler production

The price of producing different types of broilers is shown in Table 7. The average rearing expenditures of broilers kept in the treatment groups T0, T1, T2, T3, and T4 were, according to Table 4.6, 206.62 Tk, 232.29 Tk, 254.91 Tk, 236.64 Tk, and 232.87 Tk, respectively. The total cost of miscellaneous expenses, which comprised labor costs, disinfection costs, and predicted electricity costs, was 5 Tk per broiler. In groups T0, T1, T2, T3, and T4, the average live weight/ broiler was 1.425, 1.602, 1.758, 1.632, and 1.606 kg, respectively. The price of the broiler was Tk. 145/kg when sold on a live weight basis. In the T0, T1, T2, T3, and T4 groups, the net profit per kilogram of live weight was discovered to be taka 14.02, 24.02, 34.76, 26.25, and 22.45, respectively. The amount of betaine used in the diet demonstrated its impact on the broiler's profit margin. The current research backs up Zafar and Fatima's (2018) assertion that poultry is increasingly adopting organic rather than inorganic sources of minerals. They are supposed to lower feed costs by decreasing dose rates without adversely affecting performance since they are more bioavailable and effective. The organic mineral diet has a good effect on the economy, claim Abdallah et al. (2009). It was found that substituting organic minerals for inorganic ones improved bird performance and the immunological responses of chicks.

Table 5. Effect of betaine on rectal temperature and heart rate of broiler

Parameter	Dietary groups (%)					Significance
	T_0	T_1	T_2	T_3	T_4	
RT (°C)	41.7 ± 0.09^{a}	41.4 ± 0.08^{a}	41.1 ± 0.03^{a}	41.1 ± 0.02^{a}	41.1 ± 0.24^{a}	*
Heart rate	281.4 ± 8.82^{d}	256.3 ± 4.91^{c}	243.4 ±6.96^{b}	233.6 ±5.0^{a}	234.4 ±4.32^{a}	*

RT = Rectal temperature. a, b, c means having different superscript in the same row differed significantly (P<0.05). *= 5% level of significance, NS= non-significant

Table 6. Serum biochemical properties in broiler

Lipid profile (mg/dl)	Dietary groups (%)					Significance
	T_0	T_1	T_2	T_3	T_4	
Total cholesterol	175.1 ± 2.23^{e}	164.0± 1.53^{d}	145.1 ± 1.64^{b}	138.1 ± 0.91^{a}	157.4 ± 0.97^{c}	**
Triglyceride	72 ± 0.577^{b}	43± 0.577^{a}	45.6 ± 0.333^{a}	47.6 ± 0.882^{a}	42.6 ± 0.882^{a}	**
HDL	34.7 ± 0.371^{a}	41.7 ± 0.379^{b}	42.7 ± 0.309^{b}	43.7 ± 0.312^{b}	43.3 ± 0.524^{b}	**
LDL	97.4 ± 0.56^{d}	82.1± 0 .33^{c}	54.1 ± 1.16^{a}	58.1 ± 1.57^{b}	50.5 ± 0.704^{a}	**

a, b, c means having different superscript in the same row differed significantly (P<0.05). *= 5% level of significance, ** = 1% level of significance, NS = Non-significant

Table 7. Economics of broiler production kept under different treatment groups from day old chick to 28 days of age.

Parameters (Tk.)	Dietary groups with betaine				
	T_0	T_1	T_2	T_3	T_4
Chick cost	35	35	35	35	35
Litter cost / bird	4.5	4.5	4.5	4.5	4.5
Vaccine + medicine	13.8	13.8	13.8	13.8	13.8
Feed cost / broiler production	128.35	130	129	128	130
Dietary treatment cost / broiler production	0.00	5.5	6.5	7.5	8.5
Miscellaneous cost	5	5	5	5	5
Total cost/broiler	186.65	193.8	193.8	193.8	196.8
Average live weight/broiler (gr)	1425.5 ± 10.14^{a}	1602.6 ± 10.16^{b}	1758.3 ± 7.61^{d}	1632.5 ± 12.68^{c}	1606 ± 22.65^{b}
Sale price Tk./kg	145	145	145	145	145
Sale price / broiler	206.62	232.29	254.91	236.64	232.87
Net profit Tk./ broiler	19.97	38.49	61.11	42.84	36.07
Profit Tk./kg live weight	14.02	24.02	34.76	26.25	22.45

a, b, c means having different superscript in the same row differed significantly (P<0.05).

Conclusion

Utilizing varied levels of betaine also improved the quality of the carcass. Growth and meat yield performance responded favorably to betaine supplementation. With the addition of 0.06% more betaine, overall performance and quality were improved, which also improved financial gains. Conclusion: Adding betaine to a diet may be beneficial for producing broilers economically and effectively. As a result, adding betaine at a rate of 0.06% to the diet of broilers may be appropriate.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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