BIOTECHNOLOGY IN ANIMAL HUSBANDRY

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CRYOPRESERVATION OF *IN VITRO*-PRODUCED BOVINE EMBRYOS BY VITRIFICATION: IN PURSUIT OF A SIMPLIFIED, STANDARDIZED PROCEDURE THAT IMPROVES PREGNANCY RATES TO PROMOTE CATTLE INDUSTRY USE

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Abstract: The goal of cryopreservation is to retain the original stage of gametes and embryos after they have endured cooling and warming. Slow freezing is a standard method for *in vivo*-derived bovine embryo cryopreservation, three-fifths of such embryos being frozen by this method globally. However, it is evident that slow freezing is not efficient for cryopreserving *in vitro*-produced bovine embryos. Hence, only one-third of *in vitro*-produced bovine embryos are cryopreserved. Vitrification is a preferred method for storage of human embryos; consequently, it has been explored as a novel means to store *in vitro*-produced bovine embryos, for which it shows considerable promise as an alternative to slow freezing. This is due to several reasons: vitrification is often less time-consuming than slow freezing; it does not need expensive slow rate freezing machines; and it has been proven to have comparatively higher survival rates. Yet, in the cattle industry vitrification continues to present shortcomings, such as possible toxicity of vitrification solutions and failure to standardize methods, which pose a challenge for its application to *in vitro*-produced bovine embryos. Therefore, determining the most suitable procedure is crucial to make vitrification more practical in commercial settings.

Keywords: bovine, cryopreservation, cryoprotectant, embryo, slow freezing, vitrification.

Introduction

In commercial breeding of farmed livestock embryo transfer is an important tool to increase the number of elite animals in a herd or for herd replacement. In the cattle industry embryos are generated by means of either *in vivo* or *in vitro* production. With the *in vivo* method, bovine embryos are generated by a superovulation technique in which donors are given exogenous gonadotropin hormones in order to stimulate the activity of their ovaries. As a result, more oocytes are able to develop to maturity, when they are receptive to fertilization by sperm. When oocytes are fertilized in the oviduct of a cow, they migrate to the uterus and develop to the morula and blastocyst stage; hence these are known as *in vivo*-derived embryos. The alternative route of embryo production, *in vitro* to *in vivo*, involves the aspiration of either mature or immature oocytes from donor ovaries, *in vitro* fertilization, usually with frozen sperm, and subsequent *in vitro* culture to the blastocyst stage. This means of embryo production has been established since the 1980s but as late as the early 2000s the great majority of bovine embryos were still generated by the *in vivo* method (*Merton et al., 2009*).

The last decade has witnessed a pronounced shift in the practice of bovine embryo transfer such that the most recently published statistics indicate that globally the number of *in vitro*-produced embryos exceeded that of their *in vivo*-derived counterparts (742,908 to 386,133 recorded transfers, respectively) (*Viana et al., 2019*). This trend particularly reflects the increasing reliance on *in vitro* technologies in North America and South America, the two continents that are together responsible for the majority of the world's bovine assisted reproduction industry. In all regions, *in vitro*-produced embryos were transferred predominantly fresh (overall 73.2%) (*Viana et al., 2019*). Bovine embryos can be transferred into the uterus of recipients on the same day that they are harvested, when they are termed 'fresh embryos'. Typically, remaining embryos that are not transferred immediately are retained by cryopreservation for future use.

Embryo cryopreservation has become an essential aspect of assisted reproductive technology (*Leibo, 2008*). The proportion of frozen *in vitro*-produced bovine embryos transferred in 2018 decreased compared to 2017 (26.8 vs. 33.9%, respectively), breaking an annual rise observed since 2013. On the other hand, more *in vivo*-derived frozen-thawed embryos were transferred (60.1%) compared to *in vivo*-derived fresh embryos (*Viana et al., 2019*). Development of the cryopreservation procedure in cattle, as for all mammals, is facilitated by, and is closed associated with, the success of cryopreservation of sperm, oocytes and embryos (*Vajta and Nagy, 2006*). Slow freezing and vitrification are the two recognized methods for cryopreservation of each of *in vivo*-derived and *in vitro*-produced bovine embryos. Among beef cattle, animals produced from *in vitro*-produced embryos greatly exceed those from *in vivo*-derived embryos. This is due

to the intrinsically large number of follicles in beef breeds such as Nelore (*Pontes et al., 2009*).

It is apparent that understanding the cryopreservation process and identifying a simple but reliable method for *in vitro*-produced bovine embryos is necessary in order to enhance the efficiency of their production and transfer in on-farm contexts. While the published literature covers a broad scope of bovine embryo cryopreservation, including the standard slow freezing procedure, this review focuses primarily on vitrification as a method to cryopreserve *in vitro*-produced bovine embryos. It also examines theoretical aspects in regard to ice crystal formation, cooling/warming rates, and roles of cryoprotectants in protecting cells from the chilling process. Furthermore, the specific demands for storage of *in vitro*-produced bovine embryos are considered. Efforts to raise the efficiency of vitrification through modifications to *in vitro* culture and enhancements in the capacity of *in vitro*-produced embryos are also discussed. Finally, biosafety issues relating to vitrification are addressed.

Different methods of freezing embryos

Of the two methods of bovine embryo cryopreservation slow freezing was developed in the 1970s whereas vitrification was realized a decade later and has seen a rapid period of more recent development (*Hasler, 2014*). The majority of *in vivo*-derived embryos are generated in donors with the aid of exogenous gonadotropin hormones and are then frozen. *van Wagtendonk-de Leeuw et al.* (*1997*) reported that these two methods of cryopreservation led to similar conception rates of recipients. *Hasler* (*2014*) affirmed that on occasion vitrification is recommended for cryopreservation of *in vivo*-derived embryos yet maintains that slow freezing yields similar pregnancy rates among recipients.

A major objective of cryopreservation is to minimize intracellular ice formation. *Kleinhans and Mazur* (*2009*) proposed that during the slow freezing process, survivability of cells is dependent on a cooling rate slow enough for unfrozen intracellular water to dehydrate by osmosis to near the equilibrium level before reaching the temperature at which intracellular nucleation occurs. In contrast, *Kim et al.* (*2012*) and *Lawson et al.* (*2012*) each argued that vitrification negates any risk of intracellular ice crystals forming.

(a) Slow freezing

In North America 70% of superovulated bovine embryos are slow frozen, indicating that if a programmable freezer is available this method is preferred (*Hasler, 2014*). As an example, *Hasler* (*2001*) demonstrated a high overall pregnancy rate of 56.1% for recipients implanted with *in vivo* frozen embryos. Furthermore, *Lopatarova et al.* (*2010*) reported a conception rate of 48.8% for recipients implanted with frozen and biopsied *in vivo* embryos; for recipients of intact and frozen *in vivo* embryos the conception rate was 50.7%. Bovine embryos

are standardly frozen and stored in a straw with 1.8 M ethylene glycol (EG) as cryoprotectant so enabling the possibility upon thawing of direct transfer to a recipient cow (*Dochi et al., 1995; Voelkel and Hu, 1992*). The success of this method for *in vivo*-derived embryos has paved the way for its commercial use on cattle herds worldwide.

Although slow freezing is considered the conventional method of storing *in vivo*-derived embryos, several studies have shown it to be inefficient at cryopreserving *in vitro*-produced bovine embryos (*Do et al., 2017; Enright et al., 2000; Mahmoudzadeh et al., 1994; Mucci et al., 2006; Nedambale et al., 2004; Yu et al., 2010*). Prevention of intracellular ice formation is a key factor in the survival of cryopreserved cells (*Mazur et al., 2005; Mazur et al., 2007*). Ice crystals forming during cooling and warming processes are the main cause of cell damage and death (*Lee et al., 2013; Mazur and Seki, 2011*). *Seki et al.* (*2014*) observed extracellular ice crystal formation initially during the slow freezing process. An additional difficulty of cryopreserving *in vitro*-produced bovine embryos is that they contain more lipid droplets in their cytoplasm than do their *in vivo* counterparts (*Abe et al., 1999; Pryor et al., 2011*), leading to greater susceptibility to the freezing process (*Pereira et al., 2008; Seidel, 2006*).

(b) Vitrification

Characterized by rapid cooling/warming rates and high concentrations of cyoprotectants (*Jin et al., 2008*), vitrification is an alternative procedure to slow freezing for *in vitro*-produced embryo cryopreservation. *Wowk* (*2010*) stated that cells – cryopreserved by slow freezing – survive in glass between ice crystals, while cells – cryopreserved by vitrification – survive in glass as the whole sample is vitrified. In fact, since vitrification has become the main cryopreservation method to store human embryos (*Vajta et al., 2015*), it has also been recommended for cryopreservation of *in vitro*-produced bovine embryos (*Mucci et al., 2006; Hasler, 2014; Taylor-Robinson et al., 2014*).

Vitrification is a technically simple method (*Do et al., 2016a; Rios et al., 2010*), which does not require the use of a programmable freezer (*Hasler, 2014; Sinha, 2009; Vajta and Nagy, 2006*). Laboratory experiments have indicated that *in vitro*-produced bovine embryos cryopreserved by vitrification achieve a better survival rate than do those cryopreserved by slow freezing (*Do et al., 2017; Gómez et al., 2008; Mucci et al., 2006; Yu et al., 2010*). Vitrification has not yet, however, achieved widespread application in commercial cattle production. This is because most embryos are produced by superovulation, and conventional slow freezing has already become a standard method to store *in vivo* embryos, while well over half of the transfers in both Europe and the USA are from frozen embryos (Table 1). In contrast, there is a smaller proportion of embryos produced by *in vitro* fertilization, especially in Europe (Table 1). Most fresh *in vitro*-produced embryos are transferred due to the lack of a reliable cryopreservation method (*Pontes et al.,*

2011), and of an efficient method that allows the directly transfer of those embryos after being thawed (*Sanches et al., 2016*).

Table 1. Commercial bovine embryo transfer activities in Europe and the USA in 2018 *(the most recent date for which combined data are available)*

Region	Embryo types	Transferable embryos	Frozen embryos	Fresh embryo transfers	Frozen embryo transfers
* Europe	*In vivo*	141,371	N/A	52,344	70,190
	In vitro	36,832	N/A	13,928	13,233
† USA	*In vivo*	418,349	179,810	58,458	99,698
	In vitro	260,193	238,539	180,081	80,112

* *Association of Embryo Technology in Europe annual data collection 2018*
† *American Embryo Transfer Association statistics committee report 2019 (2018 data)*

Limitations of current vitrification protocols

Cryobiologists have progressively modified vitrification procedures to achieve incremental improvement but shortcomings remain, and, unlike with the slow freezing technique, no standardized method has been agreed (*Leibo, 2008; Seidel, 2006*). There is a profusion of different vitrification protocols utilizing varied timings, temperatures and cryoprotectants, so the outcome is multifactorial. In addition, the operator has a choice of vitrification carriers (for example, plastic straw, electron microscope grid microdrop, open pulled straw, cryotop, cryohook), with drop-size and straw barriers affecting cooling and warming rates. Warming protocols can differ too, which adds further to the complexity of choice for an efficient vitrification method (*Vajta et al., 2015*); this is a particular concern when vitrified samples are exchanged between laboratories. However, perhaps the most influential factor to consider when developing a highly successful vitrification method is the skill of the operator. Although described as a simple method, not requiring slow-rate freezing machines, in our experience vitrification actually requires much more skill and dexterity of the operator compared to slow freezing (*Do et al., 2019*).

While vitrification appears more favourable than slow freezing, further investigation is required to compare its efficacy with the slow freezing method (*Sinha, 2009*). Moreover, although limited data on pregnancy rates are available (*Gutnisky et al., 2013; Kim et al., 2012; Nedambale et al., 2004*), the reported sample sizes were relatively small. For instance, *Gutnisky et al.* (*2013*) transferred vitrified embryos to recipients and obtained conception rates of 46.8%. However, the number of embryos transferred, 96, was modest and thus may not reflect the

potential efficacy of the vitrification method. Of 45 confirmed pregnancies 33 calves were born (34.3%) (*Gutnisky et al., 2013*), which is not a promising outcome under commercial conditions where the ultimate goal of an embryo transfer program is to achieve a high number of healthy calves (*Do et al., 2018a*). Previously, *Enright et al.* (*2000*) reported that abortion rates of surrogate cattle receiving *in vitro*-produced embryos are raised, and that care should be taken with pregnancy interpretation.

Considerable attempts aimed at improving the quality of *in vitro*-produced embryos before cryopreservation have been made in various ways, such as modification of *in vitro* culture systems (*Do et al., 2016b; Sanches et al., 2013; Sudano et al., 2011*), enhancement of the intrinsic capacity of embryos (*Filho et al., 2011*), and other means of pretreatment of embryos before vitrification (*Min et al., 2013*). Nonetheless, a significant advancement has so far proved elusive.

Impact of cooling rates

During slow rate cooling of embryos the temperature drops at a rate of approximately 0.5°C per minute to -30°C or -35°C, at which point they are plunged into liquid nitrogen (*Leibo, 2008*). For vitrification, by comparison, cooling rates of thousands of degrees Celsius are attained, such that a sample containing embryos can reach extremely rapidly a glassy state. Two crucial characteristics of vitrification are extreme cooling rates and high concentration of cryoprotective additives (*Seki and Mazur, 2014*).

Minimum volume vitrification theory pertains to the rate of cooling and vitrification carriers. When plastic straws are used as the principal carrier to vitrify and store embryos, their survival rates of embryos following vitrification process (*Enright et al., 2000*). For example, in all experiments in which 0.25 ml plastic mini-straws were used by *Palasz et al.* (*1997*), only around 20% of vitrified embryos were recovered. Hence, cryobiologists have aimed to establish a carrier that contains the minimum volume of vitrification solution needed to raise cooling rates. *Kuwayama* (*2007*) determined that this strategy prevents breakage of zonae pellucidae.

Vajta et al. (*1998*) pioneered a novel vitrification carrier – open pulled straw – made from a standard plastic straw (typically used in slow freezing of embryos) heat-softened and pulled to reduce its internal diameter so that 1-2 μl vitrification solution containing embryos loads spontaneously due to capillary action. Cooling rates as high as 25,000°C/min were recorded over a temperature range of -25°C to -175°C (*Vajta et al.*, *1998*). These compare favourably to 2,250°C/min, ten times less, when using a larger diameter plastic straw (5 μl, typically utilized for sperm freezing). More recently, *Malenko et al.* (*2017*) modified this protocol, replacing the plastic straw with a glass capillary tube. The heat-pulled conical tip can be moulded into a protective sheath that may be moved into either a closed or open position. Initial results for survival and hatching rates post-warming were

comparable to those gained using various vitrification carriers. Further research is required to achieve high and stable survival rates with different developmental stages of *in vitro*-produced bovine embryos.

Kuwayama (*2007*) described a purpose-built carrier, named Cryotop®, a narrow and thin strip of film affixed to a hard plastic holder. This simple device enables loading of < 0.1 µl volume of vitrification solution containing the sample. As a result, cooling rates of 69,250°C/min may be achieved (*Seki and Mazur, 2014*). However, *Rios et al.* (*2010*) suggested that despite the contention that minimum volume vitrification enhances thermal conductivity and cooling rates, it does not improve hatching rates of embryos upon vitrifying/warming and subsequent culture *in vitro* (*Rios et al., 2010*).

Impact of warming rates

Recent research has highlighted the significance of warming rates to cryopreservation because recrystallization influences vitrification outcomes (*Zhou et al., 2010*). This starts at -109°C during the warming process (*Lee et al., 2013*), whereupon devitrification can cause cryo-injury to the cell (*Sansinena et al., 2014*). *Hopkins et al.* (*2012*) observed that warming rates are closely associated with cooling rates due to the presence of ice fractions inside vitrified cells. In accord with this, *Seki et al.* (*2014*) reported that during the cooling process, small ice crystals form; this causes recrystallization. Water molecules move from smaller to larger crystal size, ultimately reaching a size that proves lethal to the cell.

Faster cooling rates therefore need more rapid warming rates in order to block recrystallization (*Mazur and Seki, 2011*). *Seki et al.* (*2014*) found that combining moderate cooling rates with high warming rates of 117,500°C/min can avert recrystallization. Although it is apparent that rapid warming allows cells to avoid devitrification, insufficient cryoprotectant concentrations can promote recrystallization during the warming process (*Jin et al., 2008*). In concurrence, *Seki et al.* (*2014*) suggested that the short time, 1-2 minutes, for which embryos are exposed to equilibration solution is not enough for permeable solutes to penetrate cells to replace water molecules (by dehydration).

Morató and Mogas (*2014*) showed that keeping the warming solution of vitrified embryos at 45°C facilitates an improved survival rate compared to at 50°C, 60°C or 70°C. Corroborating this, *Caamaño et al.* (*2015*) considered that 41°C is the most suitable temperature to warm vitrified embryos.

Pros and cons of cryoprotectants

In cryobiology cryoprotectants perform a key function in preventing cryo-damage to cells. Permeating and non-permeating cryoprotectants are used frequently to cryopreserve gametes and embryos. Replacing intracellular water with a permeating compound protects a cell from ice crystals forming; the cell is then rehydrated upon warming (*Palasz and Mapletoft, 1996*). Low molecular

weight, non-electrolyte cryoprotectants penetrate cells at different rates to replace cytoplasmic water (*Leibo, 2008*). Embryos are equilibrated with each of the five most common permeable cryoprotectants – dimethyl sulfoxide (Me_2SO), glycerol, EG, methanol and propylene glycol – at half the strength of the final vitrification solution. For instance, using a Cryotop® embryos are first exposed to an equilibration solution containing 7.5% v/v EG and 7.5% v/v Me_2SO, followed by a vitrification solution (15% v/v EG and 15% v/v Me_2SO).

Permeability of cells varies with the type of cryoprotectant. EG is more permeable to bovine embryos than is glycerol (*Morató and Mogas, 2014*), so EG is typically the cryoprotectant of choice in the field using a one-step dilution (*Dochi et al., 1998*). A high concentration of cryoprotectant is required to prevent intracellular ice crystal formation (*Seki et al., 2014*), and to attain vitrification (*Lawson et al., 2012*). However, this risks causing osmotic shock to cells (*Morató and Mogas, 2014*). *Vanderzwalmen et al.* (*2013*) argued that high concentrations of cryoprotectant intracellularly and extracellularly are necessary to minimize embryo swelling, which is a consequence of water permeation during the warming process. Therefore, non-permeable cryoprotectants such as the sugars sucrose and trehalose are used to reduce osmotic stress. While cryoprotectants preserve the integrity of cellular organelles during cooling and warming (*Dobrinsky, 2002*), their toxic characteristics may be detrimental (*Fahey, 2010; Wu et al., 2013*).

Toxicity is a major drawback of cryopreservation regardless of whether slow freezing or vitrification is used, so lessening the effects on cell viability should be an ongoing priority of cryopreservation methods. In regard to bovine assisted reproduction, it is a commonly held belief that exposure to a high concentration of cryoprotectant during vitrification may be harmful to embryos. However, *Vanderzwalmen et al.* (*2013*) maintained that intracellular levels of cryoprotectant in vitrified murine embryos are significantly lower than those detected within slow frozen embryos. In supporting this perspective, *Do et al.* (*2018b*) indicated that vitrification may not be toxic to bovine embryos since no miscarriage is observed among recipients that are implanted with vitrified *in vitro*-derived embryos.

Impact of cytoplasmic lipid droplets

The presence of cytoplasmic lipid droplets in embryos is suggested to have no apparent effect on their viability after cryopreservation (*Block et al., 2010*); however, it is also argued that the survivability of cryopreserved embryos is related to their lipid contents (*Cagnone and Sirard, 2014; Seidel, 2006*). *Pryor et al.* (*2011*) and *Seidel* (*2006*) identified a correlation between lipid droplets and embryo cryotolerance. Furthermore, *Cagnone and Sirard* (*2014*) noted that *in vitro* culture affects the embryonic lipid content of embryos and their subsequent cryo-survival. Previously, in a preliminary study *Abe et al.* (*1999*) had discovered that the sensitivity of *in vitro* embryos to cryopreservation is due to the substantial cytoplasmic accumulation of lipid droplets. Embryos cultured in serum-free

medium contain predominantly lipid droplets of < 2 μm; in contrast, embryos cultured in serum-supplemented medium have lipid droplets > 62 μm. Such large lipid droplets were frequently observed in morulae and blastocysts cultured in media containing 5% v/v calf serum (*Abe et al.*, *1999*). The lipid content of *in vitro*-produced embryos cultured in a serum-free medium was similar to their *in vivo*-derived counterparts, but the lipid volume in embryos cultured with serum was almost double that of *in vivo* embryos (*Ferguson and Leese, 1999*).

The abnormal accumulation of lipid in the cytoplasm of *in vitro* embryos resulting from culture together with serum is the main factor that makes them unsuitable for cryopreservation (*Pereira et al., 2008*). *Mucci et al.* (*2006*) contended that raised numbers of lipid droplets in an embryo affect its cellular repair after cryopreservation. Moreover, *Sudano et al.* (*2011*) maintained that high concentration of fetal calf serum in *in vitro* culture media causes a rise in accumulated lipid in blastocysts, and in apoptosis, but reduces expansion of blastocoels after vitrification.

Vitrification of embryos at various developmental stages

Vitrification of embryos at different stages is controversial (*Asgari et al., 2012*). Cryopreservation of the blastocyst stage enables immediate embryo transfer after warming and to skip the repeated *in vitro* culture of embryos; moreover, advanced embryos can overcome the period of genome embryonic transition that occurs at or between the fourth and fifth cell cycle in bovine embryos (*Aono et al., 2013; Garcia-Garcia et al., 2006*). *Asgari et al.* (*2012*) revealed that a large number of 5-8 cell stage bovine embryos were arrested at the morula stage due to the negative effect of cryopreservation on the embryonic activation transition period. *Shirazi et al.* (*2009*) showed that early blastocysts have a greater cryo-tolerance than do expanded ones. However, *Sommerfeld and Niemann* (*1999*) observed that expanded blastocysts survive vitrification better than do non-expanded blastocysts; hatching rates of 42% and 12%, respectively. On the other hand, early stage embryo cryopreservation delays the *in vitro* embryo production procedure. Hence, it is important that after warming these embryos are cultured further *in vitro* to develop into blastocysts at which point they can be used as fresh embryos.

Direct transfer of vitrified *in vitro*-produced bovine embryos

Most vessels used for vitrification are not designed specifically for this purpose so they are ill suited to the direct transfer of embryos with a standard 0.25 mL plastic mini-straw (*Ha et al., 2014; Inaba et al., 2011*). Furthermore, several steps are taken when warming in order to gradually reduce the cryoprotectants left over from placing embryos in equilibrium and vitrification media before vitrification. Simplification of this process through a single warming step is required to enable the direct transfer of vitrified bovine embryos. It is thought that

warming by single or multiple steps achieves comparable results (*Caamaño et al., 2015; Morató and Mogas, 2014*). *Vajta et al.* (*1999*) designed an in-straw dilution procedure whereby a French mini-straw is loaded with holding medium and 0.2 M sucrose; however, this requires a skilled operator (*Vieira et al., 2007*). Another drawback of this modification is embryo loss during warming. *Vieira et al.* (*2007*) considered that unintentional warming in air for a few seconds before an embryo is loaded possibly reduces the warming rate, which is a critical factor in its survival (*Mazur and Seki, 2011*). Although direct transfer has provided promising results (*Caamaño et al., 2015; Morató and Mogas, 2014; Vieira et al., 2007*), the loss of embryos, evaluated by transfer, is limiting to successful outcomes.

Refined techniques to enhance cryopreservation outcomes

Prior to vitrification of *in vitro*-fertilized embryos micro-manipulation could increase their survival rates following cryopreservation. As an example, it is suggested that dehydrating blastocysts sufficiently before vitrification, through artificially collapsing the blastocoel, increases their implantation potential (*Liebermann et al., 2012*). By using this technique *Min et al.* (*2013*) observed improved survival rates of bovine *in vitro* embryos and cloned embryos after post-warming and incubation for a further 24 hours (*Min et al., 2013*). The survival of *in vitro* embryos following blastocoel forced treatments was significantly greater than for untreated controls (81.9% versus 69.8%). However, this technique is complicated due to time constraints and requires a highly skilled embryologist. *Pryor et al.* (*2011*) used a laser-assisted hatching method in which a laser micro-beam drills a hole in the zona pellucida surrounding the embryo, through which lipid droplets can exit the cytoplasm. While technically feasible, this appears a complex method.

Enhancing the intrinsic characteristics of *in vitro*-fertilized bovine embryos is another way to raise their quality prior to cryopreservation. According to *Pribenszky et al.* (*2010*) and to *Pribenszky and Vajta* (*2011*), exposing gametes and embryos to sublethal hydrostatic pressure elevates their intrinsic developmental competence to overcome physiological stress such as experienced during cooling. This improved cell performance may be due to high hydrostatic pressure causing a minimum stressful condition under which cells can synthesize heat shock proteins (*Díez et al., 2012; Pribenszky et al.; 2008*). In support of this, pretreating bovine blastocysts with high hydrostatic pressure, *Filho et al.* (*2011*) reported that re-expansion and hatching rates of treated embryos are significantly higher than those of untreated embryos. However, pregnancy rates were not detailed. This promising approach to enhancing the survivability of embryos requires further investigation.

Cryopreservation-related biosafety considerations

Biosafety is of paramount importance when importing and exporting bovine embryos internationally. Therefore, the threat posed by cryopreserved embryos of

pathogen transmission demands evaluation. The two systems of vitrification, closed and open, present different risks. Theoretically at least, there is a reduced risk of microbial contamination using a closed vitrification system, as for slow freezing in straws, compared to methods that require direct contact of embryos with liquid nitrogen (*Vajta et al., 2015*). It has been argued that it is a false assumption to consider liquid nitrogen or cryopreserved samples to be sterile (*Morris, 2005*). *Mirabet et al.* (*2012*) indicated that some viruses can survive at subzero temperatures without the need for a cryoprotectant or cryopreservation. For instance, *Bielanski et al.* (*2000*) investigated possible contamination from liquid nitrogen of three species of virus; bovine viral diarrhea virus, bovine herpesvirus and bovine immunodeficiency virus. Unsealed carriers tested either positive or negative for these viruses but no sealed source was contaminated. While 32 bacterial and one fungal species were identified in various liquid nitrogen cannisters storing semen and embryos for between 6-35 years, bovine viral diarrhea virus and bovine herpesvirus were not detected in clean semen and embryo straws kept alongside infected straws (*Bielanski et al., 2003*). Nonetheless, it is not easy to maintain long-term cleanliness of samples that are contained in the same liquid nitrogen cannister as unclean ones.

Despite the apparent low risk of microbial contamination of samples through contact with liquid nitrogen, it remains a potential hazard (*Vajta and Kuwayama, 2006*). An open system achieves sufficiently high cooling rates, but a closed system may not; hence, striking a balance between technical and sterility considerations presents a challenge to the operator. For example, *Yu et al.* (*2010*) used closed pulled straw in preference to open pulled straw to negate any threat of contamination. However, the survival rates of vitrified embryos were modest.

Conclusion

It is evident that formation of intracellular ice crystals during cooling and warming processes is the main cause of damage to cryopreserved cells. Slow freezing and vitrification both aim to reduce to a minimum, if not prevent, ice accumulation. While cryoprotectants are necessary to protect cells from chilling they are possibly cytotoxic. Although slow freezing and vitrification are each efficient for *in vivo*-derived bovine embryo cryopreservation, slow freezing may not be suited to cryopreserving *in vitro*-produced bovine embryos. This is perhaps due to the cytoplasmic lipid content of such embryos being greater than that of their *in vivo* counterparts, although improved culture conditions may mitigate this effect. Collective research indicates the advantages of vitrification as the method of choice for storing *in vitro*-produced embryos. A better understanding of cooling and warming processes, cryoprotectants, modifications to *in vitro* culture media, and to treatment of cells prior to cryopreservation is each necessary in order to improve outcomes. Furthermore, embryo biosafety should be safeguarded.

Vitrification appears a superior procedure to slow freezing for cryopreserving *in vitro*-produced bovine embryos, yet non-standardization of methods between laboratories and, consequently, inconsistent results currently hamper its applicability to the international trade in cattle genetics and the management of embryo biobanks (*Mogas, 2018*). Thus, it is important to optimize *in vitro* production of blastocysts with fewer lipid droplets, together with identifying the most efficient means to vitrify *in vitro*-produced bovine embryos. In turn, such technical advancements will both contribute to a greater understanding of bovine cryobiology and facilitate increased use of *in vitro* embryos in commercial livestock settings.

Krioprezervacija in vitro dobijenih goveđih embriona vitrifikacijom: u potrazi za pojednostavljenim, standardizovanim postupkom koji poboljšava stope graviditeta/steonosti radi unapređenja govedarstva

Van Huong Do, Andrew W. Taylor-Robinson

Rezime

Cilj krioprezervacije je zadržati prvobitnu fazu polnih ćelija i embriona nakon što izdrže hlađenje i zagrevanje. Polako zamrzavanje je standardni metod za krioprezervaciju goveđih embriona in vivo, pri čemu se tri petine takvih embriona globalno zamrzava ovom metodom. Međutim, očigledno je da sporo zamrzavanje nije efikasno za krioprezervaciju goveđih embriona proizvedenih in vitro. Dakle, samo jedna trećina goveđih embriona proizvedenih in vitro je krio-konzervirana. Vitrifikacija je poželjna metoda za skladištenje ljudskih embriona; shodno tome, istražena je kao novo sredstvo za čuvanje in vitro proizvedenih goveđih embriona, za koje se pokazala kao značajna alternativa sporom zamrzavanju, i to iz nekoliko razloga: vitrifikacija često zahteva manje vremena nego sporo zamrzavanje, nisu potrebne skupe mašine za zamrzavanje sa sporim tempom, i dokazano je da ima relativno veće stope preživljavanja. Ipak, u stočarstvu/govedarstvu, vitrifikacija i dalje pokazuje nedostatke, kao što su moguća toksičnost rastvora za vitrifikaciju i neuspeh u standardizaciji metoda, što predstavlja izazov za njegovu primenu na in vitro proizvedene embrione goveda. Zbog toga je određivanje najprikladnijeg postupka presudno kako bi se vitrifikacija učinila praktičnijom u komercijalnim uslovima.

Ključne reči: goveda, krioprezervacija, krioprotektant, embrion, sporo zamrzavanje, vitrifikacija.

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Author Contributions

VHD conceptualized the paper, which was developed further in discussion with AWTR. Both authors collated articles for review, wrote and critically reviewed various drafts, contributed to the preparation of the final version and provided consent for submission.

Conflicts of Interest

The authors declare no conflicts of interest.

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QUANTITATIVE GENETIC ANALYSIS OF VARIABILITY AND RELATIONSHIP OF LAMBS BODY WEIGHT TRAITS IN POPULATION OF INDIGENOUS PIROT SHEEP

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Abstract: Quantitative genetic analysis of variability and relationship of lambs body weight traits in indigenous pirot sheep population are done. The the examined lambs had high variability which very suitable for selection on a larger weight. There is a high and very significant correlation between the body weight of lambs with 30 and 60 days (.969 **) and the weight with 30 and 90 days (.914 **). There is also a highly significant correlation between the weights of lambs with 60 and 90 days of age (.904 **). From our research, we can see that the first month of the lamb's life is very important for the further development of the body. There is different level of correlation between weight of lambs. This gives us an idea to say that many paragenetic factors are crucial for the growth of lambs from birth to weaning. The coefficient of multiple determination (R^2) is 0.845 which means that 8.45% of the lamb's body weight variance at 90 days is determined by the variance of the set of predictor variables (PI-60, 30, 1). Each increase in lamb body weight during the observed periods of age is associated with an increase in the score of depending variable PI90. In particular, any increase in lamb body weight at birth by 1 kg is associated with an increase in lamb body weight from 90 days by 0.238 kg.

Key words: indigenous, pirot sheep, body weight, correlation, regression

Introduction

Many genetic and non-genetic factors influence body weight of lambs *(Ghafouri et al., 2008; Thiruvenkadan et al., 2008; Petrovic et al., 2011)*. On the other hand, some researchers investigate how to select lambs to have higher

marketing value *(Bromley et al., 2001; Ronny et al., 2001; Hanford et al., 2003, Caro Petrovic et al., 2013).*

There are different sheep farming systems in the world, different traditions and different breeds. There are different sheep farming systems in the world, different traditions and different breeds. In Serbia, sheep farming has always been a significant branch of livestock production. This is especially emphasized in the mountainous area, which has significant natural resources.

Stara planina, in the part that administratively belongs to the municipality of Pirot, is reasonably considered the historic center of sheep farming in Serbia. An indigenous sheep population called the Pirot pramenka has been present there for centuries. However, due to the intensive process of industrialization, there is a negative trend in the number of sheep *(Petrovic et al., 2013).*

Over time, due to better nutrition conditions, the Pirot sheep has increased its performance and, in order to preserve that trend should be created new breeding programs (*Petrovic et al., 2009, 2011).* This population of sheep is especially characterized by the quality of lamb meat. That is why the aim of this research is to determine the state and the trend of body development of lambs in the indigenous Pirot sheep, using modern mathematical and statistical methods, to define future breeding goals.

Material and Methods

Research was conducted in a population of indigenous Pirot pramenka sheep. The experiment included 350 lambs from the Pirot part of Stara planina. The examination were conducted between January and April. All mother sheep were kept in the facilities in the traditional way and fed exclusively with hay from the mountain meadows. The lambs were kept with their mothers for the first seven days, and from the eighth day they were disjoint into a separate box where they had hay and cereal concentrate available without other supplement. Lambs suckled their mothers three times a day for the first month, and later, until 90 days of age, they sucked only twice a day, morning and evening. All lambs were measured at birth (PI1) and then at 30 (PI30), 60 (PI60) and 90 (PI90) days of age. Data were processed using a software package SPSS (2012). The following parameters were calculated and determined: Mean, Std. Error, Std. Deviation and Variance. In order to determine the strength of the linear relationship or the association of lamb growth traits, data were subjected to a correlation and multiple linear regression analysis procedure. Based on the results obtained and their discussion, appropriate conclusions were drawn.

Results and Discussion

1. Lambs body weight characteristics:

The trend of body weight of lambs from birth to age at 90 days can be seen in Table 1.

Table 1. Body weight lambs from birth to weaning

	N	Minimum	Maximum	Mean		Std. Deviation	Variance
	Statistic	Statistic	Statistic	Statistic	Std. Error	Statistic	Statistic
PI1	350	2.00	5.00	3.6326	.03160	.59122	.350
PI30	350	8.00	11.10	9.8809	.04083	.76387	.584
PI60	350	13.20	18.30	15.9120	.06968	1.30354	1.699
PI90	350	18.60	28.00	21.3997	.10544	1.97253	3.891
Valid N (listwise)	350						

We can see that the population value of the lamb's body weight is within the expected range for the Pramenka as an indigenous sheep breed. Interestingly, however, the value of variance changes significantly with the age of lambs. From 0.350 at birth to 3.891 with 90 days of age. This means that the genetic potential of lambs varies, which results in greater variability in the population. Some individuals grow faster and reach more mass unlike others, which have lower body weight values. We also see that the Pirot strand is very suitable for selection on a larger weight. The recommendation for professionals and farmers is to keep this in mind when designing future breeding programs.

Mekic et al. (2008) presented similar results of Pramenka lamb development from birth to 90 days of age.The overall average for the applied linear model for birth weight was 3.52 kg, with 30 days 8.72 kg, 60 days 14.47 kg, and 90 days 20 56 kg.

Petrovic et al. (2011) examined the influence of external factors on the variability of body mass of some populations of domestic Pramenka sheep. The authors state that the body weight of lambs depends on the effects of maternal age. Lamb weight in later lactation also depends on the birth weight and the type of birth. Lambs born in the spring-summer season have a higher body mass than lambs born in the fall-winter season. Regarding of *Caro Petrovic et al. (2013)* lambs with higher birth weight had a higher weaning weight. Similar study has found by *Hanford et al. (2003)* which informed that producing lambs with heavier birth weights will tend to produce lambs with heavier weaning weights.

Suarez et al. (2000) point out that the effect of genotype, sex and birth type was significant in terms of body weight of lambs at birth, and that this difference ranged from 10.6-14.4%.

Yaqoob et al. (2004) state that the breed of the father can significantly affect the body weight of the lambs. Also, gender and date of birth were significant sources of variation in lamb body mass.

There are many papers in the literature related to the problem of lamb body weight. This in itself indicates the importance of this trait in sheep, which justifies the research we have presented in this paper.

2. Relationship of lambs body weight traits

• *Correlation between lamb body weight traits*

Table 2. Correlation between characteristics of lamb body weight

		PI1	PI30	PI60	PI90
PI1	Pearson Correlation	1	.092	.040	.137[*]
	Sig. (2-tailed)		.085	.460	.010
	Sum of Squares and Cross-products	121.989	14.528	10.653	55.783
	Covariance	.350	.042	.031	.160
	N	350	350	350	350
PI30	Pearson Correlation	.092	1	.969[**]	.914[**]
	Sig. (2-tailed)	.085		.000	.000
	Sum of Squares and Cross-products	14.528	203.642	336.700	480.458
	Covariance	.042	.584	.965	1.377
	N	350	350	350	350
PI60	Pearson Correlation	.040	.969[**]	1	.904[**]
	Sig. (2-tailed)	.460	.000		.000
	Sum of Squares and Cross-products	10.653	336.700	593.030	811.111
	Covariance	.031	.965	1.699	2.324
	N	350	350	350	350
PI90	Pearson Correlation	.137[*]	.914[**]	.904[**]	1
	Sig. (2-tailed)	.010	.000	.000	
	Sum of Squares and Cross-products	55.783	480.458	811.111	1357.910
	Covariance	.160	1.377	2.324	3.891
	N	350	350	350	350

*. Correlation is significant at the 0.05 level (2-tailed).
**. Correlation is significant at the 0.01 level (2-tailed).

Based on Table 2 we can conclude that there is a high and very significant correlation between the body weight of lambs with 30 and 60 days (.969 **) and

the weight with 30 and 90 days (.914 **). There is also a highly significant correlation between the weights of lambs with 60 and 90 days of age (.904 **). From same table we can see that there is significant correlation (.137*) between body weigt of lambs at birth and 90 days of age.

From our research, we can draw lessons about how important body weight is for lambs at 30 days of age. This weight depends on further physical development and ultimately the final weight of the lambs for the market. Management on the farm should be ensured that will allow the lambs to achieve maximum body performance in the first month of their life.

Regarding of *Caro Petrovic et al. (2013),* the correlations among the body weight of lambs in the postnatal period are ranged from low to moderate among the respective traits and ranged between 0.001 and 0.365. *Sawalha et al. (2007)* found a weak genetic correlation (0.21) between lamb viability and birth weight. *Cloete et al. (2003)* reported that body weight of lambs at birth and weaning weight were highly correlated. Regarding the research conducted by *Hanford et al. (2002),* correlation between lambs birth weight and weaning weight was moderate (0.56) in population of the Columbia sheep breed.

Generally, it can be seen that there is different level of correlation between weight of lambs. This gives us an idea to say that many paragenetic factors are crucial for the growth of lambs from birth to weaning.

• *Regression between lamb body weight traits*

90 days of lamb body weight is a key aspect of this research. In order to determine the magnitude of the expected changes in the dependent variable Y (90 day weight) for each unit of change independently X (Lamb weight at 60.30 days and at birth), we will analyze the results of the multivariate regression analysis, which are presented in the following tables.

Table 3. Results of formed model of regression analysis by genotypes

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.914[a]	.835	.834	.80292
2	.917[b]	.840	.840	.79016
3	.919[c]	.845	.844	.77917

a. Predictors: (Constant), PI30
b. Predictors: (Constant), PI30, PI60
c. Predictors: (Constant), PI30, PI60, PI1
d. Dependent Variable: PI90

Table 4. The values of the parameters of variance analysis

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	1133.559	1	1133.559	1758.312	.000[b]
	Residual	224.351	348	.645		
	Total	1357.910	349			
2	Regression	1141.258	2	570.629	913.944	.000[c]
	Residual	216.652	347	.624		
	Total	1357.910	349			
3	Regression	1147.851	3	382.617	630.230	.000[d]
	Residual	210.059	346	.607		
	Total	1357.910	349			

a. Dependent Variable: PI90
b. Predictors: (Constant), PI30
c. Predictors: (Constant), PI30, PI60
d. Predictors: (Constant), PI30, PI60, PI1

Table 3 shows us that the value of the multiple regression coefficient (R) is 0.914. This number shows us the correlations between the 90-day lamb weight values and the set of predictor variables (PI- 30, 60, 1) found in the model.

From the same table we can see that the coefficient of multiple determination (R^2) is 0.845 which means that 8.45% of the lamb's body weight variance at 90 days is determined by the variance of the set of predictor variables (PI-60, 30, 1).

From Table 3 we can see that the values of the multiple correlation coefficients in the final model are statistically very significant. It should be understood that the regression model statistically significantly predicts the values of the criterion variables.

Table 5. Results of obtained values of regression coefficient

Model		Unstandardized Coefficients		Standardized Coefficients	T	Sig.
		B	Std. Error	Beta		
1	(Constant)	-1.912	.558		-3.430	.001
	PI30	2.359	.056	.914	41.932	.000
2	(Constant)	-1.717	.552		-3.113	.002
	PI30	1.598	.224	.619	7.144	.000
	PI60	.460	.131	.304	3.511	.001
3	(Constant)	-2.377	.580		-4.102	.000
	PI30	1.436	.226	.556	6.355	.000
	PI60	.548	.132	.362	4.152	.000
	PI1	.238	.072	.071	3.296	.001

a. Dependent Variable: PI90

Table 5 shows that in the final model-3, in addition to the regression constant, there are also predictor variables PI30, PI60 and PI1. Each increase in lamb body weight during the observed periods of age is associated with an increase in the score of depending variable PI90. In particular, any increase in lamb body weight at birth by 1 kg is associated with an increase in lamb body weight from 90 days by 0.238 kg. The standardized coefficients in the table show the magnitude of the change in the standard deviation of PI90 if the values of the predictor variables would increase by one standard deviation. From table also we can see that there is a significant linear relationship between lamb body weight at 90 days of age and body weight from birth to 60 days of observation.

The association of growth traits in sheep from different populations has been examined by other authors, and more or less, we find some similarities and differences in their results. So, correlation BW with other body weight traits was estimated by *Duguma et al. (2002)* in Tygerhoek sheep, *Gowane et al. (2010)* in Malpura sheep, *Mohammadi et al. (2010, 2011)* in Sanjabi and Zandi sheep. In addition to the above, positive correlations for lamb weight traits were reported by *Eftekhari-Shahroudi et al. (2002) and Baneh et al. (2010).* Growth traits parameters have been reported by *Safari et al. (2005); Caro Petrović et al. (2012, 2013).*

Lewis and Brotherstone (2002) and *Fischer et al. (2004),* stated that regression method had a significant contribution in the prediction and assessment of growth of lambs. Correlation and regression coefficient were estimated of Bulochi sheep breed by *Bugti et al. (2016).*

Conclusion

There is a high and very significant association between lambs' weight at different ages. From our research we can see that the first month of life is very important for the further development of the body. There is a different level of correlation between lamb weights. This gives us an idea to say, that many paragenetic factors are crucial to the growth of lambs from birth to weaning. The multiple correlation coefficient indicates that any increase in lamb body weight during the observed periods is associated with an increase in the results of the dependent variable. In particular, any increase in lamb weight at birth is associated with a 90-day increase in lamb weight. A quantitative genetic analysis of the variability and relationship of lamb body weight in the indigenous Pirot sheep population shows that the genetic potential of the lambs is characterized by high variability, which is very suitable for selection to higher growth. Farmers can be advised that paragenetic factors should be taken seriously into account when designing breeding programs for this population.

Kvantitativno genetska analiza variabilnosti i povezanosti mase tela jagnjadi u autohtonoj populaciji pirotske ovce

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Rezime

Kvantitativno genetska analiza variabilnosti i povezanosti mase tela jagnjadi u autohtonoj populaciji pirotske ovce pokazuje da genetski potencijal podmlatka karakteriše visoka varijabilnost koja je veoma pogodna za selekciju na veću masu tela. Postoji visoka i veoma značajna povezanost između telesne mase janjadi sa 30 i 60 dana (.969 **) i mase sa 30 i 90 dana (.914 **). Takođe postoji vrlo značajna povezanost između mase janjadi starosti 60 i 90 dana (.904 **). Iz našeg istraživanja možemo videti da je prvi mesec života jagnjeta veoma važan za dalji razvoj tela. Postoji različit nivo korelacije između težine jagnjadi. To nam daje ideju da kažemo, da su mnogi paragenetski faktori ključni za rast janjadi od rođenja do odbijanja. Koeficijent višestruke korelacije (R2) iznosi 0,845 što znači da je 8,45% odstupanja telesne mase jagnjadi u toku 90 dana određeno varijancijom skupa prediktorskih varijabli (PI-60, 30, 1). Svako povećanje telesne mase janjeta tokom posmatranih perioda povezano je sa povećanjem rezultata zavisno promenljive PI90. Konkretno, svako povećanje telesne mase janjetine pri rođenju za 1 kg povezano je sa povećanjem telesne mase janjadi od 90 dana za 0,238 kg. Može se dati preporuka stručnjacima i farmerima da kod kreiranja odgajivačkih programa za ovu populaciju treba ozbiljno uzeti u obzir paragenetske faktore.

Acknowledgement

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SEQUENCING, POLYMORPHISM AND PHYLOGENETIC CHARACTERISATION OF KISS-1 GENE IN TWO NIGERIAN INDIGENOUS GOAT BREEDS

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Abstract: KiSS-1 gene encodes a protein product kisspeptin which are intense inducers of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in various mammalian species through its receptor GPR54 (G protein-coupled receptor-54). A total of 100 goat compromising of Red Sokoto (n = 72) and Sahel (n = 28) breeds were used to detect single nucleotide polymorphisms (SNPs) in the intronic region of the *KiSS-1* gene by sequencing and investigate their relationship with other goat breeds. Nucleotide sequence analysis revealed five novel SNPs (g.1745G>A present in Red Sokoto, g.1776G>A, g.1827A>G, g.1857T>C and g.2208T>C present in Red Sokoto and Sahel breeds). To obtain a correct phylogenetic relationship between goat breeds, nucleotide sequences were compared to other sequences in NCBI database using a BLASTn algorithm and retrieved for further analysis. Neighbour-joining phylogenetic relationship tree constructed revealed two distinct clusters with ancestral lineage of 100% identity. Nigerian goat breeds (Red Sokoto and Sahel) clustered into a clade with Indian goat breeds (Ganjam and Osmanabadi) while the second cluster involved eight other goat breeds. Genetic distance estimate revealed high genetic similarity between Red Sokoto and Sahel breeds as observed in their genetic distance value of 0.003. The nucleotide sequences of the two Nigerian goat breeds (Red Sokoto and Sahel) for *KiSS-1* gene were submitted to GenBank database and have accession numbers: MN122316 and MN122317, respectively. The analysis of polymorphism in *KiSS-1* gene indicates that genetic variation exists in the goat breeds studied. Therefore, attempts can be made to investigate the association of these polymorphism with reproductive traits in Nigerian goat breeds.

Keywords: Goat, Litter size, SNP, Genetic identity

Introduction

Goats spread all over the world because of their ability to adapt to varying environmental conditions and different regimes under which they are subsequently maintained (*Assan, 2014*) with over 300 distinct breeds to available for different purposes (*Hirst, 2018*). In Nigeria, goats constitute the largest group of small ruminant livestock totaling about 73.8 million (*FAOSTAT, 2016*). Red Sokoto and Sahel goat breeds are two well adapted and predominantly found in the Northern part of Nigeria where they are majorly managed by traditional production system with an average flock size of 3-5 goats per small holder farmers constituting a good source of protein (*Makun et al., 2006*). The Red Sokoto (RS) goat is a highly prolific breed with high incidence of multiple births and twinning rate of 54% and 43% respectively (*Akpa et al., 2010*). However, the Sahel are known to have a short fine hair with different coat colours from plain white, grey, pied, dappled, black or brown goat is a multipurpose goat breed mostly reared for meat and skin production (*Adebambo, 2012*) although reproductive potentials of this breeds have not been fully explored. Both breeds have interestingly unique adaptive traits making them strive in the hot savanna such as long leggedness, long distance walking ability, feeding behavior, heat tolerance and remarkable recovery capacity from scarcity of feed resources (*Muema et al., 2009*). With the increasing population and demand always exceeding supply for chevon (*Okewu and Iheanacho, 2015*), it is necessary to exploit ways of increasing animal protein availability other than the traditional breeding method which is by evaluating genetic variation in genes relatively affecting prolificacy in goat. Genetic markers like single nucleotide polymorphisms of some genes have been reported to be significantly associated with litter size in goats such as *POU class 1 homebox 1 (POU1F1)* gene (*Feng et al., 2011*), *KiSS-1* gene (*Cao et al., 2010; An et al., 2013a,b; Othman et al., 2015; Mekuriaw et al., 2017*) *Gonadotropin-releasing hormone receptor (GnRHR)* gene (*Yang et al., 2011; Huang et al., 2012; Bemji et al., 2018*), *KIT ligand* gene (*An et al., 2011; 2015; 2016*), *Inhibin alpha* (INHA) gene (*An et al., 2012; Sharma et al., 2015; Isa et al., 2017*). Although characterization of genetic variation in Nigerian goat breeds have been carried out using genetic markers like Microsatellite, Mitochondrial, Biochemical and SNPs (*Shoyombo et al., 2015; Awotunde et al., 2015; Ojo et al., 2017; Bemji et al., 2018; Isa et al., 2019*), there has been no published information on *KiSS-1* gene variant of Nigerian goat breeds. Therefore, this study was intended to identify single nucleotide polymorphism, genetic distance and phylogenetic relationship among two Nigerian Northern goat breeds.

Materials and Methods

Animal sampling and DNA isolation

A total of 100 goat belonging to two Nigerian indigenous breeds respectively managed semi-intensively at National Animal Production Research Institute (NAPRI), Shika-Zaria were included in this study. About 5 ml of blood sample was collected from 72 Red Sokoto goats and 28 Sahel via jugular venipuncture into vacutainer tubes containing ethylene diamine tetra acetic acid (EDTA) anticoagulant. Genomic DNA extraction was isolated from whole blood samples using Zymo Research quick-gDNA^{TM} Miniprep kit adhering to manufacturer's protocol. The concentration of DNA was evaluated by the Nanodrop spectrophotometer (ND1000; NanoDrop Technologies, USA) while the quality was virtually assessed by agarose gel electrophoresis.
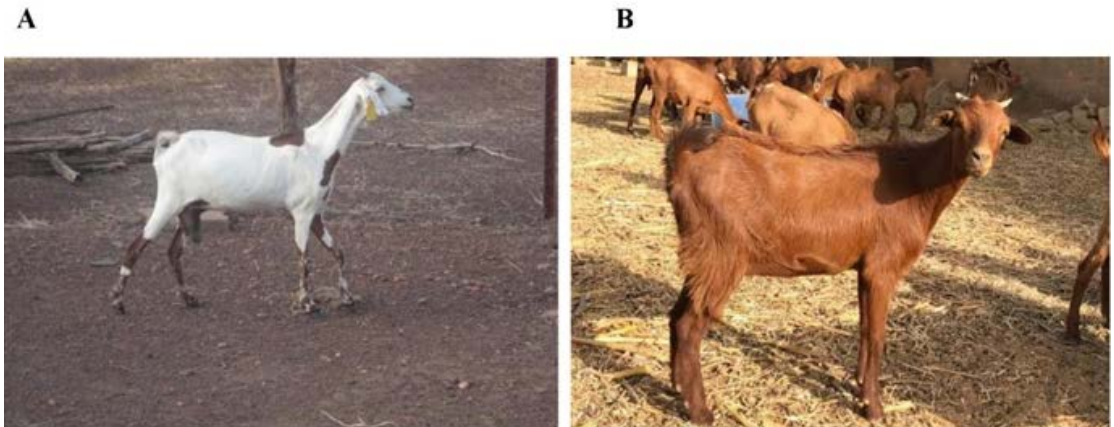
Figure 1. Pictures of two Nigerian goat breeds studied. (A) Sahel and (B) Red Sokoto

Primer design and PCR amplification

Using the caprine *KiSS-1* gene sequence (Accession number: NC_030823.1 position 1343186-1340188) a pair of primer (Table 1) was designed to amplify and sequence a 1088 bp fragment of intron 1 in Stab Vida genetics laboratory, Portugal. Polymerase chain reaction (PCR) amplification was carried out in SureCycler 8800 Thermal Cycler (Agilent Technologies, USA) with a total reaction volume of 20μL containing 50ng genomic DNA was added to a reaction mix containing 12.8μL of H2O MQ, 2.5μL of 1XPCR reaction buffer, 1μL dNTP's, 1.5μL MgCl2, pH 9, 1μL each of forward and reverse primers and 0.2 μL of *Taq* DNA polymerase. PCR

cycling protocol was accomplished by an initial denaturation at 96°C for 15 minutes followed by 40 cycles of denaturing at 95°C for 30 seconds, annealing at 58°C for 30 seconds, extension at 70°C for 1 minute and 40 seconds and final extension at 70°C for 5 minutes. The PCR products were subjected to 1% agarose gel stained with GelRed^{TM} nucleic acid stain in a Thermo EC Midicell Primo EC-330 gel system containing 1XTBE electrophoresis buffer, scored using a standard 100 bp molecular ladder and viewed under UV light and photographed using a Vilber lourmat gel documentation system.

Table 1. Primer sequences, lengths, gene region, annealing temperatures and product size

Primer	Sequence (5' – 3')	Length	Gene region	PCR size	Tm (°C)
KISS-1F	CTTTCTGGGTAAGGGAGG	18	Intron 1	1088bp	57°C
KISS-1R	AGAGAGAGGCTTTGGACC	18			

F= Forward primer, R= Reverse primer

DNA sequencing and sequence analysis

The PCR products were purified from the gel using a Carboxylate Magnetic Beads technology (MCLab, USA) and eventual sequencing was done with BigDye^® terminator cycle sequencing kit on the ABI 3730XI (Applied Biosystems, USA). The sequences were viewed and edited using Bioedit software *(Hall, 1999)*. Multiple sequence alignment of the sequences were performed using ClustaW in MEGA-X software package (*Kumar et al., 2018*). Identification of single nucleotide polymorphism in *KiSS-1* gene was carried out using codon code aligner (Codon code Corporation Dedham, MA, USA) and MEGA-X software packages. A BLAST algorithm (*Altschul et al., 1990*) search was carried out to retrieve homologous nucleotide sequences from the NCBI GenBank (http://www.ncbi.nlm.nih.gov/BLAST) database. Nucleotide sequence showing 97-100 % similarity for *KiSS-1* gene of two Nigerian goat breeds (MN122316 and MN122317), ten goat published sequences viz. San Clemente (NC_030823.1), Barki (KP835800.1), Zaraibi (KP835799.1), Xinong Saanen (JQ806381.1), Guanzhong (JQ806382.1), Ganjam (KJ425411.1), Osmanabadi (KJ425412.1), Gaddi (MH397145.1), Lezhi Black (KR065750.1), Jining Grey (GU142847.1), sheep (Barki = KP835798.1) and a Japanese medaka fish as an out-group (NC_019863.2) were assembled. Genetic distance and phylogenetic analysis of the *KiSS-1* gene were carried out for Nigerian goat breeds, other goat breeds and fish sequences applying p-distance model. A neighbor joining tree method was used to infer phylogenetic relationship between the populations following alignment of the sequence of Nigerian goat breeds and the published sequences. The reliability of the phylogenetic tree branching was estimated using a bootstrap confidence level of 1000 replications.

Results and Discussion

Identification of single nucleotide polymorphism in *KiSS-1* gene of Nigerian indigenous goat breeds

The sequence result analysis of this study among two Nigerian indigenous goat breeds revealed novel polymorphism of *KiSS-1* intron 1 gene. Five single nucleotide polymorphisms (g.1745G>A, g.1776G>A, g.1827A>G, g.1857T>C, g.2208T>C) was identified within Intron 1 of goat *KiSS-1* gene. The breed, SNPs type and the position along the reference sequence and chromosome position are presented in Table 2. Several similar studies on *KiSS-1* (intron 1) gene have identified several polymorphism in different goat populations (*Cao et al., 2010; An et al., 2013b; Maitra et al., 2014a; El-Tarabany et al., 2017*). Although these studies are focused on the non-coding regions of the gene it has been proven that mutations in intragenic (intron) regions may be involved in alternative splicing/regulation, transcript processing, gene expression and protein function, chromosomal rearrangement (*Guey-Shin and Thomas, 2007; Sjakste et al., 2011*) of varying phenotypes and have been found to affect economic traits (*Jiang et al., 2010; Ibeagha-Awemu et al., 2014*).

Table 2. Polymorphisms identified in Goat *KiSS-1* gene and their position on reference sequence

Region	SNP identified	Type of mutation	SNPs position on reference sequence (NC_ 030823.1) bp	SNPs position on chromosome 16 (bp)	Breed where identified
Intron 1	g.1745G>A	Transition	1745bp	1341442	Red Sokoto
	g.1776G>A	Transition	1776bp	1341411	Red Sokoto and Sahel
	g.1827A>G	Transition	1827bp	1341360	Red Sokoto and Sahel
	g.1857T>C	Transition	1857bp	1341330	Red Sokoto and Sahel
	g.2208T>C	Transition	2208bp	1340979	Red Sokoto and Sahel
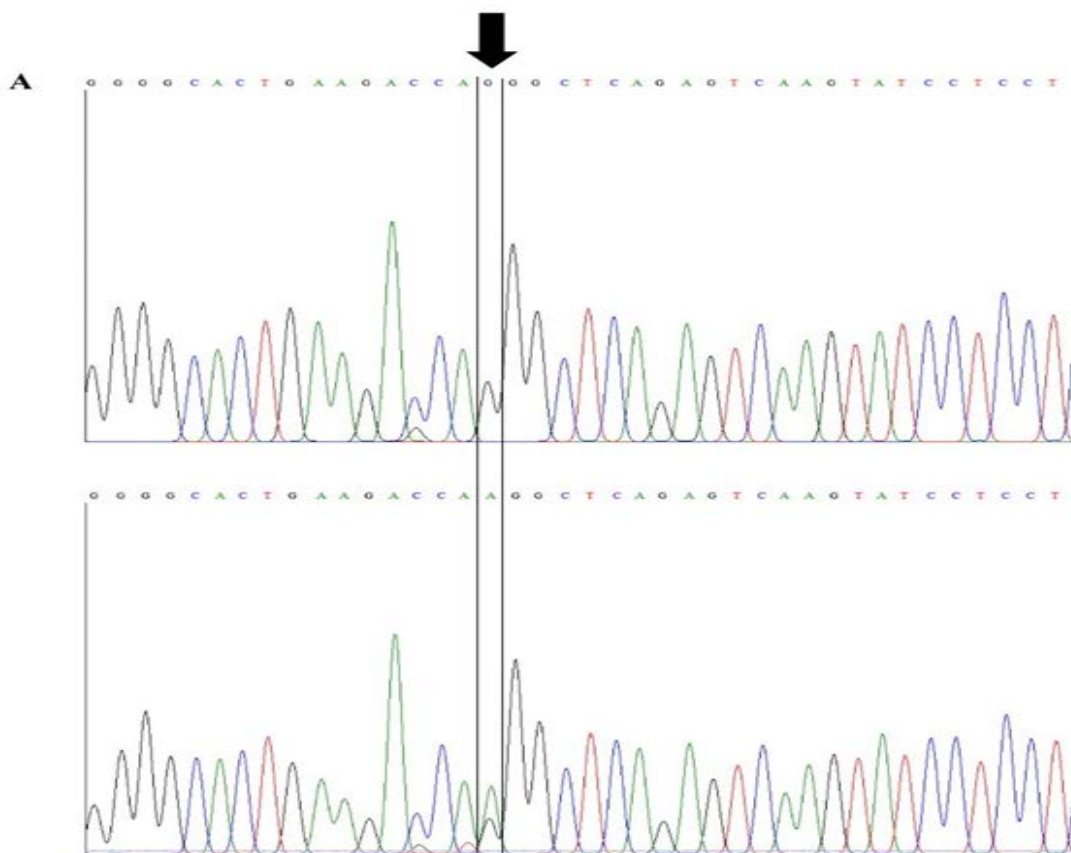
Figure 2. Single nucleotide polymorphism (SNP) variant of KiSS-1 gene in Nigerian goat breeds. (A) g.1745G>A

Genetic distance between Red Sokoto, Sahel and other goat breeds

The pair-wise estimation of genetic distance between Red Sokoto, Sahel and other goat breeds was based on sequence analysis of *KiSS-1* gene intron 1 and shown in Table 3. Among the twelve breeds sequences used for this analysis Guanzhong and Xinong Saanen had 100 percent nucleotide identity having the closest value of 0.000 and the highest was observed at 0.574 between Red Sokoto and Guanzhong, Xinong Saanen, Lehzi black. Based on the polymorphisms earlier reported in this study we observed a low pair-wise distance of 0.003 between Red Sokoto and Sahel goat breeds which compares favourable with findings of other studies estimate for Red Sokoto and Sahel (*Murital et al., 2015; Udeh, 2015; Ajibike et al., 2016; Ajayi et al., 2016; Ojo et al., 2017*). The low genetic distance estimates obtained between Red Sokoto and Sahel from different molecular markers could be attributed to migration, high degree of miscegenation or high gene flow,

geographical adaptation. Similarly, low genetic distance observed in this study would be due to shared alleles, origin and geographical adaptation between the two breeds.

Table 3. Estimated Nei genetic distance between RS, SH and other goat breeds using MEGA–X program. The standard genetic distances are below the diagonal and corrected distances are above the diagonal

	BK	ZR	XS	GZ	GJ	OS	GD	LB	RS	SH	SC	JG
BK		0.003	0.005	0.005	0.024	0.024	0.004	0.005	0.024	0.024	0.005	0.005
ZR	0.003		0.005	0.005	0.024	0.024	0.005	0.005	0.024	0.024	0.004	0.004
XS	0.011	0.013		0.000	0.024	0.024	0.004	0.003	0.024	0.024	0.004	0.005
GZ	0.011	0.013	0.000		0.024	0.024	0.004	0.003	0.024	0.024	0.004	0.005
GJ	0.564	0.564	0.569	0.569		0.005	0.024	0.024	0.003	0.003	0.024	0.024
OS	0.564	0.564	0.569	0.569	0.008		0.024	0.024	0.005	0.004	0.024	0.024
GD	0.008	0.011	0.008	0.008	0.566	0.566		0.005	0.024	0.024	0.004	0.004
LB	0.013	0.011	0.003	0.003	0.569	0.569	0.011		0.024	0.024	0.003	0.005
RS	0.569	0.569	0.574	0.574	0.005	0.008	0.572	0.574		0.003	0.024	0.024
SH	0.566	0.566	0.572	0.572	0.003	0.005	0.569	0.572	0.003		0.024	0.024
SC	0.011	0.008	0.005	0.005	0.566	0.566	0.008	0.003	0.572	0.569		0.004
JG	0.011	0.008	0.011	0.011	0.566	0.566	0.008	0.008	0.572	0.569	0.005	

BK – Barki goat breed, ZR – Zaraibi goat breed, XS – Xinong Saanen goat breed, GZ – Guanzhong goat breed, GJ – Ganjam goat breed, OS – Osmanabadi goat breed, GD – Gaddi goat breed, LB – Lezhi black goat breed, RS – Red Sokoto goat breed, SH – Sahel goat breed, SC – San clemente goat breed, JG – Jining grey goat breed

Phylogenetic relationship

The Neighbor-joining phylogenetic tree used to infer relationship between Red Sokoto, Sahel goats, some selected goat breeds and other species (*Ovis aries, Oryzias lapites*) shown in Figure 3 revealed three major clusters for which the small ruminant population had separate common ancestors at 100% reliability. First cluster estimate comprised eight exotic goat breeds and an ovine species, second cluster comprised Red Sokoto, Ganjam, Osmanabadi and Sahel, goat breeds which is indicative of these breeds having common alleles being shared and could reflect a common origin. The third clade comprised the Japanese rice (medaka) fish which from the tree had similar nucleotide sequence but branched separately showing that it is the most genetically divergent.
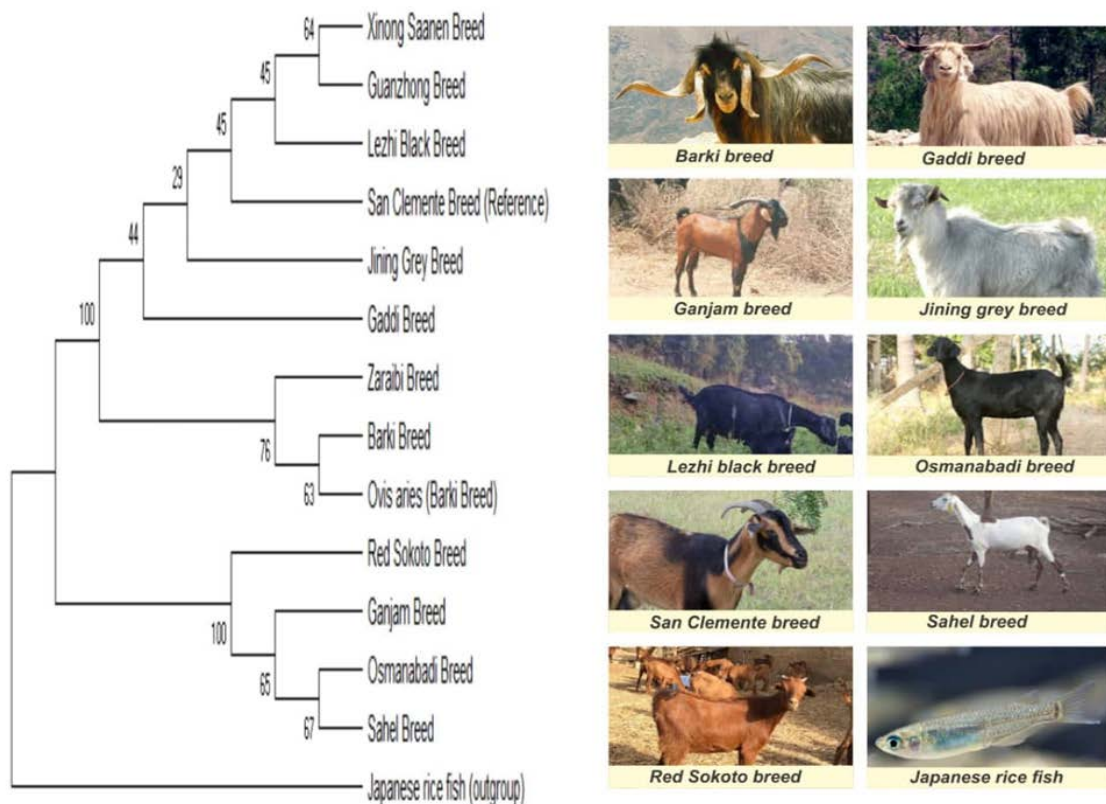
Figure 3. Neighbour-Joining phylogenetic tree between Nigerian goat breeds, other goat populations and species. Confidence bootstrap percentage values are indicated on the nodes after 1000 replications.

Conclusion

In this study, the analysis of *KiSS-1* gene of Nigerian goat breeds showed to be polymorphic. Phylogenetic relationship analysis revealed that Nigerian goat breeds (Red Sokoto and Sahel) clustered into a clade with low genetic distance (0.003) for *KiSS-1* (Intron 1) gene. The presence of genetic variation permits for genetic conservation and improvement programme of these breeds having better understanding on their genome architecture, breed relationship and phenotypic performance. It is recommended that the number of animals used in this study be increased in further studies in other to estimate genetic diversity indices and

explore the significant association of KiSS-1 gene polymorphism with litter size in Nigerian indigenous goat breeds.

Sekvenciranje, polimorfizam i filogenetska karakterizacija gena kiss-1 u dve nigerijske autohtone rase koza

Briggs Ibifiri Prekuna, Bemji Martha Nchang, Wheto Mathew, James Ikechukwu Joseph

Rezime

Gen *KiSS-1* kodira proteinski proizvod kispeptin koji je intenzivni induktor lučenja luteinizirajućeg hormona (LH) i folikul-stimulišućeg hormona (FSH) kod različitih vrsta sisara putem svog receptora GPR54 (receptor vezan za G protein-54). Ukupno 100 koza rasa crveni sokoto (n = 72) i sahel (n = 28) korišćene su za otkrivanje polimorfizama jednostrukih nukleotida (SNP) u introničnom regionu gena *KiSS-1* sekvenciranjem i istraživanjem njihove veze sa drugim rasama koza. Analiza nukleotidne sekvence otkrila je pet novih SNP-ova (g.1745G>A prisutan u crvenom sokotu, g.1776G>A, g.1827A>G, g.1857T>C i g.2208T>C prisutni u rasama crveni sokoto i sahel). Da bi se dobio tačan filogenetski odnos između rasa koza, nukleotidne sekvence su upoređene sa drugim sekvencama u bazi podataka NCBI korišćenjem BLASTn algoritma i preuzete za dalju analizu. Izgrađeno stablo filogenetskih odnosa koje se spajaju sa susedima otkrilo je dva različita klastera sa 100% identitetom loze predaka. Nigerijske rase koza (crveni sokoto i sahel) udružile su se sa indijskim rasama koza (ganjam i osmanabadi), dok je u drugom klasteru učestvovalo osam drugih rasa koza. Procena genetičke udaljenosti otkrila je veliku genetsku sličnost između rasa crveni sokoto i sahel, primećeno u njihovoj vrednosti genetske udaljenosti od 0,003. Nukleotidne sekvence dve nigerijske rase koza (crveni sokoto i sahel) za gen *KiSS-1* predate su u bazu podataka GenBank i imaju pristupne brojeve: MN122316, odnosno MN122317. Analiza polimorfizma u genu *KiSS-1* ukazuje na to da postoje genetske varijacije u proučavanim rasama koza. Stoga se mogu pokušati istražiti povezanost ovog polimorfizma sa reproduktivnim osobinama kod nigerijskih rasa koza.

Ključne reči: koza, veličina legla, SNP, genetski identitet

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EFFECT OF FARM AND BIRTH SEASON ON CALF BODY WEIGHT IN THE FIRST WEEK OF LIFE

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Abstract: The body weight of calves in the earliest period of their life (age from 0 to 7 days) is under the greatest influence of the farm and the season of birth. The farm manifests its influence most often through the conditions of nutrition and housing and the organization of the technological production process, while the influence of the birth season is manifested through microclimatic and nutritional conditions, as well. The most common deficiencies related to the nutrition of newborn calves are related to: inadequate colostrum supply in terms of quality, quantity and time of colostrum intake, which is closely related to the organization of the technological production process on the farm. Dietary deficiencies affect the body weight of calves at birth and at 8 days of age. In a study conducted over a year (4 seasons), the colostrum diet of newborn calves of the HF breed on two farms (A and B) with a tied housing system was analyzed. Calves were fed colostrum on both farms at intervals, usually up to 2 hours, less often up to 4 hours after birth. The colostrum consumed came from the mother, most often, and less often from the other cow, while on one of the farms it was also used frozen. However, the amount of colostrum consumed was deficient, it was 1-2 l on farm A, and 2.5 to 3 l on farm B. The occurence of a deficient diet or other deficiencies in the diet of calves was indicated by the average body weight, measured at birth and at the age of 8 days. On farm A, a lower average body weight of calves (37.95 and 39.68 kg) was recorded than on farm B (40.00 and 41.80 kg) by age categories, respectively. The average body weight of calves was statistically significantly ($p <0.01$) influenced by the farm and the season of birth, as well as their mutual interaction, but the effect of the farm was more pronounced.

Key words: calves, colostrum diet, body weight, season, farm

Introduction

There are numerous factors that affect the body weight of calves in intensive farming conditions. Some of them relate to the production conditions that are characteristic of each farm (farm impact). We can classify them into three groups: nutrition, housing conditions and organization of the technological process. The most common dietary deficiencies are: inadequate colostrum supply in terms of quality, quantity, and timing of colostrum intake, which is closely related to early separation from the mother; insufficient balance of whole milk diet and milk replacements, use of foods that contain allergens or are deficient in iron, excessive and deficient meals, etc. In addition to the farm, the birth season also affects the mentioned parameters through microclimate and feeding conditions.

Food is one of the key factors responsible for calf growth, both quantitatively and qualitatively. *Yang et al. (2015)* state that the best gain values are achieved by calves that consume full colostrum immediately after birth, in contrast to those that consume some type of whole milk instead of colostrum. *Ballou et al. (2013)* compare the body weight of calves of two breeds and two levels of nutrition. Regardless of the breed, higher growth and more efficient use of nutrients and energy from the meal is recorded in calves whose meal was richer in protein, fat and energy. Calves fed different diets, according to *Ververst et al. (2015),* consume different amounts of dry matter from meals, spend more or less time in feeding and achieve different gain values in the period before and after weaning, depending on the type of meal. The correlation between the body weight of calves and the ability to consume a certain amount of milk in the first 2-4 days of life is pointed out by de *Passile et al. (2015).* The amount of milk that calves consume ranges from 7.3% to 30.5% of body weight, or from 2.4 to 12 l/day. The correlation coefficient between body weight at birth and the amount of milk consumed is 0.33.

Material and Method

The study of the impact of the farm through breeding conditions and the season of birth on the body weight of calves in the period from 0 to 7 days of life, was conducted on two farms, A and B, which operated within the same production system with intensive production, capacity of about 1000 dairy cows. Both farms have a system where animals are kept tied. The calves were separated from the mother soon after birth. The number of calves included in the analysis was 596 on farm A and 572 on farm B.

During a period of one year divided into 4 seasons (autumn, winter, spring and summer), the colostrum diet of calves was followed immediately after birth.

The following were monitored: the amount of colostrum consumed, the origin (mother's or other cows') and the time period of consumption after birth.

After birth, the calves on both farms remained with their mothers for a very short time, 30-45 minutes, and then they were separated, on farm A on a specially prepared bed in the nursery, and on farm B in an individual box. After that, the calves were fed colostrum from a bucket for 1 to 4 hours. Where it was possible, calves received their mother's colostrum. If this was not feasible (death or illness of the mother, lack of milk, defective colostrum, etc.), on farm A they were fed colostrum from other cows, while on farm B there was a possibility for calves to get colostrum which was kept frozen. The amount of colostrum consumed was controlled by graduated buckets. The time when the calf received its first colostrum after birth, the type of colostrum obtained and the amount of colostrum consumed were duly recorded. Colostrum quality was controlled in only one way, organoleptically.

In the study, body weight was measured immediately after birth and at the age of 8 days. Calibrated livestock scales, located in the nursery were used on both measuring farms.

Results and Discussion

On both farms, calves received colostrum by feeding from a bucket, without the use of bottles or any other equipment. Colostrum, on farm A, was given exclusively fresh, from the mother or from another freshly calved cow. On farm B, in addition to fresh colostrum, there was a possibility of freezing excess quality colostrum, so that, in the absence of fresh, calves received colostrum which was thawed and heated to a temperature of about 39-40°C, and whose quality prior to its use was checked only by visual inspection. Colostrum intake time is very important due to the possibility of resorption of all nutrients in the calf's digestive tract. The most efficient use of colostrum is in the first few hours of life. On both farms, all calves received colostrum in the first 4 hours. On farm A, calves drank 0.5 to 2 liters of colostrum, usually 1-2 liters, and very rarely 2.5 liters. On farm B, the situation was somewhat better because calves usually received 2.5-3 l of colostrum, and in exceptional cases less (minimum 1 l) or more than 4 l (5 or 6 l).

The analysis of the results related to colostrum nutrition on farms A and B reveals several important facts. The largest number of calves received colostrum from their mother, which should have enabled the best absorption of immunoglobulins, as reported by numerous studies (*Arthington et al., 2000; Conneely et al., 2014; Yang et al., 2015*). However, the quality of colostrum is not controlled on farms, except organoleptically, therfore it is not possible to know with certainty what the concentration of antibodies was in the colostrum. The time of feeding of calves with colostrum in most cases was in the interval up to 4 h after

birth, which is in line with the recommendation of numerous authors (*Vasseur et al., 2009; 2012; Conneely et al., 2014; Klein-Jöbstl, 2015)*, who state that the optimal time to take colostrum is up to 6 h, and even up to 8 h after birth. Fewer calves received colostrum in the first 2 h, which is in concordance with the results of *Godden et al., (2012)* and *Relić et al., (2014)*. Taking into account the way calves were fed colostrum (bucket feeding), too early feeding with colostrum cannot be recommended. Namely, immediately after birth, when calves are still tired and exhausted from calving and not strong enough to stand for a long time, they are not able to consume a sufficient amount of colostrum from a bucket. It may therefore be recommended that tired and exhausted calves not be fed immediately after birth and given sufficient time to recover. Also, mothers should be allowed to lick the calves, in order to clean and dry the hair, which establishes normal thermoregulation. The biggest deficiency in colostrum supply was observed in the amount of colostrum consumed. Although it is considered that the amount of colostrum consumed should be 8-10% of the body weight of calves *(Jonić and Mirilović, 2007; Conneely, 2014)*, i.e. 3-4 l, and even more (*Weaver et al., 2000; Vasseur et al., 2009; 2010; Osaka et al., 2014; Klein-Jöbstl, 2015*), the amount that calves drank on farms A and B was lower, especially on farm A. Similar data, in their research, were also provided by *Vasseur et al., (2009; 2010)* and *Relić et al., (2014).*

Table 1. Average body weight of calves by birth season

Farm	Season	Body weight of calves, kg ($\bar{x} \pm S\bar{x}$)	
		Age	
		0	8
A	Autumn	38.64±0.156	40.38±0.166
	Winter	38.75±0.177	40.21±0.197
	Spring	37.29±0.213	39.04±0.230
	Summer	36.94±0.095	38.92±0.144
	$\bar{x} \pm S\bar{x}$ Cv (%)	37.95[a]± 0.091 5.74	39.68[a]±0.098 5.94
B	Autumn	39.17±0.222	40.92±0.230
	Winter	40.03±0.137	41.95±0.137
	Spring	40.42±0.191	42.02±0.217
	Summer	40.65±0.185	42.62±0.197
	$\bar{x} \pm S\bar{x}$ Cv (%)	39.99[b]±0.098 5.75	41.80[b]±0.106 5.92

[a,b]= statistically significant differences (p <0.05) between values with different letters in the same column

There are no statistically significant differences between the values marked with the same letters (p> 0.05)

Table 1 shows the average body weight of calves on farms A and B by rearing seasons at birth and after the first seven days. In addition to the average value, data for standard error are given, as well as an indicator of relative variability (coefficient of variation). It can be noticed that the samples were homogeneous, as the defined coefficients of variation had a lower value, i.e. did not exceed 7%.

The results presented in Table 1 show that statistically significantly higher values for body weight of calves were found on farm B compared to farm A (p <0.01).

To test the difference in body weight of calves on farms by rearing seasons, the method of two-factor analysis of variance with repeated measuring was used (Table 2).

Table 2. Value of the multivariate Wilks' lambda test

Source	Value	df	F	Significance	Partial eta square coefficient
Farm	0.7776	3	106.0	0.000	0.2224
Season	0.9562	9	5.6	0.000	0.0182
Farm x Season	0.8980	9	13.6	0.000	0.0432

The multivariate Wilks' test recorded statistically very significant differences in the achieved values of body weight of calves of different ages between farms A and B, as well as statistically significant differences in the dependence of weight on the calving season (p<0.01). Thus, the main effects of farm and rearing season factors showed statistical significance, which confirms the initial hypothesis that rearing conditions and birth season have an impact on calf body weight in the first month of life. Factor interaction (farm x season) also showed statistical significance (p<0.01), (Table 3). Based on the guidelines proposed by *Cohen (1988)* (0.01 = low impact, 0.06 = moderate impact, 0.14 = high impact), it can be said that the farm had high impact on calf body weight and the season was low impact. Namely, the effect of the farm on the change in body weight of calves was 22.24%, season 1.82%, while the effect of their interaction was 4.32%. Thus, the farm with its properties and microclimate had a significantly greater impact on the change in body weight than the rearing season.

The influence of the birth season on the body weight of calves (Duncan test) is given in Table 3.

Table 3. Analysis of the influence of the birth season on the body weight of calves (Duncan test)

Season	Average body weight of calves (kg)	
	Age 0	Age 8
Autumn	38.905[b]	40.649[ab]
Winter	39.371[a]	41.057[a]
Spring	38.744[b]	40.425[b]
Summer	38.787[b]	40.763[ab]

[a,b]= statistically significant differences ($p < 0.05$) between values with different letters in the same column

There are no statistically significant differences between the values marked with the same letters ($p > 0.05$).

The season had a statistically significant effect on the change in body weight of calves, as follows:
- at birth of calves (initial age) in the winter period (season 2) the weight of calves (39.371 kg) was statistically significantly higher than the weight of calves born in autumn (38.905 kg), $p < 0.05$, and statistically very significantly higher in relation to the weight of calves born in the spring (38.744 kg) and summer (38.787 kg) seasons, ($p < 0.01$).
- at the age of eight days, the highest average body weight value in the winter period (second season) of 41.057 kg was recorded again. However, it was statistically significantly higher only than the body weight of calves of the same age born in the spring period (third season) ($p < 0.01$), while in relation to the body weight of calves born in the autumn and summer period it was not statistically significantly higher ($p > 0.05$).

Analyzing the average body weight of calves on farms A and B, it is observed that it did not deviate from the value stated for the Holstein Frieian breed by *Olson et al. (2009)* and *Ballou et al. (2013),* but the obtained values were lower compared to values reported by *Heins et al. (2010).* However, analyzed by farms, it was observed that the average body weight of calves, at all ages measured on farm B, was statistically significantly higher than the values recorded on farm A, which confirms the initial hypothesis that rearing, feeding and housing conditions of calves affect BW in the first 8 days of life. These results were consistent with a number of studies related to rearing conditions (*Tapki et al., 2006; Wojcik et al., 2012; Costa et al., 2015; Bazeley et al., 2016)*; nutrition *(Thickket et al., 1981; Kertz et al., 1987; de Passillé et al., 2015)* and the breeder's attitude toward calves *(Lürzel et al., 2015).* In addition to the significant influence exerted by the farm, the body weight of calves was statistically significantly affected by the birth season through the action of climatic factors on food consumption and calf growth, which is also in line with the initial hypothesis that rearing conditions, i.e. farm, and the birth season influence the body weight of calves in the first week of life, as

evidenced by other authors in their studies *(Coleman et al., 1996; Silanikove, 2000; Avendaño-Reyes et al., 2006).*

Conclusion

Colostrum consumption on farms A and B was performed during the first four hours after birth, in most cases up to 2 hours after birth of calves. On farm A, calves consumed colostrum most often from the mother, and less from other newly calved cows; on farm B, the mother's colostrum was also most often used to feed newborn calves, but calves also received colostrum from other cows, as well as frozen colostrum. The amount of colostrum consumed on farms A and B was less than that recommended by calf feeding technology, as well as the amounts reported in the studies of other authors. The problem was especially pronounced on farm A, where calves consumed 1-2 l of colostrum most often in the first feeding, while on farm B they received between 2.5 and 3 l of colostrum. Colostrum quality was assessed by organoleptic method, while laboratory analyzes of chemical composition and biological values of colostrum were not performed. The colostrum feeding period lasted for the first four days after the birth of calves on both examined farms.

The body weight of calves was measured at birth and at 8 days of age, on both observed farms. On farm A, a lower average body weight of calves (37.95 and 39.68 kg) was recorded than on farm B (40.00 and 41.80 kg) by age categories, respectively. The farm and the season of birth had a statistically very significant effect on the average body weight of calves (p<0.01, but the influence of the season was less pronounced compared to the influence of the farm. The difference in average body weight between farms was statistically highly significant (p<0.01) at both ages, while the difference between seasons was statistically highly significant (p<0.01) at birth only.

Regarding the nutrition of calves with colostrum, it is necessary to solve a number of problems, starting with training breeders on the importance of colostrum feeding and enhanced control of the feeding process, through determining the quality of colostrum and forming a colostrum supply, to changing the way colostrum is given (artificial pacifiers, probes, etc.), in order for calves to consume a sufficient amount of high quality colostrum in a timely manner. A better diet with colostrum would inevitably lead to an improvement in the body weight of calves in the first days of life.

Uticaj farme i sezone rođenja na telesnu masu teladi u prvoj nedelji života

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Rezime

Telesna masa teladi u najranijem periodu života teladi (uzrast od 0 do 7 dana) je pod najvećim uticajem farme i sezone rođenja. Farma svoj uticaj ispoljava najčešće kroz uslove ishrane i držanja i organizaciju tehnološkog procesa proizvodnje, dok se uticaj sezone rođenja ispoljava kroz mikroklimatske i uslove ishrane, takođe.

Najčešći nedostaci vezani za ishranu novorođene teladi odnose se na: neadekvatno napajanje kolostrumom u smislu kvaliteta, količine i vremena uzimanja kolostruma, što je u tesnoj vezi sa organizacijom tehnološkog procesa proizodnje na farmi. Nedostaci u ishrani odražavaju se na telesnu masu teladi na rođenju i sa 8 dana života.

U ispitivanju sprovedenom tokom 2013-2014 godine (4 sezone) analizirana je ishrana kolostrumom novorođenimh teladi HF rase na dve farme (A i B) sa vezanim sistemom držanja.

Telad su napajana kolostrumom na obe farme u intervalu, najčešće do 2 sata, ređe do 4 sata nakon rođenja. Konzumirani kolostrum je poticao od majke, najčešće, a ređe od druge krave, dok se na jednoj od farmi koristio i zamrznut. Međutim, količina konzumiranog kolostruma bila je deficitarna, iznosila je 1-2 l na farmi A, a 2,5 do 3 l na farmi B.

Na postojanje deficitarne ishrane ili drugih propusta u ishrani teladi ukazivala je prosečna telesna masa, merena na rođenju i u uzrastu od 8 dana života. Na farmi A je zabeležena manja prosečna telesna masa teladi (37,95 i 39,68kg) nego na farmi B (40,00 i 41,80kg) po starosnim kategorijama, redom. Na prosečnu telesnu masu teladi statistički veoma značajno (p<0,01) su uticali farma i sezona rođenja, kao i njihova međusobna interakcija, ali je efekat farme bio izraženiji.

Ključne reči: telad, ishrana kolostrumom, telesna masa, sezona, farma

Acknowlegments

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EFFECT OF BREED OF PERFORMANCE TESTED BOARS ON EJACULATE TRAITS

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Abstract: The main objective of the study was to determine the influence of breed on the traits of boar ejaculate: ejaculate volume (VOL, ml), sperm concentration (CON, $x10^6$ sperm/ml), total sperm count in ejaculate (TNS, $x10^9$ spermatozoa), sperm motility in native ejaculate (MON, %), sperm motility after dilution (MOD, %) and number of doses produced (NPD). The aim was also to evaluate the correlation of the boar performance test traits: average life daily gain (g), backfat thickness measured in two locations (mm), depth of longissimus dorsi muscle (mm) and carcass meat content (%) with ejaculate traits. Total of 931 ejaculates of 36 boars during reproductive exploitation were analysed (16 Landrace boars and 20 Large White boars). The effect was assessed using the procedure of the general linear model of the statistical package SAS 9.1.3 (*SAS Inst. Inc., 2002-2003*). The model for analysis included the influence of breed, season and the linear regression influence of body weight at the end of the performance test. The correlation of the traits was determined by applying the Pearson's correlation coefficient. Most of the examined ejaculate traits (VOL, CON, MOD and NPD) varied under the influence of boar breed ($p<0.01$; $p<0.001$). Weight at the end of the test ($p<0.05$; $p<0.01$; $p<0.001$) affected all examined traits, except CON and TNS. A weak association was found between production performance and ejaculate traits.

Key words: pig, boar, breed, performance test, ejaculate traits

Introduction

Modern pig breeds are characterized by intensive growth, high meat content in the carcass and high fertility. The primary goals in the selection of male

heads are focused on traits that have economic significance such as growth, weight at a certain age and productivity of their daughters *(Robinson and Buhr, 2005)*.

Intensive selection for meat content caused a significant reduction in the content of subcutaneous and even the content of intramuscular fat *(Bahelka et al., 2007)*. Therefore, it is necessary to know whether the improvement of boar production performance (growth and meatiness) can have a negative impact on sperm quality *(Wolf, 2009)*.

The use of high-value, genetically superior boars for artificial insemination has a great economic impact on intensive pig production *(Gadea et al., 2004)*. The maximum number of insemination doses per ejaculate (or per boar per year) should be obtained from such boars, which is the main indicator of successful reproductive exploitation of boars. The average manifestation and variability of boar ejaculate traits is influenced by breed, age, season, intensity of use, and other factors *(Kondracki et al., 2009; Smital, 2010; Kunowska-Slósarz and Makowska, 2011)*.

As important as it is to pay attention to the reproductive traits of the boar, it is also important to know how they relate to the boar performance test traits, in order to prevent the consequences of unilateral selection. The aim of this study was to determine the influence of the boar breed on ejaculate traits and whether there is a correlation between production performance and ejaculate traits.

Material and Method

The research was conducted on a pig farm, which has its own reproductive and commercial herd. Total of 931 ejaculates collected from 36 boars of two fertile meat breeds were analysed (Landrace, n = 16 with 450 ejaculates; Large White, n = 20 with 481 ejaculates). The influence of season was examined as a cold (October-March) and warm (April-September) season.

The boars were kept in a separate building, in boxes measuring 2×4 m, with a partial grid concrete floor. The microclimatic conditions in the boar housing facility were under semi-automatic control, with vertical and horizontal ventilation. The animals were fed balanced feed mixtures, and fresh water was available to them at will. The average body weight at the end of the performance test was 110 kg at the age of 175 days.

Following performance test traits were analysed: ALDG- average life daily gain (g), FT- backfat thickness (mm), DLD- depth of longissimus dorsi muscle (mm) and CMC- carcass meat content (%). At the end of the performance test of the boar, certain ultrasound measuring was performed using the ultrasonic device PIGLOG 105. Back fat thickness was measured in two places: the first measurement in the loin area (FT1) between the 3rd and 4th lumbar vertebrae (measured from the last lumbar vertebra), 7 cm lateral to the dorsal line, and the second measurement of back fat thickness in the lumbar region (FT2) between the

3rd and 4th ribs counting from the last, 7 cm lateral to the dorsal line. At the FT2 measurement site, DLD was also measured.

In order for a boar to be included in this analysis, it was necessary to have a minimum of 5 ejaculates during reproductive use. The average interval between two ejaculations was 10 days. The study included ejaculate traits: ejaculate volume (VOL, ml), sperm concentration (CON, x10^6 spermatozoa/ml), total sperm count in ejaculate (TNS, x10^9 spermatozoa), sperm motility in native ejaculate (MON, %), sperm motility after dilution (MOD, %) and number of doses produced (NPD). Ejaculates were collected by the standard manual method, by introducing the boar into the box with the phantom. Ejaculate volume was measured with a graduated cylinder, with an accuracy of ± 2 ml. The concentration of native sperm was assessed using a photo-colorimeter. All ejaculates with subjectively estimated motility of sperm mass in the native state less than 70% were excluded from use, so they were not included in the research. Insemination doses are standardized to a volume of 100 ml and 3.5 billion sperm per dose. The total number of sperm in the ejaculate was obtained by multiplying the sperm concentration by the ejaculate volume. Evaluation of sperm mass motility in native ejaculate and after dilution was performed by subjective assessment, observation under a microscope.

The effect was assessed using the procedure of the general linear model of the statistical package SAS 9.1.3 (*SAS Inst. Inc., 2002-2003*), using the following model:

$$y_{ijk} = \mu + B_i + S_j + b(x_{ijk} - \bar{x}) + e_{ijk},$$

where: y_{ijk}- analysed ejaculate trait, μ- general population average, B_i- influence of the breed ($i = 1,2$), S_j- effect of the season ($j=1,2$), $b(x_{ijk} - \bar{x})$ - linear regression effect of body weight at the end of the test and e_{ijk}- random error. Testing of differences between Least Square Means (LSMeans) values was performed by t-test.

The correlation of traits was assessed by applying Pearson's correlation coefficient. The strength of the correlation was interpreted on the basis of a rough approximation of the height of the correlation according to *Petz (2004):* 0.0-0.2 (slight correlation), 0.2-0.4 (weak correlation), 0.4-0.7 (medium correlation) and 0.7-1.0 (strong correlation).

Results and Discussion

Ejaculate traits vary under the influence of breed and boar body weight at the end of the performance test (Table 1).

Table 1. Statistical significance (p) of the influence of factors included in the model

Trait	Breed	Season	Body weight at the end of the test		R^2
			b	p	
VOL	***	ns	1.608	*	0.035
CON	**	ns	1.417	ns	0.022
TNS	ns	*	0.762	ns	0.017
MON	ns	ns	-0.185	***	0.024
MOD	**	ns	-0.164	***	0.031
NPD	***	ns	-0.144	**	0.039

VOL-ejaculate volume (ml), CON-concentration of sperm (x10^6 spermatozoa/ml), TNS -total number of spermatozoa in ejaculate (x10^9 spermatozoa), MON-motility of spermatozoa in native ejaculate (%), MOD-motility of spermatozoa after dilution (%), NPD-number of doses produced, ns=p>0.05; * = p<0.05; ** = p<0.01; *** = p<0.001, b- regression coefficient, R^2- determination coefficient.

Ejaculate volume increased by 1.61 ml, i.e. the total number of sperm in the ejaculate by 0.76x10^9 for each kg of increase in body weight of boars at the end of the test. However, the increase in body weight at the end of the test resulted in a decrease in the motility of the sperm mass in the native state and after dilution, as well as the number of doses, which is shown by negative regression coefficients. Low values of the coefficients of determination indicate that the factors included in the model to a small extent explain the variability of the examined traits.

The results of this study are partially similar to those of *Smital (2010)* and *Wierzbicki et al. (2010)* in which the influence of different genetic and non-genetic influences on sperm traits was determined. Also, this research partly correspond to the research of *Savić et al. (2013)* in wich the effect of all analyzed factors for both sperm traits (volume and motility) was significant. In this study, the season influenced only on the TNS, which is in contrast to the study of *Savić et al. (2015)* in which all analyzed ejaculate traits varied under the influence of the season.

Table 2. Average LSM ± SE values of ejaculate traits by breeds

Trait	Breed	
	Landrace	Large White
VOL	294.59±4.90[A]	319.48± 4.62[B]
CON	271.24±7.03[a]	239.71±6.64[b]
TNS	84.75±2.87	77.99± 2.70
MON	86.02±0.33	85.70±0.31
MOD	82.12±0.29[a]	80.85±0.27[b]
NPD	22.14±0.37[A]	19.53±0.35[B]

VOL-ejaculate volume (ml), CON-concentration of sperm (x10^6 spermatozoa/ml), TNS -total number of spermatozoa in ejaculate (x10^9 spermatozoa), MON-motility of spermatozoa in native ejaculate (%), MOD-motility of spermatozoa after dilution (%), NPD-number of doses produced; Statistical significance: [a, b]=p<0.01; [A, B]=p<0.001.

Large White boars had a higher ejaculate volume (+24.89 ml), but less doses per ejaculate (-2.61) compared to Landrace boars (Table 2). The sperm concentration of Landrace boars was higher by 31.53×10^6 spermatozoa per ml of ejaculate, which had a crucial effect on obtaining a higher number of doses per ejaculate.

Contrary to our study, *Wolf and Smital (2009)* have found slight differences in ejaculate traits, with Large White boars having lower ejaculate volume and higher sperm concentration compared to Landrace boars. Contrary to our study, *Banaszewska and Kondracki (2012)* have established lower ejaculate volume of Large White boars compared to Landrace (247.03: 257.03 ml). The average values of Large White boar ejaculate traits determined by this research are lower in compared to the results of the research of *Savić et al. (2015)*. In our study, an average of 22.14 and 19.53 doses were obtained from ejaculates with an average volume of 294.59 and 319.48 ml, respectively, while in the study of *Wierzbicki et al. (2010)* significantly more doses were obtained from the ejaculate with an average volume of 221.15 ml (on average 24.52 doses). This difference in the exploitation of boars is most likely a consequence of the different number of spermatozoa per dose produced. In recent years, sperm plasma research are actual, so *Ivanova et al. (2015)* established a correlation between high level of one specific sperm plasma protein with the low cryotolerance and low motility of boar spermatozoa.

A slight to weak correlation of performance traits and ejaculate traits was found within both breeds (Table 3 and Table 4).

Table 3. Correlations between production traits and ejaculate traits of Landrace boars

Trait	ALDG	FT1	FT2	DLD	CMC
VOL	0.09^{ns}	0.00^{ns}	0.45***	-0.14**	-0.37***
CON	0.20***	0.23***	0.18**	-0.16**	-0.12**
TNS	0.17**	0.17**	0.30***	-0.19**	-0.24***
MON	0.00^{ns}	-0.12**	-0.18**	0.20***	0.24***
MOD	0.00^{ns}	-0.09^{ns}	-0.11**	0.14**	0.20***
NPD	0.04^{ns}	0.08^{ns}	0.24***	-0.13**	-0.11**

VOL-ejaculate volume (ml), CON-concentration of sperm ($x10^6$ spermatozoa/ml), TNS-total number of spermatozoa in ejaculate ($x10^9$ spermatozoa), MON-motility of spermatozoa in native ejaculate (%), MOD-motility of spermatozoa after dilution (%), NPD-number of doses produced. ALDG-average life daily gain (g), FT-backfat thickness (mm), DLD-depth of longissimus dorsi muscle (mm), CMC-carcass meat content (%); Statistical significance: ns=p>0.05; **=p<0.01; ***=p<0.001.

In Landrace boars, a positive correlation between back fat thickness (FT1 and FT2) and ejaculate traits (VOL, CON, TNS and NPD) was found, and on the other hand, meatiness traits (DLD and CMC) were slightly or weakly negatively correlated to ejaculate traits, with the exception of sperm mass motility (Table 3).

Table 4. Correlations between production traits and ejaculate traits of Large White boars

Trait	ALDG	FT1	FT2	DLD	CMC
VOL	-0.09^{ns}	-0.14^{**}	-0.14^{**}	0.14^{**}	-0.05^{ns}
CON	0.00^{ns}	0.02^{ns}	0.01^{ns}	0.05^{ns}	-0.04^{ns}
TNS	-0.06^{ns}	-0.05^{ns}	-0.05^{ns}	0.13^{**}	-0.05^{ns}
MON	0.15^{**}	0.14^{**}	0.12^{**}	0.00^{ns}	-0.07^{ns}
MOD	0.14^{**}	0.18^{**}	0.17^{**}	-0.03^{ns}	-0.11^{**}
NPD	0.05^{ns}	0.12^{**}	0.12^{**}	0.07^{ns}	-0.11^{**}

VOL-ejaculate volume (ml), CON-concentration of sperm ($x10^6$ spermatozoa/ml), TNS-total number of spermatozoa in ejaculate ($x10^9$ spermatozoa), MON-motility of spermatozoa in native ejaculate (%), MOD-motility of spermatozoa after dilution (%), NPD-number of doses produced. ALDG-average life daily gain (g), FT-backfat thickness (mm), DLD-depth of longissimus dorsi muscle (mm), CMC-carcass meat content (%); Statistical significance: ns=p>0.05; **=p<0.01.

Contrary to Landrace, Large White boars were found to have a weak positive correlation between sperm mass motility traits and back fat thickness and average daily life gain (Table 4). Similar to Landrace, the higher meat content in the carcass of Large White boars implied obtaining less doses during reproductive exploitation.

The weak correlation between the examined traits is similar to the results of the study by *Wolf (2009),* who has found low values of genetic correlation coefficients between production traits and boar sperm traits (0.00-0.13). Similar to the above, *Oh et al. (2005)* have found a weak phenotypic correlation of growth and ejaculate traits (sperm volume and concentration; -0.02 and 0.11). One of the important factors that could have influenced the strength of the correlation between these two groups of traits is the age of the boars when measuring the phenotypic values of these traits. Production performance of boars was determined at the age of about 6 months, and the characteristics of ejaculate later, during reproductive use. A similar conclusion was reached by *Wolf (2009).*

Conclusion

The traits of boar ejaculate varied under the effect of breed. Body weight at the end of the test affected the variability and average manifestation of all examined traits, except sperm concentration and total sperm count in ejaculate. There was a slight to weak correlation between the traits from the performance test and the ejaculate traits, so that the selection with the aim of improving the carcass will not have a great negative impact on the later reproductive performance of the boar. Production traits (growth, food utilization and carcass quality) are the ones that will have priority in selection work in the future, but it is important to monitor ejaculate traits, so that selection does not lead to worsening of the sperm traits by improving production performance.

Uticaj rase performans testiranih nerasta na osobine ejakulata

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Rezime

Osnovni cilj istraživanja bio je da se utvrdi uticaj rase na osobine ejakulata nerasta: volumen ejakulata (VOL, ml), koncentracija sperme (CON, x106 spermatozoida/ml), ukupan broj spermatozoida u ejakulatu (TNS, x109 spermatozoida) pokretljivost spermatozoida u nativnom ejakulatu (MON, %), pokretljivost spermatozoida nakon razređenja (MOD, %) i broj proizvedenih doza (NPD). Cilj je bio i da se oceni povezanost osobina iz performans testa nerasta: prosečan životni dnevni prirast (g), debljina slanine merena na dva mesta (mm), dubina dugog leđnog mišića (mm) i sadržaj mesa u trupu (%) sa osobinama ejakulata. Analiziran je 931 ejakulat od 36 nerasta tokom reproduktivne eksploatacije (16 nerasta landrasa i 20 nerasta velikog jorkšira). Procena uticaja izvršena je primenom procedure opšteg linearnog modela statističkog paketa SAS 9.1.3 (SAS Inst. Inc., 2002-2003). Model za analizu obuhvatao je uticaj rase, sezone i linearni regresijski uticaj telesne mase na kraju performans testa. Povezanost osobina utvrđena je primenom Pirsonovog koeficijenta korelacije. Većina ispitivanih osobina ejakulata (VOL, CON, MOD i NPD) varirala je pod uticajem rase nerasta ($p<0,01$; $p<0,001$). Masa na kraju testa ($p<0,05$; $p<0,01$; $p<0,001$) uticala je na sve ispitivane osobine, osim na CON i TNS. Između proizvodnih performansi i osobina ejakulata utvrđena je slaba povezanost.

Ključne reči: svinja, nerast, rasa, performans test, osobine ejakulata

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EFFECT OF REPLACEMENT OF SOYBEAN RESIDUES FOR GROUNDNUT CAKE ON CARCASS YIELD OF BROILER CHICKENS

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Abstract: A feeding trial was conducted with two hundred (200) Arbor Acre strain of broiler chicks to determine the effect of soybean residue (SBR) on carcass characteristics and visceral organs. Birds were fed for 28 days (starter phase) with formulated diet containing 22% crude protein and 3000Kcal/kg (ME). Five iso-nitrogenous experimental diets were formulated which contain SBR. In the control diet (T_1) groundnut cake was served as the only protein source. Four (4) test diets designated as: T_2, T_3, T_4 and T_5 were formulated with SBR replacing 25, 50, 75 and 100% of groundnut cake respectively in finisher phase. The birds were randomly grouped into five (5) experimental treatment groups in four (4) replicates of 40 birds per treatment in a completely randomized design. The finisher phase lasted for five (5) weeks and the birds were fed and given drinking water *ad-libitum*. All carcass characteristics evaluated differ significantly (P<0.05) among treatment groups except breast and wings. No significant (P>0.05) difference were shown for organs, while shanks was only the residue that was significantly affected by dietary treatment. Soybean residue can be used up to 100% to replace groundnut cake in the diet of broiler chickens thus providing a productive use for this hither to neglected agro allied waste.

Key words: Soybean residue, carcass yield, organs, offal, finisher phase.

Introduction

Soybean is a popular crop globally, which is usually processed for the extraction of soybean oil mainly used in food industries. After the extraction of the oil, the remaining mass obtained is the soybean meal which has a very high amount of nitrogen (*Chen et al., 2010).* This protein rich soybean meal is basically used as an animal feed for poultry and other livestock etc. Soybean residue a by-product of

soymilk, is produced in large volumes by the soy food industry and is often discarded due to its undesirable flavour. However, the use of the residue in the supplementation for other protein sources is of high importance in animal research (*Esonu, 2006*). The supply of animal protein in human diets is important since it provides essential amino acids (particularly lysine, methionine) and B-vitamins which the body cannot synthesize from vegetable sources (*Aduku and Olukosi, 2000*). The need to provide feed is basic to any livestock enterprise including poultry; however, making the feed cheaply available is more compelling to profitability and sustainable livestock development (*Ayuk et al., 2009*).

Ogundipe et al. (2002) and Tuleun et al. (2011) reported that, in most of the diets prepared for poultry, the conventional dietary source of lysine in the fish meal and soybean meal, when available, are very expensive. In the event of the global feed crisis therefore, the only better approach to solving the escalating prices of feed ingredients is the use of alternative feed ingredients to the conventional ingredients that can partly or wholly replace them without compromising on the health status and performance of the animals (Poultry).

The alternatives to the high cost conventional ingredients are the discovery, processing and harnessing of unconventional sources of poultry feedstuffs for which there is little or no competition from human (*Ogundipe et al., 1992*). Grains residue or wastes like soybean residue is cheaper and represent unutilized protein sources.

Soybean residue (SBR) has higher lysine content (2.8%) than groundnut cake (1.6%), (*Esonu, 2006*) and is a good source of protein which makes a good protein (44%) concentrate in poultry ration *Abimiku et al. (2017).*

The utilization of SBR as a feed resource may help in reducing the pressure on conventional feedstuffs, control environmental pollution caused as a result of indiscriminate discarding of waste. This study was designed to evaluate the effect of replacement of soybean residue for groundnut cake on carcass characteristics and visceral organs of broiler chickens.

Materials and Methods

Experimental Site

The feeding trial was carried out at the Livestock complex, College of Agriculture, Lafia in Nasarawa State of Nigeria. Lafia is located within the Guinea Savanna zone of Central Nigeria. The area is between latitude 07^0 $52^/$ N - 08^0 $56^/$ N and longitude 07^0 $25^/$ E - 90^0 $37^/$ E. The mean monthly temperature is between 20^0C and 34^0C, with the hottest months being March and April, and the coolest months being December and January *(Layam, 2000).*

Sources of experimental soy bean residues

Soybean residue a by-products of soya bean milk or soya bean cheese ("Awara") production which was collected from the producer in Lafia Local Government Area of Nasarawa state. Soybean seeds are soaked in water for about six to eight hours depending on the temperature of water. The rehydrated beans then undergo milling and filtering to obtain soybean residue and paste for making "Awara" (Soya cheese). The processing method used by the producers was presented in Fig 1. The wet (residue) material was collected and sun-dried to about 10% moisture. The extraction rate of soybean residue is about 0.52kg per 3kg of soybean seed processed into Awara or soymilk. The soybean residue was ground into 0.73mm as recommended by *Beneletti et al. (2011)* to obtain a suitable meal for chemical analysis and broiler chicken diets.

Experimental birds and management

A total of two hundred (200), 28 days old Arbor Acres strain broiler chickens were randomly allotted to five dietary treatment groups replicated four times with ten (10) birds each to give randomized complete design.

The experimental birds were fed a common broiler starter containing 22% CP and 300Kcal/kg (ME) for a period of 4 weeks. Five Iso-nitrogenous diets containing 20% CP broiler finisher were formulated with inclusion varying levels (0%, 25%, 50%, 75%, and 100%) of soybean. residue (Table 1). Feed and water were provided ad libitum. The feeding trial lasted for 35 days.

Data Collection

Parameters measured include carcass yield cuts, organs, and offal. The experiment lasted for thirty five (35) days. At the end of thirty-fifth day, four birds per treatment group were randomly removed and starved (feed only) for 18 hours and were used for carcass studies. Parameter evaluated were expressed as percentage of live weight which include:
  (a) Carcass yield cuts: thigh, drum stick, breast, back and wing
  (b) Organs: liver, gizzard, proventiculus, heart lungs spleen, bursa of fibricus and kidney.
  (c) Offal: head, nick shank and abdominal fat
Data collected were subjected to one-way analysis of variance (ANOVA) using SPSS (2010).
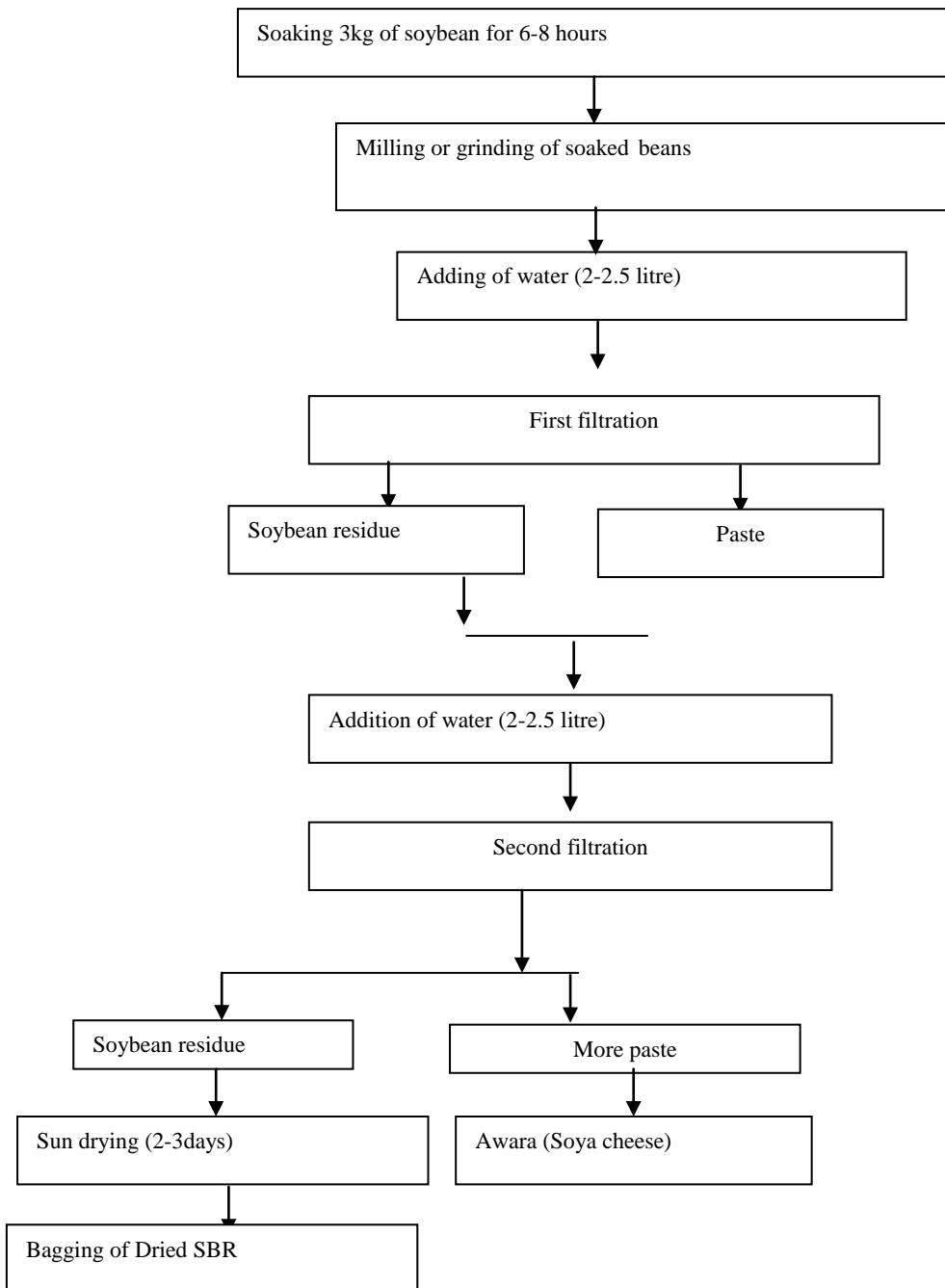
Figure 1. A Flow Chart Showing the Production of Soybean Residue.

Table 1. Composition of experimental diets for finisher broiler chickens (5 –9 weeks) containing graded levels of soybean residue as replacement for groundnut cake

Ingredients	Dietary levels of soybean residue				
	T_1 (0%)	T_2 (25%)	T_3 (50%)	T_4 (75%)	T_5 (100%)
Maize	42.92	42.92	42.92	42.92	42.92
Maize offal	14.30	14.30	14.40	14.30	14.30
GNC	31.78	23.84	17.89	7.94	0.00
SBR	0.00	7.94	17.89	23.84	31.78
Rice offal	5.00	5.00	5.00	5.00	5.00
Bone meal	2.00	2.00	2.00	2.00	2.00
Limestone	1.00	1.00	1.00	1.00	1.00
Palm oil	2.10	2.10	2.10	2.10	2.10
Premix'	0.25	0.25	0.25	0.25	0.25
Common Salt	0.25	0.25	0.25	0.25	0.25
Lysine	0.20	0.20	0.20	0.20	0.20
Methionine	0.20	0.20	0.20	0.20	0.20
Total	100	100	100	100	100

Calculated Nutrient

ME (Kcal/Kg)	2908	2942	2979	3014	2908
CP (%)	20.00	19.92	19.84	19.80	19.70
CF (%)	4.02	4.19	4.35	4.52	4.68
Ca (%)	1.16	1.17	1.17	1.19	1.19
P (%)	0.64	0.64	0.64	0.64	0.64
Lysine (%)	1.34	1.35	1.55	1.76	1.96
Methionine (%)	0.74	0.58	0.61	0.64	0.67
Feed cost Kg N/kg)	78.66	71.55	67.72	57.31	50.21

T_1 (0%) control diet, T_2 Diet contained 25% SBR, T_3 = Diet contained 50% SBR T_4 = Diet contained 75% SBR and T_5 = Diet contained 100% SBR GNC = Groundnut cake, SBR = Soybean residue, ME = Metabolizable energy. 'Vitamin – mineral premix (Biomix®) will supply per Kg diet, vit. A 500iU, vit. D_3 888iU, vit. E12, 000mg, vit. K_3 (500mg), niacin 12000mg, pantothenic acid 2000mg, Biotin 1000mg, vit. B_{12} 300mg, folic acid 1,500kg, choline, chloride 600mg, manganese 1000mg, iron 1,500mg, zinc 800mg, copper 400mg, iodine 80mg, cobalt 400mg and selenium 800mg. ME (kcal/ kg) = 35 x CP% + 81.8 x EE % + 35.5 x NFE % *(Pauzenga, 1985)*.

Table 2. Nutrient composition and metabolizable energy of soybean residue

Nutrient	% Content
Dry matter	71.50
Crude protein	44.00
Crude fibre	5.90
Nitrogen free extract	39.80
Ether extract	4.50
Total ash	5.80
Calcium	0.30
Phosphorus	0.60
*Energy (KcalME kg^{-1})	2973.10

*Metabolizable energy (kcalkg^{-1}) calculated by method described by *Pauzenga, 1985*

Statistical analysis

All statistical analyses was done using standard spreadsheet software of excel and one-way analysis of variance of the Statistical Package for Social Sciences (SPSS version 22). The level of statistical significance was defined as (P<0.05). Fisher's Least Significance difference (LSD) was used for separating the treatment means.

Results and Discussion

The mean relative weight of all carcass cuts: thigh, drum stick, breast, back and wing were expressed as percentage (%) of live weight (Table 3). The carcass yield showed significant difference (P<0.05) among treatment means for thigh, drum stick and back. However, there was no particular pattern of variation across the dietary groups. The highest thigh value was observed in T_3 (11.14%) and the lowest in T2 (8.74%).

The result of the current study is in congruous with the work of *Lukić et al. (2012)* whose results indicate that there were no statistically significant differences in carcass quality between the control and trial groups, in regard to obtained dressing percentages (yields) as well as the amount of abdominal fat in broiler carcass. *Perić et al. (2018)* reported significant difference in the carcass qualities however their report is not in congruous with the result of this finding.

Dosković et al. (2012) reported that the use of different protein levels and enzyme supplementation in broiler diet showed no statistical significance (P>0.05) in dressing percentage of conventionally dressed carcass and percentage of abdominal fat between the experimental groups and it sagree with the result of this findings.

Petričević et al. (2015) have found significantly lower values of carcass yield with an increase in the share of raw soybean in the final mixtures for chickens. Comparing raw and heat-treated soy in the chicken diet, *Beuković et al. (2012)* have found statistically significantly higher yield in case of conventional carcass dressing, carcass „ready to roast" and „ready to grill" and share of breast in the carcass of chickens fed heat-treated soybeans compared to raw soybeans. These results also agree with the findings of *Mustafa et al. (2012)* who fed broilers with diets including different percentage of animal protein and plant protein sources and obtained significant (P < 0.05) effects on their carcass yield.

Table 3. Effect of experimental diet containing soybean residue for groundnut cake on carcass characteristics of broiler chickens

Carcass indices	$T_{1(0\%)}$	$T_{2(25\%)}$	$T_{3(50\%)}$	$T_{4(75\%)}$	$T_{5(100\%)}$	SEM
	Experimental diets		
Final live weight (g)	2105.00[ab]	1995.00[b]	2287.50[a]	2062.50[ab]	2025.00[ab]	41.72
Dressed wt (g)	1762.50[ab]	1600.00[bc]	1895.00[a]	1450.00[c]	1675.00[ab]	44.33*
Dressing (% of LW)	84.49[a]	79.95[ab]	83.33[a]	70.42[b]	81.85[ab]	3.70*
Thigh (% of LW)	10.22[ab]	8.74[b]	11.14[a]	10.37[ab]	9.66[ab]	0.31*
Drumstick (%of LW)	27.98[ab]	22.52[b]	28.17[ab]	29.79[a]	24.67[ab]	1.04*
Breast (% of LW)	28.61	23.48	33.44	23.29	24.77	1.57[ns]
Back (% of LW)	14.49[a]	14.55[a]	13.77[ab]	12.50[ab]	11.84[b]	0.37*
Wing (% of LW)	8.42	8.11	9.04	8.74	8.52	0.18[ns]

*=(p<0.05), a,b,c Means on the same row with different superscripts are significantly different (P<0.05), ns = (P>0.05), SEM = Standard error of mean, T_1 = Control diet, T_2= Diet containing 25% of soybean residue, T_3 = Diet containing 50% of soybean residue, T_4 = Diet containing 75% of soybean residue, T_5 = Diet containing 100% of soybean residue, % LW = Percent of live weight.

The values obtained for organs were not significantly altered by the dietary treatments (Table 4). This suggests that the processing method in the study i.e. soaking of SBR was adequate and efficient to reduce the anti-nutritional factors in soybean to the required level for raising poultry. This agrees with earlier report by *Church and Pond (1988)* that feeds may be processed to alter the physical form of particle size, to isolate specific parts to preserve, to improve palatability or digestibility and to improve nutrient composition. It is common practice in feeding trials to use weights of some internal organs like liver and kidney as indicators of toxicity. *Bone (1979)* reported that if there was any toxic element in the feed, abnormalities will be observed in the weight of liver and kidney.

Table 4. Effect of experimental diet containing soybean residue for groundnut cake on internal organs of broiler chickens

Internal organs (% of LW)	Experimental diets					SEM
	$T_{1(0\%)}$	$T_{2(25\%)}$	$T_{3(50\%)}$	$T_{4(75\%)}$	$T_{5(100\%)}$	
Liver	1.73	1.44	1.25	1.81	1.73	0.10^{ns}
Gizzard	2.40	2.57	2.36	2.65	2.86	0.12^{ns}
Proventriculus	0.57	0.44	0.51	0.55	0.56	0.02^{ns}
Heart	0.40	0.35	0.42	0.40	0.36	0.01^{ns}
Lungs	0.47	0.54	0.60	0.58	0.61	0.03^{ns}
Spleen	0.70	0.55	0.54	0.56	0.69	0.04^{ns}
Bursa of fibricus	0.09	0.12	0.09	0.12	0.08	0.01^{ns}
Kidney	0.13	0.08	0.09	0.07	0.08	0.01^{ns}

ns = Not significant (P>0.05), SEM = Standard error of mean, T_1 = Control diet, T_2= Diet containing 25% of soybean residue, T_3 = Diet containing 50% of soybean residue, T_4 = Diet containing 75% of soybean residue, T_5 = Diet containing 100% of soybean residue, % LW = Percent of live weight.

The mean value of offals namely head, neck, shank, and abdominal fat were not significantly influenced by dietary treatments except the shank (Table 5). Though the result showed significant difference in shank, the values obtained did not follow any particular trend.

The test ingredient (SBR) in this trial did not impact any negative effect on carcass yield of finisher broiler chickens.

Table 5. Effect of experimental diet containing soybean residue for groundnut cake on carcass offals of broiler chickens

Carcass offal (% of LW)	Experimental diets					SEM
	$T_{1(0\%)}$	$T_{2(25\%)}$	$T_{3(50\%)}$	$T_{4(75\%)}$	$T_{5(100\%)}$	
Head	2.53	2.70	2.85	2.53	2.74	0.07^{ns}
Neck	4.31	3.66	2.62	3.44	3.35	0.24^{ns}
Shank	3.55^{ab}	3.01^{b}	3.71^{a}	3.67^{a}	3.56^{ab}	0.10^{*}
Abdominal fat	2.23	2.46	1.50	1.50	1.94	0.17^{ns}

*=(p<0.05), a,b, Means on the same row with different superscripts are significantly different (P<0.05), ns = (P>0.05), SEM = Standard error of mean, T_1 = Control diet, T_2= Diet containing 25% of soybean residue, T_3 = Diet containing 50% of soybean residue, T_4 = Diet containing 75% of soybean residue, T_5 = Diet containing 100% of soybean residue, % LW = Percent of live weight.

Conclusion

The outcome of this study showed that Soybean residue can be used up to 100% to replace groundnut cake in the diet of broiler chickens thus providing a productive use for this hither to neglected agro allied waste.

Efekat zamene rezidua soje sa pogačom od kikirikija na prinos trupa brojlerskih pilića

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Rezime

Sprovedeno je istraživanje ishrane sa dve stotine (200) pilića brojlera Arbor Acre kako bi se utvrdio efekat rezidua soje (SBR) na karakteristike trupa i unutrašnje organe. Ptice su hranjene 28 dana (početna faza) formulisanom hranom koja sadrži 22% sirovih proteina i 3000Kcal/kg (ME). Formulisano je pet izoazotnih eksperimentalnih obroka koji sadrže SBR. U kontrolnoj ishrani (T1) pogača od kikirikija služila je kao jedini izvor proteina. Četiri (4) ogledne ishrane/obroka, označene kao: T2, T3, T4 i T5, formulisane su sa SBR koji je zamenio 25, 50, 75 i 100% pogače od kikirikija u finišer fazi. Pilići su nasumično grupisani u pet (5) eksperimentalnih grupa u četiri (4) ponavljanja, 40 pilića po tretmanu, u potpuno slučajnom dizajnu. Finišer faza je trajala pet (5) nedelja i ptice su hranjene i dobijale pitku vodu *ad-libitum*. Sve procenjene karakteristike trupa značajno se razlikuju (P<0,05) među grupama tretmana, osim grudi i krila. Nije utvrđena značajna razlika (P>0,05) za organe. Rezidue soje mogu se koristiti do 100% da zamene pogaču od kikirikija u ishrani pilića brojlera, pružajući na taj način produktivnu upotrebu zanemarenom srodnom otpadu.

Ključne reči: rezidue soje, prinos trupa, organi, iznutrice, finišer faza

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Conflict of interest
Authors have affirmed that no competing interests exist regarding the manuscript

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SERUM BIOCHEMICAL PROPERTIES OF VIETNAMESE INDIGENOUS NOI CHICKEN AT 56 DAYS OLD

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Abstract: Serum profile is generally considered as comprehensive data which directly reflected animal health and their potential resistance to environmental, nutritional and pathological stress. The present study aimed to provide physiological reference values for selected biochemical parameters in Noi chickens, a famous Vietnamese native breed, at 56 days old. The collected blood samples of 355 Noi birds (164 males and 191 females) were used to evaluate biochemical serum profile. Glucose, total protein, albumin, globulin, albumin/globulin ratio, triglycerides, cholesterol, creatinine, and uric acid in the serum were measured. The variation in these values arising from different sexes was also investigated. The results indicated that there were no differences (P>0.05) in the assessed indices between male and female Noi chickens. In overall for the whole group, these values were obtained as 250.86 mg/dL, 7.34 g/dL, 3.40 g/dL, 3.94 g/dL, 1.75, 148.54 mg/dL, 190.86 mg/dL, 0.44 mg/dL, and 1.44 mg/dL, respectively. The relationship among the biochemical parameters showed relatively low coefficients (r=0.79 in maximum). The females obtained higher correlation coefficients between levels of total protein and glucose (r=0.42) as well as between total protein and globulin (r=0.79), compared to the males. It was concluded that the male and female chickens performed similar values of serum indices. The

contribution of this study might expand the knowledge on the biochemical profile and improved breeding strategies of Vietnamese indigenous Noi chickens.

Key words: Noi chickens, biochemical parameters, serum indices, correlation coefficients.

Introduction

Indigenous chickens are an important source of high quality protein, accounting for the majority of the local production of chicken meat as well as contributing to food security in the rural and peri-urban areas (*Assan, 2015*). Local population of Noi chickens which has been long-established in Vietnam, is known to be well-adapted to the harsh environment conditions (*Quyen, 2008*). The growth performances and some quantitative genetic traits of this breed were reported by various researchers. However, these traits are greatly influenced by sex, age and season (*Giang et al., 2018; Giang et al., 2019; Khoa et al. 2019a,b*). At the stage of 0-28 days old, Noi chick daily consumed 16.54 g of feed to gain a weight of 6.98 g /day, with the feed conversion ratio was 2.37. Their skeleton (wings and keel lengths) rapidly developed within the first week of life, followed by the development of muscle tissues (breast and thigh diameters) in the second week of age (*Khoa et al., 2019a*). At latter stage of age (28-84 days old), males and females of Noi chickens performed significant differences in feed intake, body weight, and some morphological dimensions, such as lengths of beak, thigh, shank, as well as diameters of breast and thigh (*Khoa et al., 2019b*). According to *Giang et al. (2018, 2019)*, Noi chickens were adapted and grew well in both semi-intensive backyard and confined systems. After 14 weeks of age, the chickens weighed at 1,779 g/bird and gained around 17.82 g/day in the whole period. Males presented higher yield of carcass but there are no significant differences in carcass ratio as well as the proportion of other edible parts between males and females. At the stage of 0-28 days old, Noi chick daily consumed 16.54 g of feed to gain a weight of 6.98 g /day, with the feed conversion ratio was 2.37. Their skeleton (wings and keel lengths) rapidly developed within the first week of life, followed by the development of muscle tissues (breast and thigh diameters) in the second week of age (*Khoa et al., 2019a*).

Wide spread distribution of Noi chickens demonstrates a need to improve appropriate breeding approaches based on their morphological and physiological characteristics. Blood haematological and biochemical profile is generally considered as a comprehensive data and an ideal indicator of production and health status of many species. Changes in haematological and biochemical traits, in turn, directly reflected potential resistance of animal to environmental, nutritional and pathological stress (*Chikumba et al., 2013; Shanmugam et al., 2017; Kongroi and*

Likitdacharote, 2019). These indicators are different among bird genotypes, sex, age, nutrition, stocking density and other stress-creating factors (*Pires et al., 2007; Ibrahim et al., 2012; Khawaja et al., 2013; Attia et al., 2017; Parveen et al., 2017; Mosleh et al., 2018*). Although the reference ranges for avian haematological and biochemical profiles has been well published, mainly in commercial broilers and layers (*Gyenis et al., 2006; Tabeekh, 2016; Adeleyea et al., 2018; Al-Rubaee, 2018*), there are still limited data on the biochemical profile of Vietnamese indigenous Noi chicken breed. This study, therefore, aimed at determining normal baseline values for some biochemical parameters in male and female Noi chickens at 56 days of age.

Materials and Methods

A total of 355 broilers (164 males and 191 females) of Noi breed were used in the study. The birds were raised at the Can Tho Center for Breeds of Seed, Livestock and Fish as previously described by *Khoa et al. (2019a; 2019b)*. At the time point of 56 days old, approximately 3.0 mL blood specimens were taken from the brachial vein of each bird by using a syringe needle and gently mixed in a heparinized tube. The samples were, then, kept on ice in a cool container to avoid protein denaturation. All they were used to analyze serum biochemical properties such as glucose, total protein, albumin, globulin, triglycerides, cholesterol, creatinine, and uric acid within 2 hours after sampling by using the Biochemical Systems 3000 Evolution (Biomedical Systems International-BSI, Italia) with chemicals and kits of Erba Mannheim (Germany) according to theirs protocols and instructions at Veterinary Clinic, Can Tho University. The ratio of albumin to globulin (A/G) was calculated by dividing the concentration of albumin and globulin fractions.

Briefly, serum was separated by centrifuging at 900 rpm for 2 minutes and stored in the freezer at $-20^{o}C$ for further analysis. The serum was allowed to thaw under room temperatures before subsequent analyses.

The different biochemical parameters in male and female chickens were statistically analyzed using GLM procedures within Minitab 16 software. Data were expressed as means ± standard deviation (x ± SD) for each sex. The means were considered significant when the P-value were less than 0.05. Pearson's correlation coefficients (r) were used to evaluate the relationship among the biochemical parameters of Noi chickens.

Results and discussion

Biochemical Parameters

The concentration of different serum constituents is shown in Table 1. There were no differences (P>0.05) in glucose, total protein, albumin, globulin,

albumin/globulin ratio, triglycerides, cholesterol, creatinine, and uric acid and between male and female Noi chickens. In overall for the whole group, these values were obtained as 250.86 mg/dL, 7.34 g/dL, 3.40 g/dL, 3.94 g/dL, 1.75, 148.54 mg/dL, 190.86 mg/dL, 0.44 mg/dL, and 1.44 mg/dL, respectively. The main metabolite of animal metabolism is glucose, which is the primary energy substrate for animal activities, especially for the brain functions, is stored as glycogen in 1 - 5% in liver 1% in muscles (*Fuller, 2004*). In this study, sex did not affect the levels of serum glucose. However, the mean values for serum glucose was within the normal range as earlier reported by *Dong et al. (2015)* and *Kalita et al. (2018)*. It also found that commercial broiler chickens had a significantly higher glucose content (253.28 mg/dL) than that of indigenous chickens (210.55 mg/dL) which might be due to the consumption of rich-grain diet (*Kalita et al., 2018*).

Table 1. Difference of observed between genders

Traits	**Male** (n = 164)	**Female** (n = 191)	SEM	P	Overall
Glucose (mg/dL)	249.01 ± 45.23	252.49 ± 45.12	3.41	0.471	250.86 ± 45.14
Total protein (g/dL)	7.25 ± 1.54	7.41 ± 1.47	0.11	0.317	7.34 ± 1.50
Albumin (g/dL)	3.33 ± 1.28	3.46 ± 1.19	0.09	0.316	3.40 ± 1.23
Globulin (g/dL)	3.93 ± 1.72	3.95 ± 1.93	0.14	0.886	3.94 ± 1.84
A/G ratio	1.64 ± 3.42	1.85 ± 6.28	0.39	0.701	1.75 ± 5.11
Triglycerides (mg/dL)	148.34 ± 14.74	148.72 ± 14.48	1.10	0.805	148.54 ± 14.58
Cholesterol (mg/dL)	190.27 ± 45.14	191.39 ± 44.85	3.39	0.815	190.86 ± 44.93
Creatinine (mg/dL)	0.45 ± 0.10	0.44 ± 0.10	0.01	0.537	0.44 ± 0.10
Uric acid (mg/dL)	1.45 ± 0.49	1.43 ± 0.43	0.03	0.729	1.44 ± 0.46

A/G: Albumin/Globulin ratio.
Values bearing different superscripts within sex differed significantly ($P<0.05$).

While the total protein is one of the criteria to identify the status of the animal body, serum protein that plays a key role in maintaining the osmotic pressure, is considered as an immediate alternative source of essential amino acids. Level of serum albumin also directly contributes to the process of tissue regeneration in the animal growth stage. The level of total protein found in Noi chickens (7.25-7.41 g/dL) is similar to that shown in five breeds of Nigerian indigenous chickens (6.43-7.93 g/dL) reported by *Ibrahim et al. (2012)* but higher than those shown in Ross 308 (3.2-3.4 g/dL) (*Al-Rekabi et al., 2018*) and in local Saudi chickens during summer season (3.3-3.8 g/dL) (*Albokhadaim, 2012*). As shown in Table 1, the total protein of female Noi birds was comparable to males. The result is disagreeing with finding of *Simaraks et al. (2004)*, who found that total protein of female Thai indigenous chickens was higher than that in males.

The total serum or plasma protein and its fractions can be extremely variable in avian species. Changes in the levels of the fractions depend on both internal and external, revealing the physiological role of total protein (*Tóthová et al., 2019*). In term of quality, albumin is the most important protein in the serum or plasma and therefore, this is the favourable source of essential amino acids for the tissue synthesis to increase body mass, particularly during fattening (*Filipović et al., 2007; Tóthová et al., 2019*). The present study on Noi chickens revealed a relatively constant level of serum albumin between males and females (P>0.05), ranging from 3.33 to 3.46 g/dL (Table 1). According to *Kalita et al. (2018)*, serum albumin might increase when the amount of protein exceeds the amount required for growth and maintenance. Globulin level has been used as indicator of immune responses and sources of antibody production (*Tothova et al., 2016*). The similar values in serum globulin found in males and females suggest that both sexes have the similar health status at the age examined. The higher level of globulin concentration (3.94 g/dL) might confer higher capacity in disease resistance of indigenous Noi chickens compared to broiler in other studies (*Dong et al., 2015; Rasheed and Olusegun, 2017; Al-Rubaee, 2018*). The changed proportion of albumin and globulin in the evaluated sex groups of Noi chickens was reflected in the changed A/G ratio with higher value was found in the females (1.85) compared to the males (1.64). However, the difference was not significant (P>0.05).

Lipid metabolites are strongly associated to energy metabolism. According to *Attia et al. (2017)*, level of plasma cholesterol of slow-growing chickens was decreased by feeding restriction regime (85% and 70 % of the energy compared to the control diet). The concentration of serum triglycerides and cholesterol of Noi chickens was 148.54 mg/dL and 190.86 mg/dL, respectively, with no significant difference between sexes (Table 1), similar to the report in pheasant by *Simaraks et al. (2004).* However, these levels were higher than those in the indigenous Iran chickens (152.60-167.60 mg/dL) (*Abdi-Hachesoo et al., 2011*). All chickens in this study were fed by the same diets which might result in the similar values of triglycerides and cholesterol in both sexes. *Saklani et al. (2019)*, on contrary observed significant difference in male and female birds of native chicken of Himachal Pradesh.

Like urea, creatinine, a waste substance generated from the muscle metabolism, which range of normalcy is substantially maintained by kidney (*Wyss and Kaddurah-Daouk, 2000*). The interaction of sex on the serum creatinine is not significant (P>0.05) and in overall the creatinine of Noi chickens valued at 0.44 mg/dL (Table 1), suggesting a better utilization of protein in the diet. Different from mammals, in birds, uric acid, the main end-product of protein and purine metabolism, is excreted via faeces. It is relatively less toxic in comparison to ammonia and urea (*Barsoum and El-Khatib, 2017*). The results showed sex did not affect (P>0.05) the concentration of serum uric (1.45 mg/dL in males *vs.* 1.43 mg/dL in females). Contradictory result is found by *Simaraks et al. (2004)*, who

found that sex may result in the increased concentration of serum uric in Thai indigenous birds. The disagreement with these previous studies may be due to difference in the genetic variation among breeds and ages of the broiler chickens used in the current experiment.

Table 2. Pearson's correlation coefficient (r) among the biochemical parameters of Noi chickens at 56 days of age

Traits	Glucose	Total protein	Album.	Globulin	A/G ratio	Triglycer.	Cholester.	Creatinine	Uric acid
Male and female animals									
Glucose	**1.00**	0.36***	-0.03[NS]	0.34***	-0.16*	0.20**	-0.06[NS]	0.02[NS]	0.10[NS]
Total protein	0.42***	**1.00**	0.26***	0.69***	-0.17*	0.39***	-0.02[NS]	0.01[NS]	0.24**
Albumin	-0.06[NS]	-0.05[NS]	**1.00**	-0.51***	0.52***	0.24**	0.12[NS]	-0.04[NS]	0.13[NS]
Globulin	0.36***	0.79***	-0.65***	**1.00**	-0.54***	0.17*	-0.12[NS]	0.04[NS]	0.12[NS]
A/G ratio	-0.14[NS]	-0.24***	0.22**	-0.32***	**1.00**	0.01[NS]	0.14[NS]	-0.05[NS]	0.03[NS]
Triglycerides	0.27***	0.24***	0.09[NS]	0.13[NS]	-0.01[NS]	**1.00**	0.05[NS]	-0.16*	0.20**
Cholesterol	0.24***	0.13[NS]	0.05[NS]	0.06[NS]	0.04[NS]	0.23***	**1.00**	0.02[NS]	0.04[NS]
Creatinine	-0.10[NS]	-0.18**	0.14*	-0.22**	0.05[NS]	-0.20**	0.06[NS]	**1.00**	-0.04[NS]
Uric acid	0.32***	0.18**	-0.16*	0.23***	-0.02[NS]	0.02**	0.08[NS]	-0.08[NS]	**1.00**
Overall (male + female)									
Glucose	**1.00**								
Total protein	0.39***	**1.00**							
Albumin	-0.04[NS]	0.11*	**1.00**						
Globulin	0.35***	0.74***	-0.58***	**1.00**					
A/G ratio	-0.14**	-0.21***	0.30***	-0.37***	**1.00**				
Triglycerides	0.24***	0.31***	0.16**	0.14**	-0.001[NS]	**1.00**			
Cholesterol	0.10[NS]	0.06[NS]	0.09[NS]	-0.01[NS]	0.07[NS]	0.14**	**1.00**		
Creatinine	-0.05[NS]	-0.09[NS]	0.05[NS]	-0.11*	0.02[NS]	-0.18***	0.04[NS]	**1.00**	
Uric acid	0.21***	0.21***	-0.01[NS]	0.18***	-0.004[NS]	0.20***	0.06[NS]	-0.06[NS]	**1.00**

A/G: Albumin/Globulin ratio.
Values bearing different superscripts within sex differed significantly ($P<0.05$).
*=significant at $P<0.05$, **=significant at $P<0.01$, ***=significant at $P<0.001$, [NS]= non-significant.
Male: above diagonal line break; Female: below the diagonal break.

Correlation of the biochemical parameters

The relationship among the biochemical parameters are presented in Table 2, showing relatively low coefficients (0.79 in maximum). Although sex was

unclear factor, female chickens obtained higher coefficients between levels of total protein and glucose (r=0.42, P<0.001) as well as between total protein and globulin (r=0.79, P<0.001), compared to the males. It also showed that Tables 2 also revealed the correlations among the serum characteristics in overall for both sexes. In this study, it was not noticed any significant relationships among the values of A/G ratio, cholesterol and creatinine. However, it is found that the serum globulin was possibly correlated to the total protein (r=0.74, P<0.001), rather than the albumin levels (r=0.11, P<0.05). There was a positive correlation between A:G ratio and albumin (r=0.30, P<0.001), demonstrating that the decrease in albumin is also related to decrease in A:G ratio. On the contrary, globulin was negative correlate to the A/G ratio (r=-0.37, P<0.001).

Conclusion

The results provide new information about selected biochemical parameters in clinically. The results suggested that sex of Noi chickens was not influence the metabolic values. Therefore, these biochemistry values could be used as additional information for evaluation of Noi chickens.

Biohemijska svojstva seruma vijetnamske autohtone rase živine Noi u uzrastu od 56 dana

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Rezime

Profil seruma se obično smatra sveobuhvatnom informacijom koja direktno odražava zdravstveno stanje životinja i njihovu potencijalnu otpornost na okruženje, prehrambene i patološke stresove. Ova studija imala je za cilj da pruži fiziološke referentne vrednosti za odabrane biohemijske parametre kod pilića Noi, poznate nativne rase iz Vijetnama, u uzrastu od 56 dana. Sakupljeni uzorci krvi 355 pilića Noi (164 muških i 191 ženskih grla) korišćeni su za procenu biohemijskog profila seruma. Izmereni su glukoza, ukupni protein, albumin, globulin, odnos albumina i globulina, trigliceridi, holesterol, kreatinin i mokraćna kiselina u serumu. Takođe je ispitivana varijacija u tim vrednostima kod različitih polova.

Rezultati su pokazali da nisu postojale razlike (P> 0,05) u procenjenim indeksima između muških i ženskih pilića Noi. Generalno, za celu grupu, dobijene su sledeće vrednosti: 250.86 mg/dL, 7.34 g/dL, 3.40 g/dL, 3.94 g/dL, 1.75, 148.54 mg/dL, 190.86 mg/dL, 0.44 mg/dL, i 1.44 mg/dL, respektivno. Odnos među biohemijskim parametrima pokazao je relativno niske koeficijente (r = 0,79 maksimalno). Ženska grla su imala veće koeficijente korelacije između nivoa ukupnog proteina i glukoze (r = 0,42), kao i između ukupnog proteina i globulina (r = 0,79), u poređenju sa muškim pilićima. Zaključeno je da su muški i ženski pilići imali slične vrednosti indeksa u serumu. Doprinos ove studije mogao bi proširiti znanje o biohemijskom profilu i poboljšati strategije uzgoja autohtonih vijetnamskih Noi pilića.

Ključne reči: Noi pilići, biohemijski parametri, indeksi seruma, koeficijenti korelacije.

Acknowledgements

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THE EFFECTS OF DIFFERENT LEVELS OF PROTEIN AND SILYMARIN ON THE POPULATION GROWTH AND HYPOPHARYNGEAL GLAND SURFACE OF HONEY BEE WORKERS (*Apis mellifera meda*)

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Abstract: A pollen substitute is a valuable resource to maintain bee colonies strong and healthy, in the absence of pollen in sufficient quantities in nature. Hence, the current study was performed to investigate the effects of different levels of dietary proteins and silymarin (SM) as a natural antioxidant, on honey bee worker colonies. The study was carried out as a completely randomized design in an experiment conducted using 36 honey bee colonies in a completely randomized design with nine experimental treatments (four levels of crude protein 0, 20, 30 and 40%, two levels of silymarin 0 and 0.2 mM and pure pollen (control)), and four replications at Agricultural Sciences and Natural Resources University of Khuzestan in autumn 2015. In current study parameters such as workers in sealed broods, newborn workers bee weight, body protein and, the amount of development of Hypopharyngeal gland surface were studied. Soybean meal, maize and wheat gluten were included as pollen substitutes. Based on the results of the pre-experiment, SM supplement at a dose of two-tenths mM improved the survival of bees. Capped broods number using the divided box into squares with 2×2 cm, newborn workers bee weight using the balance, body protein based on the percentage of body weight and development of the Hypopharyngeal gland surface using microscopes and micrometers were measured. The results showed that there was a significant difference between treatments in terms of laying eggs ($P < 0.05$). The highest and lowest rate of workers in sealed broods were related to treatment containing 30% protein and SM (12467 cells) and sucrose treatment (2042 cells), respectively. Also, the highest and lowest newborn workers bee weight were related to pollen treatment and the sucrose treatment,

respectively (P <0.05). Body protein of worker bees in studied treatments had significant differences (P <0.05), so that the widest and narrowest percent body protein were observed in a diet containing 30% protein and SM and sucrose treatment, respectively. The Hypopharyngeal gland surface in the colonies fed with different diets was significantly different (P<0.05) and the widest and narrowest of its surface were observed in the diet containing 30% protein and SM and sucrose treatment, respectively. According to the current results, to maximize the reproduction of bees, a diet containing 30% crude protein is proposed.

Keywords: antioxidants, diet protein, pollen substitute, longevity, sealed broods.

Introduction

According to the study of *van Engelsdorp et al. (2009)* honey bees population as major pollinators of many edible plants have declined. A poor diet, due to land use changes reducing the availability and diversity of floral resources, might help drive these declines *(Vanbergen and Initiative, 2013)*. In keeping with this idea, nutrition is a key determinant of honeybee survival *(Altaye et al., 2010; Archer et al., 2014b)*. Understanding the association between diet and honeybee survival is essential to protect declining and threatened honeybee populations *(Pirk et al., 2014)*.

Oxidative stress suppresses animal health, performance, and production, subsequently impacting economic feasibility; hence, maintaining and improving oxidative status especially through natural nutrition strategy is essential for normal physiological process in animals *(Li et al., 2012)*. Antioxidants, which neutralize Reactive Oxygen Species (ROS), help to maintain this balance and a poor diet could, in theory, disrupt it. Diets, high or low in proteins could push cells into oxidative stress by increasing ROS production from the mitochondria, impairing antioxidant defenses against ROS or reducing the repair of oxidized molecules *(López-Torres and Barja, 2008)*. Silymarin (SM) as a natural antioxidant can be considered as one of the most promising materials used in animal diets in various forms *(Surai, 2015)*. SM is the bioactive extract from *Silybum marianum* L. seeds (*Asteraceae*) and contains 65-85% flavonolignans like silychristin, isosilychristin, silydianin, silybin A and B, isosilybin A and B, and also 20- 35% fatty acids, flavonoids, and other polyphenolics. The major source of SM is fruits and seeds from this plant, but traces of these compounds can occur in all plant parts *(Ramasamy and Agarwal, 2008)*.

Honey bee workers start to consume pollen just a few hours after emerging *(Hagedorn and Moeller, 1967; Dietz, 1969)* and enough supply of proteins particularly during the first two weeks after emergence is required to sustain their

normal growth and development, and for them to be able to rear larvae *(Haydak, 1963)*. Pollen as a natural and protein-rich food source for honey bees *(Schäfer et al., 2006)* is essential for the production of royal jelly; a high-protein food used to feed bee larvae and adult queens *(Crailsheim, 1992)*. Royal jelly is secreted by two hypopharyngeal glands (HPGs), situated in the head, reaching maximum development in nurse bees, around 6–12 days after emergence, and afterward degenerate in forager bees *(Deseyn and Billen, 2005)*. A decline in accessibility and proteins content of bee-collected pollen might result in the lower development of HPGs *(DeGrandi-Hoffman et al., 2010; Di Pasquale et al., 2013)*, less brood reared *(Herbert et al., 1977; DeGrandi-Hoffman et al., 2008)*, shorter longevity *(Schmidt et al., 1987; Di Pasquale et al., 2013)* and recruitment of bees at a young age *(Sagili and Pankiw, 2007)*, eventually entailing a decreased lifespan *(Khoury et al., 2011)*.

During times of pollen scarcity, the pollen reserves in the combs and protein reserves in bees are quickly expended. Consequently, supplementary pollen or pollen substitutes are needed to preserve the colony's strength for pollination services or honey production *(Herbert et al., 1977)*. The availability of pollen depends on the plants' growing seasons during the year. Brood production could decrease or even stop completely in the colonies if there is no pollen or not available at desirable pollen substitute.

The pollen might contain spores of pathogens causing diseases such as chalk brood *(Flores et al., 2005)* or American foulbrood in bees and larvae. Provision of artificial diets is a safe way to feed bees protein. Beekeepers often provide pollen substitute diets to colonies, although these are often formulated without considering the costs of the diet components versus the benefits of providing such diets *(Herbert et al., 1977; Li et al., 2012; Morais et al., 2013)*.

This study aims to investigate the effects of dietary proteins levels and antioxidants (silymarin) on honey bee colonies, especially the population growth, hypopharyngeal gland, emergent weight and proteins content of honey bee workers during fall.

Material and methods

Experiment 1: Evaluation of silymarin dose on the survival of honey bee workers fed with sucrose solution

To identify an appropriate dose of SM supplementation, a pre-trial was conducted based on completely randomized design with six treatments and three replications in the cage rearing and an incubator. The cages volume was 2000 cm3 (12.5 × 10.7 × 15 cm), enclosed by wire mesh on one side. They contained two larger holes for inserting 40.0 ml Eppendorf tubes, each with fifteen small holes

through which bees could feed. One of these tubes was used to provide water and the other was used to provide one of the 6 liquid diets. We established three replicate cages, for each of the six treatments and three colonies. In this experiment, survival was monitored for six weeks. Brood frames were collected from three different colonies at the Department of the honey bee, Animal Sciences Research Institute of Iran and incubated at 34°C in constant darkness. On the day of their emergence from the brood comb, freshly emerged (<24 h) honey bee workers were caged in groups of 100 individuals. Each group received a diet consisting of 0.68 M sucrose solution and one of six SM doses (0, 0.2, 0.4, 0.6, 0.8 and 2.4 mM). Diets were made every week to ensure that the silymarins did not deteriorate and were frozen in aliquots at -20 °C and defrosted on the day of use. Each colony received all six diets; therefore, a total of 18 groups were fed in standard laboratory hoarding cages *(Köhler et al., 2013)* following standard procedures *(Köhler et al., 2012)*. The liquid diet and water were provided fresh daily when survival was also measured and dead bees removed from cages.

Experiment 2: Evaluation of different levels of protein and silymarin on physiological processes

The current study was performed at Agricultural Sciences and Natural Resources University of Khuzestan, Ahwaz, Iran from November to February 2015. A set of equalized honey bee *(Apis mellifera Meda)* colonies was selected during the fall. The colonies were randomly assigned to different protein-level treatments. Before the experiment, each colony consisted of a sorority 6-month-old queen that had been reared and mated naturally and the same quantity of bees. Three empty comb frames were applied for each brood chamber, and two comb frames were full of honey. According to *Burgett (1985),* the population bees were evaluated. To prevent pollen from entering the ventilation, all of the hives were installed with a pollen trap.

Experimental diets

The effects of four diets containing different levels of crude protein (CP) were tested (Table 1). The dietary formulas and the approximate compositions of four isocaloric test diets (diet 1-diet 4); two levels of SM (0 or 0.2 Mm). The CP of the diets was measured using the Kjeldahl nitrogen procedure. The experiment was consisted of testing 32 colonies (8 diets by four colonies). The defatted soybean meal, corn gluten, wheat gluten and mixed pollen components of the diet were sieved through a 185 μm mesh. The diets were supplemented by providing the colonies with prepared feed patties weighing 400 g each consisting of the dry

feeds. The patties were wrapped in ordinary waxed paper and placed on top of the frames over the brood clusters. Also, the honey and patties were checked every 3-4 d, and new honey and patties were supplied when the levels were inadequate.

Table 1. Ingredients and chemical compositions of the experimental diets (on dry matter basis)

Ingredients	Dietary proteins levels				
Ingredients (%)	0% (diet 1)	20% (diet 2)	30% (diet 3)	40% (diet 4)	Control diet (control)
Mixed pollen	0	5.00	5.00	5.00	100
sucrose	100	65.0	49.5	33.5	0
Wheat gluten	0	10.0	15.1	20.5	0
Corn gluten	0	10.0	15.2	20.5	0
Soybean meal	0	10.0	15.2	20.5	0
Total Proximate analysis	0	100	100	100	100
Dry matter (%)	99.5	96.4	95.2	93.9	86.7
Crude protein (%)	0	20.1	30.0	39.8	22.5
Gross energy (MJ/kg)[a]	16.9	17.7	18.0	18.4	19.5

[a] Gross energy (kJ/g diet) = (% Crude protein $\times$ 23.6) + (% Crude lipids $\times$ 39.5) + (% Carbohydrates $\times$ 17.3).

Coding and sampling the bees

Emerging brood combs were put in single-combs isolator from the colonies, to obtain bees of defined ages. Fifty newly emerged adult honey bee workers were collected per colony within 6 h, and their thoraces were marked with shellac paint *(von Frisch, 1965)*. To minimally disturb the colonies, the sampling was carried out without the use of smoke, inducing honey bee workers to feed on honey *(Free, 1968; Hrassnigg and Crailsheim, 1998b)* and affected the trophallactic behavior of the honey bee workers *(Farina and Núñez, 1991).*

Monitoring brood rearing activity in the colonies

Capped broods number was evaluated to determine the honey bee workers number reared in each colony. Assessments were made at 12-d intervals, once before and four times after treatment. The pupating honey bee workers remained in the sealed cells for 12 d *(Winston 1991)*, and the sealed broods mortality was so little (generally, the honey bee workers mortality in sealed broods was lower than 3%; according to *Fukuda and Sakagami (1968)*. Thus, a new set of pupating honey bee workers was counted in each assessment. The capped brood number was

monitored via a modified square grid system *(Mattila and Otis, 2007)*. The grids with a characteristic of 189 squares and each with an area of 4 cm^{2}were placed over the brood frames, and the comb area occupied by the capped brood was measured. The total number of bees reared in each colony was evaluated using a factor of 4.29 worker cells per square centimeter *(Seeley and Visscher, 1985)*.

Newly emerged honey bee workers

The wet weight of 20 bees per colony was obtained within 2 h after emergence. Nutrition quality was measured via CP level and this index is correlated with the physiological conditions of honey bee workers *(Standifer et al., 1960; Pernal and Currie, 2000)*.

Hypopharyngeal gland measurements

Five honey bee workers from each colony were selected at 9 d of age to assess HPG development. The HPGs were removed and placed in a Petri dish with wax depressions each containing a droplet of ice-cold sodium chloride solution (0.85%, isotonic to the hemolymph). Micrographs of the HPGs were taken using a microscope equipped with a camera. For calibrating, an image of a 1µm scale bar was obtained at the same magnification. The analysis of HPG acini was carried out by measuring the areas of five acini cells selected randomly for each bee using the Photoshop (Adobe) pixel counting routine. Hereupon, 25 acini cells were surveyed for each colony for a total of 100 acini cells per treatment.

Statistical analysis

The data were analyzed by completely randomized design by one-way ANOVA using Proc MIXED. All statistical analyses were performed with SAS. Protein level and SM was the main effect and colony in each group was the random factor. The significance difference among treatments (P < 0.05) was tested using Duncan's new multiple range test of SAS.

$$\mu ij = \mu + \alpha i + \beta j$$

Results

Effects of silymarin dose on survival of honey bee workers fed with a single, pure carbohydrate diet

In current study, the effect of silymarin was evaluated on the lifespan (Fig. 1), survival (Fig. 2) and overall survival of honey bees. The dose of SM that bees were fed affected their survival (Fig. 1). SM with dosage of 0.2 mM was improved survival, compared to other SM doses and had significant effect on lifespan (Fig. 2).
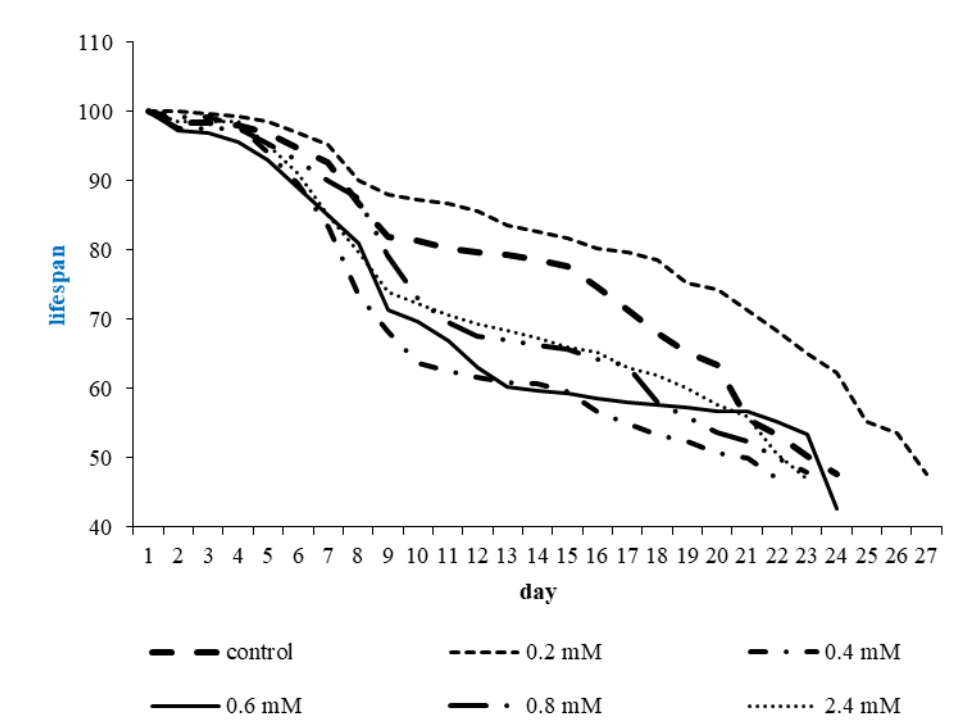

Figure 1. Honey bee workers survival fed with 0.68 M sucrose without SM (control) and with five SM concentrations until bee population decline by half. Diet 1: sucrose, diet 2: sucrose with 0.2 Mm SM, diet 3: sucrose with 0. 4 Mm SM, diet 4: sucrose with 0. 6 Mm SM, diet 5: sucrose with 0.8 Mm SM and diet 6: sucrose with 2.4 Mm SM.
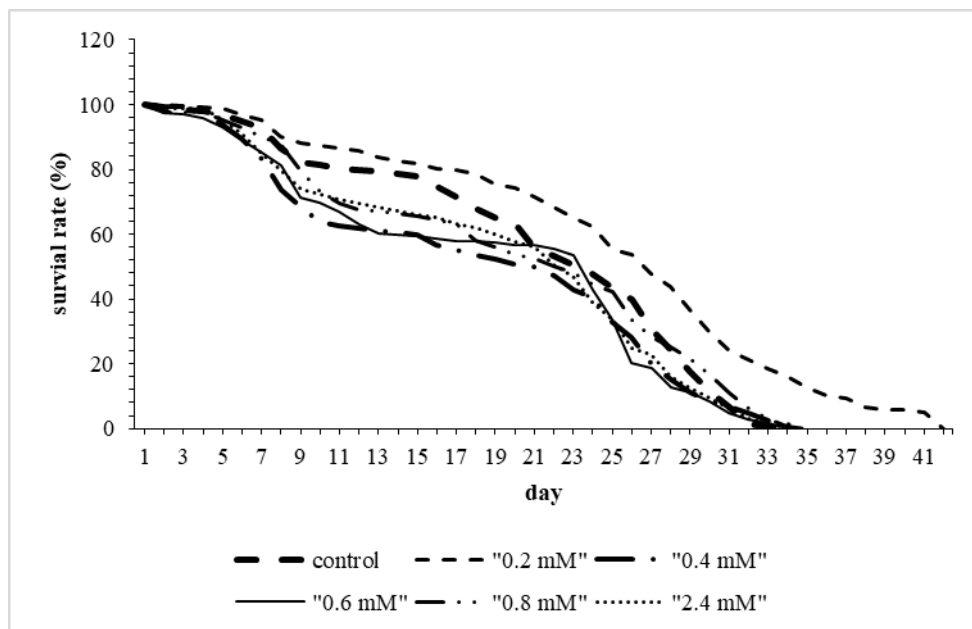
Figure 2. Honey bee workers survival with fed 0.68 M sucrose without SM (control) and with five SM concentrations. Diet 1: sucrose, diet 2: sucrose with 0.2 Mm SM, diet 3: sucrose with 0. 4 Mm SM, diet 4: sucrose with 0. 6 Mm SM, diet 5: sucrose with 0.8 Mm SM and diet 6: sucrose with 2.4 Mm SM.

Number in sealed broods

The brood rearing number in sealed broods during the experiment is shown in Fig. 3. Furnishing diets with different CP levels to the experimental colonies had a significant effect on the timing of the increase in brood rearing activity as the season progressed ($P < 0.05$). The average number of honey bee workers brood cell by the colonies quickly increased. At the beginning of the experiment, all of the brood colonies were similar. Treatments supplements with 30% CP with SM and 30% CP colonies tended to have the greatest number of honey bee workers in sealed broods at each colony census, and brood rearing activity was not significantly different from that observed with other treatments ($P > 0.05$). Treatment of 20% CP and 40% CP colonies represent more honey bee workers than sucrose treatment, but were statistically insignificant ($P > 0.05$).
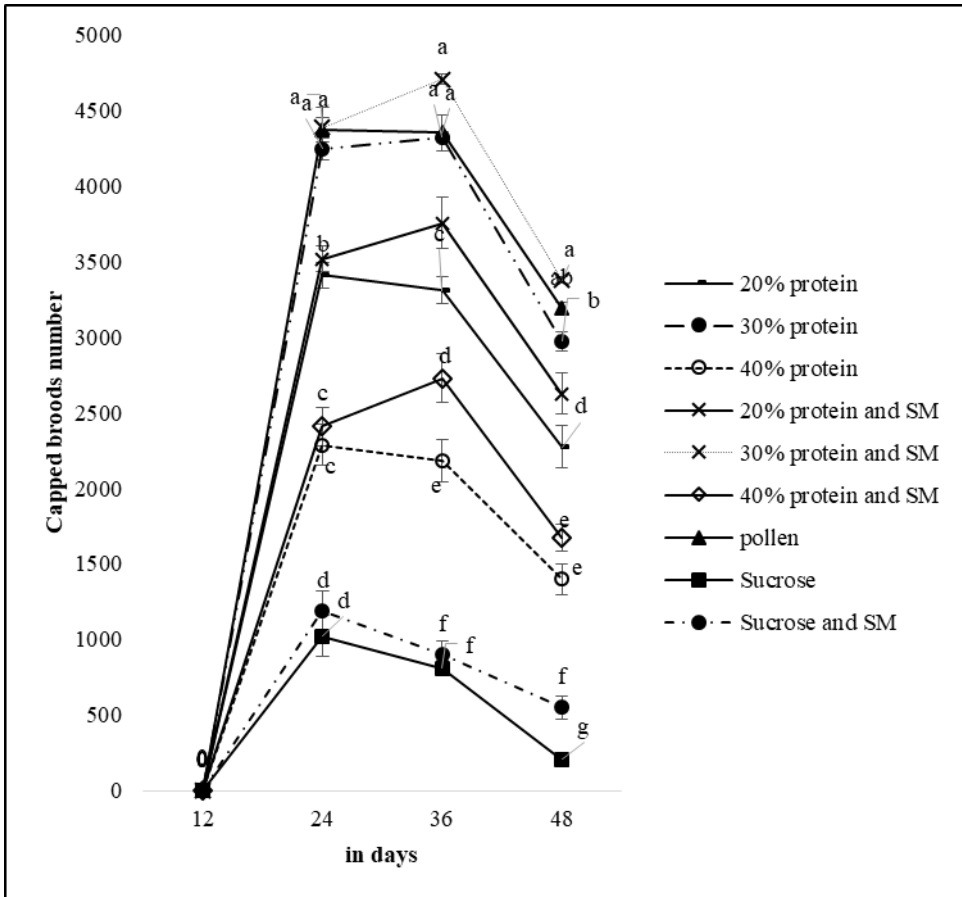
Figure 3. Mean number of Sealed pupa by colonies fed mix pollen and diets with different protein levels (0, 20, 30 and 40 %) during fall (N = 4 colonies per treatment). Different letters signify significant differences at P < 0.05.

Newly Emerged honey bee workers-Emergent Worker Weight

The average emergent worker weight was significantly affected by dietary proteins levels (P < 0.05; Fig. 4). The greatest emergent worker weights were obtained with honey bees fed with 30% CP diet (0.115 g), but there was no significant difference between the weights of honey bees fed with 30% CP and 30% CP with SM diet, and the lowest emergent worker weights (0.098 g) was observed in the treatment of sucrose, in compared to other treatments (P > 0.05).
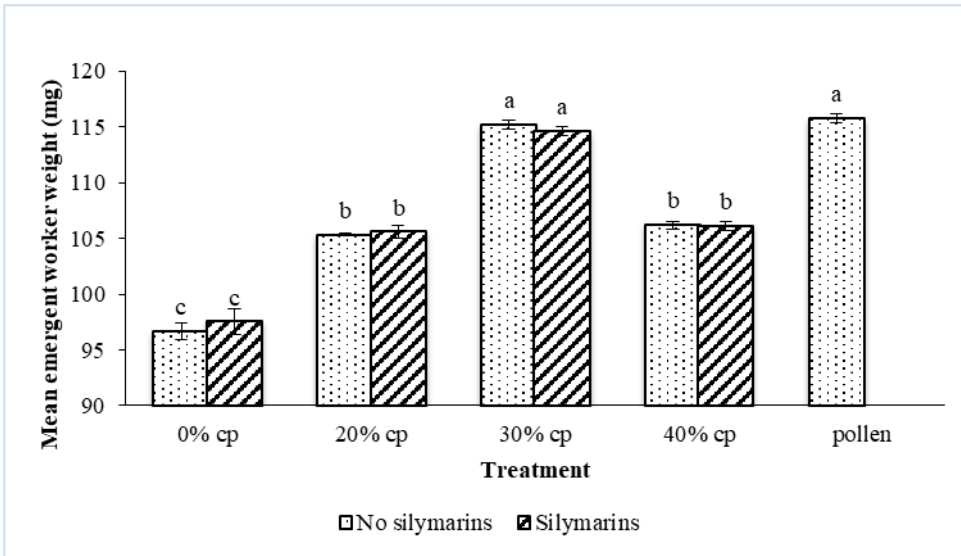
Figure 4. Mean emergent worker weight (±SE) bees fed pollen and diets containing different protein levels (0, 20, 30, and 40), two levels of silymarin (0 or 0.2 Mm) N=4 colonies per treatment). Different letters signify significant differences at P<0.05.

Protein Concentration

The highest protein concentrations in body were obtained using the 30% CP with SM diet (Fig. 5), but there was no significant difference between protein in body of the bees fed with 30% CP diet and 20% CP diet. There was a significant difference between the protein in the body, the 40% CP diet and 0% diet against other treatments.
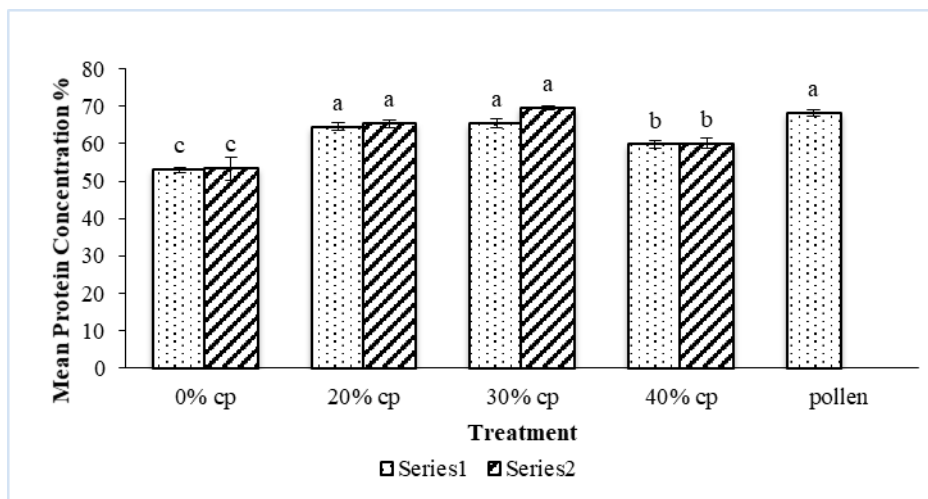
Figure 5. The mean Protein Concentration (±SE) of workers fed pollen and the various dietary proteins levels (0, 20., 30, and 40%), two levels of silymarin (0 or 0.2 Mm) N=4 colonies per treatment). Different letters signify significant differences at P<0.05.

Development of hypopharyngeal glands

The largest HPG acini in the nurse bees were obtained at 30.0% CP (P < 0.05). There were significant differences in HPG development in bees fed with 30.0% CP with other treatments (P > 0.05; Fig. 6).
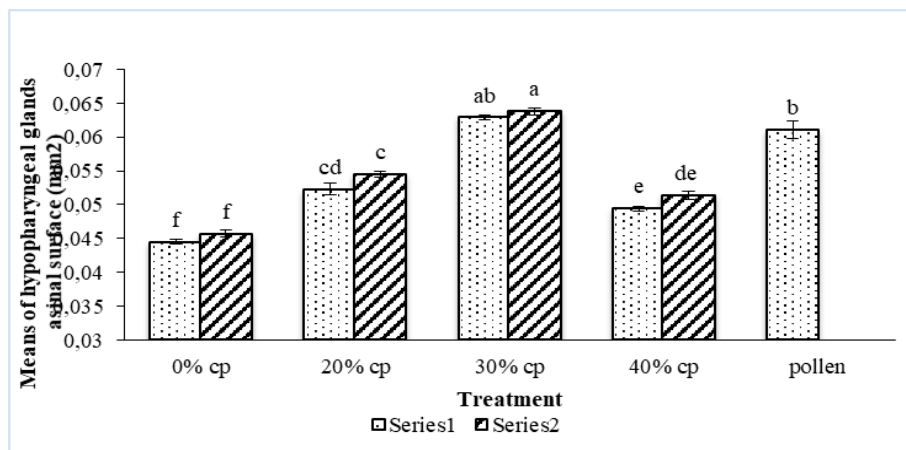
Figure 6. Development of hypopharyngeal glands during the nursing period of honey bee workers (at 9 d; N=4 colonies per treatment). The control diet is mixed pollen, and the dietary proteins (0, 20., 30, and 40%), silymarin (0 or 0.2 Mm). Different letters signify significant differences at P<0.05.

Discussion

Caged honey bees survived favorably when fed with sucrose and 0.2 mM SM solutions. Antioxidant supplementation at low doses improved honey bee survival (Fig. 1 and Fig. 2). Similar to our results using the major antioxidant in green tea epigallocatechin-3-gallate supplementation improved honeybee survival but only at an intermediate dose (0.3–0.5 mM) *(Archer et al., 2014a)*. It seems likely that antioxidants are among the major regulators of many physiological processes and, therefore, a redox balance between antioxidants and prooxidants in the diet, gastro-intestinal tract, plasma and tissues is an important determinant of the state of our health. Plants consumed by humans and animals contain thousands of phenolic compounds. Among them, the effects of dietary polyphenols including SM are of great current interest. Indeed, various phytochemicals, including flavonoids, are an essential part of our diet, which is responsible for turning on and maintaining the optimal status of our antioxidant defenses. Since flavonoids are not well absorbed in the gut, their active concentration in the plasma and target tissues are comparatively low, but probably sufficient for Nrf2 activation and NF-κB suppression as well as vitagene activation.

In the current study, we provided experimental evidence for a link between dietary proteins and important parameters in honey bees. Based on the current findings, there is a strong association between dietary proteins content and worker proteins content.

Current results showed that an increase in dietary proteins from 20% to 30% consequently caused an increase in the number of honey bee workers in sealed broods at each colony census. Also, brood rearing activity was noticeable. However, increase in dietary proteins from 30% to 40% was associated with decrease number of honey bee workers in sealed broods at each colony census, and brood rearing activity was observed (Fig. 3). Providing pollen substitutes had good impact on colony growth parameters like honey production, pollen storage, sealed brood and adult population *(Mitta and M R, 2016)*. Autumn is a critical period for honey bee colonies and the weak colonies probably die during winter. The colonies need good pollen sources during this period to foster enough brood and increase the survive-ability of colony during winter *(Abou-Shaara, 2015)*. Similar findings were observed by *Zheng et al. (2014)* and *Morais et al. (2013)*, reporting the colonies fed with artificial diets had a significant improvement in population development, colony weight, honey production and increases the efficiency of the colonies. *Somerville* (2000) showed that what is probably more important for the growth rate and development of bees is the total protein intake of a colony but not simply food consumption. The high-protein diets also increased colony growth parameters during periods of scarcity of pollen resources, in the field experiments, as also reported by *Mattila and Otis (2007)*. Bee bread and the artificial protein diets were well accepted by the bees. *Cremonz et al. (1998)* also found the highest

protein values in bees fed with bee bread. *Garcia et al. (1986)* supplied various protein foods with 20, 30 and 40% crude protein; they reported that CP negatively affects food collection. *Herbert et al. (1977)* reported that pollen substitutes containing 50% protein depressed brood rearing. Although the 40% CP diet is protein rich, the excess protein might inhibit the absorption of other nutrients or otherwise result in fitness costs. The current study demonstrated that 30% protein level is optimal for meeting the nutritional requirements for brood rearing during fall.

In the current study, average emergent bee honey worker weight fed with 30% protein and protein with silymarin ($P > 0.05$) was heavier than other treatments (Fig. 4). The heaviest honey bee workers were reared when pollen is readily available *(Kunert and Crailsheim, 1988)*. The bee development enhancement with increase in protein intake is probably due to honey bees requiring protein for producing cuticle *(Campbell, 1929)*, muscle, and other tissues *(Somerville 2000)*.

Proteins are responsible for 66-74% of the dry matter of adult honey bee workers *(Hrassnigg and Crailsheim, 1998a)*. This proteins content increases during the first days due to protein anabolism and decreases as the honey bee workers age *(Crailsheim, 1992)*. Measurement proteins content in honey bees is an effective method to evaluate the dietary proteins quality *(De Jong et al., 2009)*. To evaluate the diet efficiency precisely, total proteins content in newly emerged honey bee workers was tested. Increase in dietary proteins from 20% to 30% of colonies in the present study had a significantly positive effect in the protein concentrations of body, but increase in dietary proteins from 30% to 40% substantially decreased the protein concentrations of body. A significant difference was not observed in the protein concentrations of body between protein supplement of 30% treatment (Fig. 5). Similar to current results bees fed with 15% protein supplement diet had lower body protein concentration than bees fed with 30.5% and 35% protein supplement diet, indicating that protein level in protein supplement 15% diet was not supportive for the optimum growth and development of bees *(Li et al., 2012)*

The results indicated that an increase in dietary proteins from 20% to 30% of colonies had a significant effect on HPG acini, whereas an increase in dietary proteins from 30% to 40% significantly decreased the HPG acinal surface. *Di Pasquale et al. (2013)* found that certain aspects of nurse bee physiology, such as HPG development, were affected by pollen quality. Similar results were obtained when comparing honey bees fed pollen patties versus Mega Bee patties *(DeGrandi-Hoffman et al., 2010)*. Contrary to current results, *Zheng et al. (2014)* reported that nurse bees fed with pollen supplements had significantly larger HPG acini than the control group, which was fed pure pollen and confirmed that the superficial area of HPG acini increased with the bee's age regardless of whether the colonies were fed pollen or pollen supplements. It seems that the pollen supplements activated HPG development similarly to that activated by bee bread.

Our observations indicated that bees fed with 30% CP diet had the largest HPG acini and brood rearing activities. Current results suggest that an adequate provision of protein is required to sustain normal development of bees. The results of the current study demonstrate that dietary proteins levels strongly affect the population growth, performance, and physiological status of worker bees.

Conclusion

In present study, protein supplements can closely resemble pollen in nutritional value and because of their effects on protein concentrations and HPG development can play a significant part in colony. Diet containing 30% protein was recognized as an excellent one to promote honey bee colonies development. The optimum proteins content in field applications might differ according to dietary ingredient compositions and feeding methodology. These findings are particularly important for the successful bee keeping (colonies management) using pollen supplements when natural pollen is unavailable. In conclusion, population growth, body proteins content and surface HPG and worker quality of emerging honey bee workers were significantly affected by dietary composition and could be manipulated as metabolic tools to assess the optimal concentration of dietary proteins in the honey bees feeding.

Efekti različitih nivoa proteina i silimarina na rast populacije i površinu hipofaringealne žlezde pčela radilica (*Apis mellifera meda*)

Sayed Mohammad Reza Hossaini, Mohsen Sari, Gholamhosein Tahmasbi, Morteza Chaji

Rezime

Zamena za polen je dragoceni resurs za održavanje pčelinjih društava jakim i zdravim, u odsustvu polena u dovoljnim količinama u prirodi. Stoga je ovo istraživanje sprovedeno kako bi se istražili efekti različitih nivoa dijetetskih proteina i silimarina (SM) kao prirodnog antioksidansa na kolonije medonosnih pčela radilica. Istraživanje je izvedeno u ogledu sa 36 društava medonosnih pčela u potpuno randomiziranom dizajnu sa devet eksperimentalnih tretmana (četiri nivoa sirovih proteina 0, 20, 30 i 40%, dva nivoa silimarina 0 i 0,2 mM i čisti polen

(kontrola), i četiri ponavljanja na Univerzitetu za poljoprivredne nauke i prirodne resurse u Khuzestanu u jesen 2015. U navedenim parametrima studije, kao što su radilice u zapečaćenom leglu, masa novorođenih pčela radilica, telesni proteini i razvoj površine hipofaringealne žlezde su proučavani. Sojino brašno, kukuruz i pšenični gluten bili su uključeni kao zamene polena. Na osnovu rezultata prethodnog eksperimenta, dodatak sojine sačme u dozi od dve desetine mM poboljšao je preživljavanje pčela. Brojnost omeđenog legla pomoću podeljenog okvira na kvadrate veličine 2 × 2 cm, težina novorođenih radilica pomoću vage, telesni proteini na osnovu procenta telesne mase i razvoj površine hipofaringealne žlezde su mereni pomoću mikroskopa i mikrometara. Rezultati su pokazali da postoji značajna razlika između tretmana u pogledu polaganja jaja (P<0,05). Najveća i najniža stopa radilica u zatvorenom leglu odnosila se na tretman koji sadrži 30% proteina i SM (12467 ćelija), odnosno na tretman saharozom (2042 ćelije). Takođe, najveća i najmanja masa novorođenih pčela radilica bile su povezane sa tretmanom sa polenom, odnosno sa saharozom (P<0,05). Proteini u telu pčela radilica u ispitivanim tretmanima imali su značajne razlike (P <0,05), tako da su najveći i najniži procenat telesnih proteina primećeni u ishrani koja sadrži 30% proteina, odnosno SM i saharozi. Površina hipofaringealne žlezde u kolonijama hranjenim različitim obrocima bila je značajno različita (P <0,05), a najšira i najuža njena površina zabeležene su u ishrani koja sadrži 30% proteina, odnosno SM i saharozu. Prema trenutnim rezultatima, kako bi se reprodukcija pčela maksimizirala, predlaže se ishrana koja sadrži 30% sirovih proteina.

Ključne reči: antioksidanti, protein u obroku, zamena za polen, dugovečnost, zatvorene kolonije

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SEROPREVALENCE OF *COXIELLA BURNETII* IN CATTLE IN THE BELGRADE EPIZOOTIOLOGICAL AREA

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Abstract: Q-fever is antropozoonosis which is caused by *Coxiella burnetii,* obligate intracellular pathogen. The most significant characteristics of this pathogen are resistance and stability in the environment, possibility of aerosol dissemination, and very low infective dose. *C. burnetii* can infect domestic and wild animals, rodents, birds and ticks. Q fever in animals is generally asymptomatic, although it can lead to reproductive disorders during pregnancy. The main route of infection in humans is inhalation of contaminated aerosol and dust. Serological studies have shown the presence of antibodies to *C. burnetii* in the serum samples of cattle in Belgrade epizootiological area. Seroprevalence of 18% was found in farm bred cattle, while it was only 1.5% in individual breeding. In farm bred cows that have suffered abortion prevalence was 49%, and only 1.9% in individual breeding. The overall results indicate that the circulation of this pathogen in cattle, in Belgrade epizootiological area, poses a health risk, not only to the cattle, but also to the humans, especially persons working with animals.
Q fever control programs most often recommend serological research and vaccination of animals. Accordingly, it is necessary to define a strategy for the implementation of biosecurity measures and preventive measures against Q fever.

Key words: Q-fever, *Coxiella burnetii*, abortus, serological surveys, antibody, inhalation.

Introduction

Q fever is one of the most well-known anthropozoonoses, present worldwide, with the exception of New Zealand (*Angelakis and Raoult, 2010*). The disease has also been recorded in Serbia, both in humans and in various species of animals. It was established for the first time in humans in 1937, in Australia (*Derrick, 1937*). It was clinically manifested by fever, and due to ignorance about

the nature of the disease, it was called *Q-query fever*. In the same year, the causative agent was isolated from infected people and named *Rickettsia burnetii (Burnet and Freeman, 1937)*. A year later, in the USA, the causative agent was isolated from ticks and named *Rickettsia diaporica (Davis and Cox, 1938)*. Out of gratitude to the researchers, the causative agent was renamed *Coxiella burnetii (C. burnetii)*.

C. burnetii is an obligatory intracellular bacterium, which belongs to the family *Coxiellaceae*, order *Legionellales*, class *Gamaproteobacteria* type *Proteobacteria (Raoult et al., 2005; Shaw and Voth, 2019)*. The species *C. burnetii* is the only member of the genus *Coxiella*. This bacterium is immobile, small, polymorphic, most often cocoid in shape and Gram negative (*Eldin et al., 2017*). The size is 0.2 - 0.4 μm x 0.4 -1 μm (*Abnave et al., 2017*). *C. burnetii* shows a two-phase life cycle that manifests itself in two different morphological forms - as a large cell variant (LCV) and a small cell variant (SCV). The SCV is 0.2 to 0.5 μm in size, while the LCV is larger in size > 0.5 μm (*Maurin and Raoult, 1999; Eldin et al., 2017*). SCV appears in a spore like form, with pronounced resistance to osmotic, physical, chemical and drying factors. This property ensures its long survival in external conditions and gives ecological stability (*Arricau-Bouvery and Rodolakis, 2005; Clark et al., 2018*). The SCV morphotype is metabolically inactive and represents the main infectious stage. The LCV morphotype is metabolically active and allows replication within the vacuole in the host cell (*Maurin and Raoult, 1999; Shaw and Voth, 2019*).

According to the antigenic structure, *C. burnetii* is divided into two antigenic phases, namely virulent phase I and less virulent phase II (*Arricau-Bouvery and Rodolakis, 2005; Abnave, 2017*). Phases I and II are morphologically identical, but differ in some biochemical properties, including their lipopolysaccharide composition. Strains isolated from infected organisms have complete LPS, express antigen phase I and show high infectivity, while those with antigen phase II are less infectious and have incomplete LPS (*Porter et al, 2011; Gwida et al., 2012*).

C. burnetii shows exceptional resistance in the environment. The SCV type survives for 7-10 months on wool at room temperature, for more than one month in fresh meat and over 40 months in milk (*Angelakis and Raoult, 2010*). It is resistant to the action of 1% formalin and 1% phenol for 24 hours, but it is inactivated by 0.05% hypochlorite and 1% lysol. Heating at 63°C only partially destroys *C. burnetii*. Pasteurization for 15 seconds at 71.5°C is required for safe destruction in milk *(Arricau-Bouvery and Rodolakis, 2005; Valčić et al., 2014)*.

Due to its pronounced resistance, stability in the environment, aerosol transmission, wind transmission and significantly low dose of infectivity, the Centres for Disease Control (CDC) has classified *C. burnetii* as a group of potential bioterrorism agents of category B *(Eldin et al, 2017; CDC, 2018)*. Also, the stated characteristics of the causative agent indicate the need for strict control when

working in the laboratory in accordance with the biosafety level standard 3 (*OIE, 2018*).

C. burnetii is a highly virulent bacterium (*Shrestha, 2020*). Research has shown that 1–10 viable bacteria (*Sawyer et al., 1987*), and even one bacterium, is sufficient to cause infection (*Waag, 2007*).

Q fever used to be rare and regionally limited. At the beginning of the 21st century, the disease has spread as a re-emergent zoonosis in many European countries *(Gwida et al., 2012; Dijkstra et al., 2012: Pandit et al., 2016).* It is assumed that the spread of the disease is due to increased virulence of the pathogen, changes in the clinical picture, application of more reliable tests in diagnostics, as well as changes in epidemiological characteristics (*Aricau-Bouvery and Rodolakis, 2005*).

C. burnetii can infect a variety of animal species, including domestic animals such as cattle, sheep, goats, dogs, cats, also rodents, wildlife, reptiles, birds, fish, and ticks (*Angelakis and Raoult, 2010; Gwida et al., 2012).* Ticks play a significant role as reservoirs of pathogens, but also as vectors in the transmission of pathogens, especially from wild to domestic animals (*Cantas et al., 2011*).

In animals, Q fever is mostly asymptomatic, although reproductive disorders such as abortion, stillbirth, placental abruption, and foetal underdevelopment may occur during pregnancy *(Gwida et al., 2012; Pexara et al., 2018).*

By monitoring of the epidemiological data, it was concluded that humans are most often infected by inhalation of contaminated aerosols and dust particles containing bacteria from infected animals. Most often, this contamination occurs during the birth of infected animals through products such as placenta, amniotic fluid, colostrum, etc. (*Maurin and Raoult, 1999; Vidić et al., 2008; Angelakis and Raoult, 2010*).

Consumption of contaminated raw milk and dairy products is also considered to be a potential source of infection for humans (*Boboš et al., 2013; Radinović et al., 2014; Pexara et al., 2018*). For people who come in contact with animals, such as veterinarians, livestock breeders, slaughterhouse staff, but also laboratory workers, Q fever is considered an occupational disease (*Maurin and Raoul, 1999; OIE, 2018*). According to the data of the City Institute for Public Health of Belgrade, in the period from 1984 to 2018, 78 cases of Q fever in humans were recorded in the area of the city (*https://www.zdravlje.org.rs/*).

The aim of this study was to determine the seroprevalence of *C. burnetii* in cattle in the Belgrade epizootiological area, whether they come from dairy farms or individual households. Based on the obtained research results, it will be possible to assess the epizootiological situation, and accordingly develop Q fever control programs.

Materials and Methods

Serological surveillance is carried out in many countries with the aim of assessing the prevalence of *C. burnetii* in domestic ruminants. The Rulebook on the Program of Animal Health Measures adopted annually by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, Veterinary Directorate, stipulates diagnostic tests in cases of abortion of domestic ruminants to Q fever. In accordance with that, the Scientific Veterinary Institute of Serbia conducts serological tests of cows that have had abortions. In addition, animals that had other reproductive disorders or were in circulation were also examined. The samples originated from commercial farms or individual agricultural households with extensive production, and are located in the epizootiological area of the city of Belgrade.

Blood samples for diagnostic tests were delivered during 2017, 2018 and 2019 to the Scientific Veterinary Institute of Serbia, Belgrade, by the competent veterinary stations. Blood sera were tested for the presence of antibodies against *C. burnetii* using a commercial ELISA test, ID Screen® Q Fever Indirect Multi-species/ ID Vet, Grabels, France. A total of 862 samples were tested, of which 226 originated from aborted cows. Serological tests were performed by stipulated methods performed in accordance with the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (*OIE, 2018*), and the test results were interpreted according to the instructions of the diagnostic kit manufacturer.

Results and Discusion

Our three-year research included the examination of bovine serum for the presence of antibodies against *C. burnetii* in cattle from the epizootiological area of the city of Belgrade. The tested sera originated from cattle reared on three commercial farms and cattle from individual agricultural households. A total of 862 samples were analyzed, of which 145 (16.8%) showed the presence of antibodies to *C. burnetii*. Significantly higher seroprevalence was found in cattle from farms than in cattle from individual agricultural households. The prevalence on farms 1, 2 and 3 was 17.4%, 9.0% and 39.2%, respectively. The antibodies to *C. burnetii* were found in only one sample of a total of 65 tested samples from individual agricultural households. The results (number/percentage) are shown in Table 1.

Table 1. Results of testing for the presence of antibodies to *C. burnetii* in bovine sera

	Year	2017	2018	2019	Total
Farm 1	Examined	412	245	101	758
	Positive/%	25/6	99/40	8/7.9	132/17.4
Farm 2	Examined	4	3	4	11
	Positive/%	0	0	1/25	1/9
Farm 3	Examined	2	25	1	28
	Positive/%	0	10/40	1	11/39.2
Ind. ag. households	Examined	37	12	16	65
	Positive/%	0	1/8.3	0	1/1.5
Total	Examined	455	285	122	862
	Positive/%	25/5.4	110/38.5	10/8.1	145/16.8

226 samples of a total of 862 were from cows after abortion. Of those 226 sera, 175 were from farm animals and 51 were samples of animals from the individual sector. The seroprevalence of Q fever was 38% in cows after abortion. Out of 175 farm animals, 86 (49%) were positive and only 1 sample of 51 cows from individual sector. The results of the analysis (number/percentage) are shown in Table 2.

Table 2. Results of testing the presence of antibodies to *C. burnetii* in the sera of cows after abortion

	Year	2017	2018	2019	Total
Farm 1	Examined	19	117	6	142
	Positive/%	9/47.3	61/52.1	4/66.6	74/52.1
Farm 2	Examined	0	3	4	7
	Positive/%	0	0	1/25	1/14.2
Farm 3	Examined	0	25	1	26
	Positive/%	0	10/40	1	11/42.3
Ind. ag. households	Examined	25	12	14	51
	Positive/%	0	1/8.3	0	1/1.9
Total	Examined	44	157	25	226
	Positive/%	9/20.4	72/45.8	6/24.0	87/38.4

The obtained results show that the highest number of positive animals was established during 2018, as a consequence of the epizootic incidence on Farm 1. We assume that the occurrence of the disease is related to the position of Farm 1, which geographically gravitates to the endemic area of the South Banat district. A milder form of the disease was found on Farm 3, also during 2018, with the remark that fewer blood samples were sampled from this farm. The prevalence of Q fever in the animals with abortion ranged from 47.3% to 66.6% on Farm 1, which was most exposed to the infection. At the same time, only 1 of 51 samples from individual rearing was serologically positive, which indicates a low prevalence, i.e. almost complete absence of Q fever in private sector. Previous research in Serbia in the area of Vojvodina showed that Q fever was found in 9.5% of herds (*Vidić et al., 2008*), while the percentage of infected heads in the population ranged from 5 to 80% (*Radinović et al., 2014*).

Serological surveillance of Q fever revealed significant differences in prevalence, not only between countries but also between individual regions. Thus, 82% of seropositive tests were found in the Netherlands (*van Engelen et al., 2014*), 72% in Germany (*Böttcher et al., 2011*), 48% in the Great Britain (*McCaughey et al., 2010*), 40% in Poland (*Jodełko et al., 2015*), 38% in Italy (*Galluzzo et al., 2019*) and 36% in France (*Gache et al., 2017*). A significantly lower percentage was recorded in northern European countries: 0.24% in Finland (*EVIRA, 2016*) and 8% in Sweden (*Ohlson et al., 2014*). Similarly, 3.4% of seropositive tests were found in the United States (*Angelakis and Raoult, 2010*). There are also differences in the number of infected animals in herds. There were 30% positive cattle in Saudi Arabia *(Jarelnabi et al., 2018)* and 15% in Turkey (*Gulmez and Sahin, 2016*). Lower percentages were found in cattle in Iran 3.23% (*Ghasemi et al., 2018*) and in Italy 5.28% (*Galluzzo et al., 2019*). In Great Britain, 12.5% of positive cows were determined among dairy cows (*McCaughey et al., 2010*), while in China the percentage for the same species was 33 (*El-Mahallawy et al., 2016*). Also, only 2.1% of positive cattle were found in Great Britain (*McCaughey et al., 2010*).

The results of the study indicate a correlation between ruminant abortions with *C. burnetii* infection (*Žutić et al., 2019*). In Cyprus, 35% of aborted cows, 33% of sheep, and 50% of goats were positive for *C. burnetii* (*Cantas et al., 2011*). Lower percentages were found in Italy, where 11.6% of cows and 21.5% of sheep and goats that had aborted were positive for Q fever (*Parisi et al., 2006*). In France, 2,695 cows, 658 sheep, and 105 goats that had aborted, seroprevalence of *C. burnetii* was found in 36%, 55.7%, and 61% of the herds, respectively (*Gache et al., 2017*). In Latvia, seroprevalence was recorded in 13.4% of herds where abortions occurred in dairy cattle (*Boroduske et al., 2017*). In the Netherlands, 3,264 cases of Q fever in humans were reported during the 2007–2010 epidemic (*Dijkstra et al., 2012*). Research has shown that the epidemic appeared in a narrower area where dairy goat farms were located, where abortions occurred in

waves. The cause of the epidemic is believed to have been the airborne transfer of contaminated dust particles from farms to densely populated areas.

Q fever is a very complex disease in both humans and animals, so control and eradication measures require a series of procedures over a long period of time. Q fever control programs most often recommend serological testing and vaccination of animals. Serological examination can identify positive herds and thus determine risk levels on farms and in the regions (*Valčić et al., 2014*). Rodents and ticks as reservoirs of pathogens should be systematically destroyed in natural habitats and on farms (*Vidić et al., 2012; Vidić et al., 2014*). By applying preventive and biosafety control measures, it is possible to reduce environmental contamination, and thus the risk to human and animal health.

Conclusion

The established seroprevalence of Q fever in our research indicates that *C. burnetii* circulates in cattle breeding in the Belgrade epizootiological area. A significantly higher percentage of infection was found in cattle on farms than on individual agricultural households. The presence of this pathogen represents a significant risk to human and animal health, in which the reproductive system is particularly endangered.

The implementation of serological surveillance is one of the most important preventive measures for mass testing and detection of infected animals. The obtained results impose the need for the application of remedial, preventive, biosafety and other animal health measures. But also research has to be continued on a larger number of samples for the future period. Control measures need to be coordinated with those in the region and/or surrounding countries in order to achieve better and geographically broader program to protect animals and humans from Q fever, as a global anthropozoonosis.

Seroprevalencija *Coxiella burnetii* kod goveda na beogradskom epizootiološkom području

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Rezime

Q groznica je antropozoonoza prisutna u celom svetu, izuzimajući Novi Zeland. Uzročnik bolesti je *Coxiella burnetii* (*C. burnetii*), obligatno intracelularna Gram negativna bakterija.

Najznačajnije karakteristike ovog patogena su otpornost i stabilnost u spoljašnjoj sredini, prenošenje putem aerosoli i veoma niske doze infektivnosti. *C. burnetii* može inficirati razne vrste životinja, uključujući domaće životinje, zatim glodare, divlje životinje, ptice i krpelje. Q groznica kod životinja prolazi uglavnom asimptomatski, mada tokom gravidnosti može dovesti do različitih reproduktivnih poremećaja i steriliteta. Ljudi se inficiraju inhalacijom kontaminiranih aerosoli i čestica kontaminirane prašine. Sprovedenim serološkim istraživanjima utvrdili smo prisustvo antitela za *C. burnetii* u serumima goveda sa beogradskog epizootiološkog područja. Ukupno je analizirano 862 seruma, od kojih su 16,8% bili pozitivni na Q groznicu. Znatno viša seroprevalencija, od 18% utvrđena je kod goveda na farmama nego kod onih iz individualnih uzgoja. U svega 1 od 65 seruma goveda iz individualnog uzgoja ustanovljena su antitela za *C. burnetii*. Od ukupno 862 seruma, njih 226 je bilo poreklom od krava koje su pobacile, i to 175 iz farmskog i 51 iz privatnog uzgoja. Seroprevalencija Q groznice iznosila je 49% kod krava poreklom sa farmi, a 1,9% kod krava iz individualnih uzgoja. Rezultati pokazuju da je najveći broj pozitivnih grla ustanovljen tokom 2018. godine kao posledica pojave epizootije na Farmi 1. Dobijeni rezultati ukazuju na cirkulaciju ovog patogena u populaciji goveda beogradskog epizootiološkog područja, što predstavlja rizik, ne samo za zdravlje goveda, već i za zdravlje ljudi, posebno onih koji rade sa životinjama. Programi kontrole Q groznice najčešće preporučuju serološka istraživanja i vakcinaciju životinja. Na osnovu rezultata ispitivanja i epizootiološke procene, potrebno je uraditi program sprovođenja preventivnih i biosigurnosnih mera radi kontrole Q groznice.

Ključne reči: Q groznica, Coxiella burnetii, pobačaj, serološki pregled, antitela, inhalacija.

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GASTROINTESTINAL PARASITES OF SHEEP AND GOAT IN AND AROUND GONDAR TOWN, NORTHWEST, ETHIOPIA

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Abstract: A cross sectional study was conducted from November 2018 to April 2019 to determine the prevalence and risk factors associated with sheep and goat gastrointestinal (GIT) parasites in and around Gondar town, Northwest of Ethiopia. A total of 384 sheep and goats fecal samples (313 sheep and 71 goats) were collected and examined using standard parasitological techniques. The present study revealed that an overall prevalence of 56.77% with prevalence rate of 185 (59.11%) and 33(46.48%) in sheep and goats respectively. From the examined samples mixed infection was recorded with about 21.35%. The parasitic species identified were *Strongyle spps* (22.14%), *Trichuris spps* (0.52%), *Monezia* (2.5%), *Emeria spps* (0.260%). Female animals were found with higher prevalence of helminthes infection rate than male animals with a prevalence of 48.98% and 61.60%, respectively. Higher prevalence was observed in young animals (69.61%) than adult animals (45.32%). Body conditions and production system also showed significance difference (P<0.05) in the occurrence of GI parasites of sheep and goats in the study area. It can be concluded that, in the study area there was high prevalence of GIT parasites in sheep and goats with age, sex, body condition and production system as important risk factors. Therefore, strategic uses of anthelmintics drugs and good management should be given for the control and prevention of GIT parasites as well as further studies to determine burden of the parasites and seasonal variation is recommended.

Keywords: GIT parasite, Gondar town, Risk Factor, prevalence, Ethiopia

Introduction

In Ethiopia small ruminants represent the most important part of the Ethiopian livestock system. These animals are almost entirely managed by the poor small-holder farmers and pastoralists (*Sissay, 2007*). Sheep and goat are playing an important role in the livelihood of resource poor farmers and provide a vast range

of products and services such as meat, milk, skin, hair, manure and food security, gifts, religious rituals and medicine. However, the full exploration of this production was hindered due to traditional husbandry and management system, poor genetic potential of local breeds and the presence of numerous animal diseases (*Mtenga et al., 1994*).

Gastrointestinal parasite infections are a world-wide problem for both small and large-scale farmers, but their impact is greater in sub-Saharan Africa in general and particularly in Ethiopia due to the availability of a wide range of agro-ecological factors suitable for diversified hosts and parasite species. Gastrointestinal parasites causes economic losses due to lowered fertility, reduced work capacity, involuntary culling, lower weight gains, treatment costs and mortality in heavily parasitized animals (*Fikru, 2006*).

Helminthes and coccidia are mentioned to be the most common and important gastro-intestinal parasites in small ruminants. In the tropics, the most important nematode species affecting small ruminants are *Haemonchus contortus*, *Trichostrongylus* species, *Nematodirus species, Cooperia species, Bunostomum species* and *Oesophagostomum species* (*Khan, 2005; Smith, 2009*). Coccidian parasites of the genus *Eimeria* contribute to enteric disease especially in young or stressed goats under poor farm management that leads to high mortality in goat kids (*Ratanapob et al., 2012*).

Even some work has been done on gastrointestinal parasites of sheep and goats in northern Ethiopia, most of the studies were restricted to estimate its prevalence than determination of potential risk factors and effect of management system on occurrence of GIT parasites to develop possible prevention and control strategies. Therefore, this study was designed with the objectives of:

- ✓ To estimate status of GIT parasites of sheep and goat in Gondar town

- ✓ To determine effect of management system and other possible risk factors on prevalence of GIT parasites in the study area

Materials and Methods

The Study Area

The present study was conducted in and around Gondar town which is located 750 Km away from Addis Ababa, in north Gondar zone, northwest Ethiopia. Geographically, the study area is located on 35°7' N and 13°8' E and an altitude of 2200 meter above sea level. The annual mean temperature of the area was between 15°C and 26°C respectively. It receives a bimodal rainfall with short rainy season occur during the months of March, April, and May while the long ones extend from June to September with mean annual rain fall of 1172 mm (CSA, 2012). The soil type of the area consists of vertisoil and sandy type of soil with vegetation type

which varies from larger tree to bushes. The area is characterized by mixed crop livestock production farming system.

Study Animals and management
The study animals were local breeds of small ruminants (sheep= 313 and goat=71) kept in and around Gondar town. Species, sex, body condition, production system and age groups of local origin were included in this study. Adults are above one year with young less than one year was considered in the study according to (*Yami and Merkel, 2008*) and owner information. Body condition can be also classified as poor, medium and good according to (*Yami and Merkel, 2008*). The production system is classified based as intensive, semi intensive and extensive production system.

Despite the presence of studies on gastrointestinal parasites of sheep and goats in the study area, there was no comprehensive data showing the status of all GIT parasites in different management systems. Thus, the sample size was determined based on formula given by *Thrusfield, (2007)* with expected prevalence of 50% and 5% absolute precision at a 95% confidence interval (CI). Accordingly, 384 sheep and goat were sampled (313 sheep and 71goats).

Study design
A cross-sectional study design was used to estimate the prevalence of GIT parasite of sheep and goat in and around Gondar town based on coprological examination. Households were selected purposively based on easy of accessibility and interest of owners. Simple random sampling technique was used to select study animals in examined flocks. Species, age, sex, body condition and production system considered as risk factors for the occurrence of GIT parasite in sheep and goat.

Sample Collection and Laboratory Examination
A fresh fecal sample of approximately 5-10 gram was collected directly from the rectum of sheep and goat by using gloved fingers. Collected fecal samples placed in plastic bottles and transported to the University of Gondar parasitology laboratory for fecal examination. All the necessary information including body condition, sex, age and management systems were labeled. Samples were immediately stored in the refrigerator at 4°C until it was processed. The collected samples were subjected to qualitative flotation and sedimentation parasitological techniques. Identification of egg/ oocysts was performed by their characteristic morphological features as described by (*Soulsby, 1982*).

Data Management and Analysis
The collected data from field level and laboratory investigation was organized and entered in to MS excel work sheet. Data analysis was done using STATA version

12. Descriptive statistics were used to determine the prevalence of the parasites and Chi-square test ($\chi2$) was used to determine any association between the prevalence of GIT parasites with risk factors. In all data analyses, confidence level was held at 95% and P<0.05 for significance value.

Results

In the present study, the species prevalence of 185 (59.11%) and 33(46.48%) in sheep and goats, respectively were infected at least by one parasite species, and these give an overall prevalence of 56.77% in both species. Females and males were found to be infested with a significant variation (P < 0.05); accordingly, higher prevalence of GIT parasites was observed in female animals (61.60%) as compared to males (48.98%) between the two sexes. Young and adult animals were found to be infested with a prevalence of 69.61% and 45.32%, respectively with Significant difference (P < 0.05). Body condition scores and production system also significantly affect the outcome of GIT prevalence (Table 1).

Table 1. Overall prevalence of GIT parasite and its associated factors in sheep and goat

Risk factors	No. examined animals	Number of positive (%)	χ^2	p-value
Species				
Sheep	313	185 (59.11)	3.7596	0.053
Goat	71	33 (46.48)		
Sex				
Male	147	72 (48.98)	5.8913	0.015
Female	237	146 (61.60)		
Age				
Young	181	126 (69.61)	23.009	0.000
Adult	203	92 (45.32)		
Body Condition				
Poor	139	125 (89.93)	128.22	0.000
Medium	169	84 (49.70)		
Good	76	9 (11.84)		
Production System				
Extensive	246	204 (82.93)		
Semi Intensive	84	12 (14.29)	192.32	0.000
Intensive	54	2 (3.70)		
Total	384	218(56.77)		

The predominant GI parasites identified in sheep and goats were *strongyle types*, *trichuris spps, monesia* and, *Emeria spps* as a single and mixed infection. *Strongyle types* were the most frequently 85(22.14%) recovered GIT parasites eggs followed by *Emeria spps* 48(12.35%), *Trichuris spps* 2(0.26%) and *Monesia* 1(0.26%) (Table 2).

Table 2. Prevalence of GIT parasites of sheep and goat with egg /oocyst types

Parasite species	No. examined animals	Positive samples	Prevalence (%)
Strongyle types	384	85	22.14
Trichuris spps	384	2	0.52
Monesaia spps	384	1	0.26
Emeria spps	384	48	12.50
Mixed type	384	82	21.35
Total	384	218	56.77

Out of the total 219 positive cases, 136 (35.42%) were infected with one parasite eggs type and 82 (21.35%) were infected with two or more parasite eggs type. Among these, *strongyle type* eggs and *Emeria oocyst* coexist most of the time, with an overall prevalence of 69 (17.97%) followed by *strongyle types* and *monesia* 4 (1.04%).

Discussion

In the present study, the overall prevalence of GIT parasites was 56.77%. The current finding is lower than the report of *Tefera et al.(2010)* from Bedelle, *Nuraddis et al. (2014)* from Jimma, *Bikila et al. (2013)* from Illubabor, *Dabasa et al. (2017)* from *Bale and Tesfaye et al. (2019)* from Benishangul Gumuz who indicated prevalence rate of 91.9%, 87.2%, 77.8% and 67.7%, respectively. However, it is slightly higher than the report *of Kedir and Asfew (2017)* from south eastern Ethiopia, *Negasi et al. (2012)* from North Ethiopia and *Dagnachew et al. (2012)* from northwest Gondar, who closed prevalence rate of 52.78%, 48.21 and 47.67% respectively. This variation may be due to management system and level of production, individual animal factors, origin, season and differences in study methods.

The study showed that higher prevalence of GIT parasite was observed in poor body condition animals as compared to medium and good body condition animals and the difference was statically significant (p<0.05). This agrees with previous reports of *Welemehret et al. (2012)* in Northern Ethiopia, *Diriba and Birhanu (2013)* in south eastern Ethiopia, *Kedir and Asfew (2017)* in South Eastern Ethiopia and *Tesfaye et al. (2019)* in Northwest Ethiopia. This might be due to either well-fed animals have good immunity for parasitic infection.

The higher prevalence of GIT parasites reported in females than in males (P<0.05). This finding agrees with the report of different researchers (*Mihreteab and Aman, 2011; Bashir et al., 2012; Emiru et al. 2013*). This may be due to female animals are exposed to more stress than male animals in different times such as during pregnancy and peri-parturient period as stress decreased immune status (*Gauly, 2006).*

The study was also under taken to observe the prevalence of GIT parasites in age groups and the finding revealed that young animals were highly infected when compared to that of adult animals (p<0.05). This finding was in agreement with the finding of some researcher in different parts of Ethiopia (*Fikru et al., 2006; Diriba, 2013; Dilgasa et al., 2015*) who reported higher prevalence in younger animals. This is due to young animals are more susceptible to parasite infection due to immunological immaturity and immunological unresponsiveness (*Urquhart et al., 1996*).

The study showed that higher prevalence of GIT parasite was observed in extensive production system as compared to semi intensive and intensive production system and the difference was statically significant (p<0.05). This is in agreement with different findings (*Waller, 2004; Iyad, 2012; Sangama et al., 2013*) who reported high prevalence rate in extensive as compared to semi-intensive and intensive production systems. The possible explanation is that animals kept under extensive production systems, there is continuous infection and re-infection from heavily contaminated pastures rendering anthelmintic treatment of limited value compared to the situation under semi intensive and intensive production systems .This could be due the fact that the animals kept under semi-intensive system and intensive production system are properly managed and routinely dewormed.

The major GIT parasite that has been observed in this study were *Strongyle type* of species *trichuris spps*, *monesia spps* and *coccidian spps* of parasites. The current prevalence of *Strongyles sppswas* 22.14% which is lower with reports of previous studies conducted in different parts of Ethiopia (*Temesgen 2008; Ragassa et al., 2006*). The prevalence of coccidian parasites in the present study was 12.50% which is in line with the findings of *Nuraddis et al. (2014)* in and around Jimma town who reported 11.7% prevalence. Among the Cestoda, Monezia was the only observed species in the present study with prevalence of 0.26%. This difference in prevalence rate may be attributed to the difference in agro ecology and variation in management practice of anima.

The current study has shown the presence of mixed infection (polyparasitism) characterized by the presence of more than one GIT parasite in sheep and goat of the study area. This agrees with the findings of other researchers in the country (*Abebe and Esayasu, 2001; Regassa et al., 2006; Tefera et al., 2010*). These mixed infections have been suggested to be an important cause of morbidity and loss of production in sheep and goat (*Kumsa et al., 2011*). Moreover, the presence of interaction and compromization of the immune system of the host by mixed infections increase their susceptibility to other diseases or parasites (*Wang et al., 2006*).

Conclusion

Gastrointestinal parasites are the major animal health constraints in sheep and goat production and contributing loss in productivity and economy. In the present study an overall prevalence of GIT parasites were high with occurrence of mixed infection. The predominant GIT parasites identified were *Strongyle spps*, *Trichuris spps*, *Monesia spps* and *Emeria species*. Risk factor like ages, sex, body condition and production system were found determinant factors for the occurrence of GIT parasites. From the above findings and conclusions, strategic use of anthelmintics and good management should be practice to control the gastrointestinal parasites infection with further study on Seasonal variation and burden of parasites in the study area.

Gastrointestinalni paraziti ovaca i koza u gradu Gondar i okolini, severozapadna Etiopija

Olifan Fayisa, Ararsa Duguma, Melkamu Temesgen, Fethu Lemma

Rezime

Istraživanje je sprovedeno od novembra 2018. do aprila 2019. godine kako bi se utvrdila prevalenca i faktori rizika povezani sa gastrointestinalnim parazitima ovaca i koza (GIT) u i oko grada Gondar, severozapadna Etiopije. Ukupno su prikupljena i ispitana 384 uzorka fekalija ovaca i koza (313 ovaca i 71 koza), koji su korišćeni standardnim parazitološkim tehnikama. Ova studija je otkrila da je ukupna prevalenca od 56,77% sa stopom prevalence 185 (59,11%) i 33 (46,48%) kod ovaca i koza. Od ispitivanih uzoraka zabeležena je mešovita infekcija sa oko 21,35%. Identifikovane parazitske vrste su *Strongile spps* (22,14%), *Trichuris spps* (0,52%), *Monezia* (2,5%), *Emeria spps* (0,260%). Utvrđeno je da su ženska grla sa većom stopom prevalence helmintsa u odnosu na muška grla sa prevalencom od 48,98%, odnosno 61,60%. Veća prevalenca primećena je kod mladih životinja (69,61%) od odraslih životinja (45,32%). Stanje tela i proizvodni sistem takođe su pokazali značajnu razliku (P <0,05) u pojavi GI parazita ovaca i koza na istraživanom području. Može se zaključiti da je u istraživanom području bila velika prevalenca GIT parazita kod ovaca i koza, uzrast, pol, stanje tela i proizvodni sistem kao važni faktori rizika. Zbog toga treba obezbediti stratešku upotrebu lekova protiv glista i dobro upravljanje za kontrolu i prevenciju GIT parazita, kao i dalje studije za utvrđivanje opterećenja parazita i sezonskih varijacija.

Ključne reči: GIT parazit, Gondar, faktor rizika, prevalenca, Etiopija

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POTENTIALS OF SERBIAN LIVESTOCK PRODUCTION – OUTLOOK AND FUTURE

Milan M. Petrović[1], Stevica Aleksić[1], Milan P. Petrović[1], Milica Petrović[2], Vlada Pantelić[1], Željko Novaković[1], Dragana Ružić-Muslić[1]

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[2]University of Belgrade, Faculty of Agriculture, Nemanjina 6, 11080 Zemun, Serbia
Corresponding author: Milan M.Petrović, **e-mail address**
Review paper

Example 2

EFFECTS OF REARING SYSTEM AND BODY WEIGHT OF REDBRO BROILERS ON THE FREQUENCY AND SEVERITY OF FOOTPAD DERMATITIS

Zdenka Škrbić, Zlatica Pavlovski, Miloš Lukić, Veselin Petričević

Institute for Animal Husbandry, Autoput 16, 11080 Belgrade, Serbia
Corresponding author: Zdenka Škrbić, **e-mail address**
Original scientific paper

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Text and titles of tables, figures and graphs, Times New Roman, font size 9, **bold**, in the following form:

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Milan M. Petrović, Stevica Aleksić, Milan P.Petrović, Milica Petrović, Vlada Pantelić, Željko Novaković, Dragana Ružić-Muslić

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In scientific journals:

PETROVIĆ M. M., SRETENOVIĆ LJ., BOGDANOVIĆ V., PERIŠIĆ P., ALEKSIĆ S., PANTELIĆ V., PETROVIĆ D. M., NOVAKOVIĆ Ž. (2009): Quantitative analysis of genetic improvement of milk production phenotypes in Simmental cows. Biotechnology in Animal Husbandry, 25,1-2, 45-51.

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Editor's office

Institute for Animal Husbandry, Belgrade-Zemun
13th International Symposium
"Modern Trends in Livestock Production"
6th – 8th October 2021., Belgrade, Republic of Serbia

FIRST ANNOUNCEMENT

Institute for Animal Husbandry, Belgrade-Zemun is organizing traditional International Symposium, "Modern Trends in Livestock Production" with following topics:

1. Genetics and Selection of Farm Animals
2. Breeding and Reproduction of Farm Animals
3. Physiology and Nutrition of Farm Animals
4. Organic Livestock Production
5. Livestock Feed and Ecology
6. Animal Welfare and Health Care
7. Technology and Quality of Animal Products
8. Protection of the Environment and Biodiversity in Animal Production
9. Alternative Production Methods in Livestock Production

The work of the Symposium will be divided in sessions according to animal species.

Full paper submission deadline is **April 30th 2021**. Authors should prepare full paper according to the "Instruction for Authors" of journal "Biotechnology in Animal Husbandry" (www.istocar.bg.ac.rs), otherwise the paper will not be considered.

All submitted symposium papers will be peer reviewed. Members of the International Scientific Committee will select papers for oral presentations, other papers will be presented in poster sessions. Oral and poster presentations should be prepared in English. All accepted papers will be published in the Proceedings.

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On behalf of Organizing Committee

Dr Čedomir Radović,
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