Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Food Safety and Infection Biology

Philosophiae Doctor (PhD)
Thesis 2019:29

Reproductive problems in cattle in Bosnia and Herzegovina

Reproduksjonproblemer hos storfe i Bosnia-Herzegovina

Adis Softic

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Adamstuen, Oslo (2019)

Thesis number 2019:29

ISSN 1894-6402

ISBN 978-82-575-1774-8

Content

Acknowledgements

This PhD project was performed between March 2014 and August 2018 at the Department of Food Safety and Infection Biology, Faculty of Veterinary Medicine, Norwegian University of Life Sciences (NMBU), Oslo, Norway. The project was funded by the Norwegian State Educational Loan Fund (Lånekassen) through the Quota scheme. The financial support was obtained from the institutional cooperation between Norwegian University of Life Sciences and Veterinary Faculty of the University of Sarajevo, Bosnia and Herzegovina. This funding is greatly appreciated.

In the study period, I have worked with great people and received much support both in Norway and in Bosnia and Herzegovina. Herewith I would like to acknowledge my supervisor and co-supervisors for their trust and support and for enabling me to complete the PhD. First and foremost, I would like to express my sincere gratitude to my main supervisor Prof. Dr Eystein Skjerve for the continuous support of my PhD research and related studies, for his patience, motivation, and immense knowledge, but also for efforts to widen my research from various perspectives. I could not have imagined having a better supervisor for my PhD study. Eystein, it is a great pleasure to have you not only as a supervisor but also as a lifelong friend. Thank you very much. I would like to thank my co-supervisors: Prof. Dr Nihad Fejzic for giving me initial recommendation as a good candidate for this project and offering his indispensable academic, logistic and moral support; Prof. Dr Kassahun Asmare Wondim for giving his insightful comments and help, useful tips and encouragement, Dr Erik Georg Granquist for his help and kindness, and for admirable guidance and lectures in the field of animal reproduction; and Prof. Dr Jacques Godfroid for his generous support, and essential contribution to planning and realization of studies in my PhD.

My sincere thanks go to the staff of the Department of Infectious Animal Diseases and Epizootiology, Veterinary Faculty of the University of Sarajevo: Prof. Dr Tarik Bajrovic, Prof. Dr Behija Dukic, Associate Prof. Dr Lejla Velic, Mr. sci. Toni Eterovic, Esmir Bajric, Admira Bogunic, Maja Ivkovic, and Slavoljub Tomic who provided me an opportunity to join their team as intern, and who gave access to their laboratory and research facilities. Without their precious help, it would not be possible to conduct this PhD. Also, I would like to thank Prof. Dr Teufik Goletic and Prof. Dr Aida Kustura for their generous help in laboratory work and analyses, as well as their appreciated contribution to the manuscripts for publication. My sincere thanks go to Associate Prof. Sabina Seric-Haracic for her generous help during my internship at the Veterinary Faculty in Sarajevo, as well as for the encouragement she given me in the planning and realisation of this thesis. My special thanks go to the staff of the Veterinary Institute in Bihac, especially Mr. sci. Ermina Nogic and Mr. sci Zlatko Jusufhodzic for all the hard logistic work they have done for me. I would like to acknowledge Dr Adam Dunstan Martin for his generous contribution to the production of the thesis and manuscript for publication and for keeping me on the right track to realising this PhD.

I wish to extend my deepest thanks to the current and former academic and research staff located at the EpiCentre: Prof. Dr Stig Larsen, Ass. Prof. Dr Rolf Bjerke Larsen, Dr Paul Midtlyng, Dr Jostein Mulder Pettersen, Dr Chisoni Mumba, Dr Luke Nyakarahuka, Dr Doreen Sitali, Dr Ann-Katrin Llarena, Dr Elibariki Reuben Mwakapeje, and the PhD students Sol Høgseth, Gudrun Seeberg Boge, Sveinung Eskeland, Trond Holland, Letemichael Negash Welekidan and David Persson. I will always be happy to remember our shared lunch breaks and funny discussion on various topics of our every day and research life. You were an excellent company to have, and I was very privileged to work with such academic group. Also, I would like to thank my Bosnian fellow Dr Selma Hurem and Sabina Sibcic for nice collegiality. We went together through all ups and downs of our PhD journey, and it was very nice to have you with myself.

I am deeply indebted to 62 of my colleagues, veterinarians who participated in this PhD project. Dear all, you are greatly acknowledged for your support during the fieldwork. I will always remember our inspirational discussions, and your marvellous help in obtaining necessary materials, animating farmers to participate and dedicated follow-ups. I wish to extend my due appreciation to all farmers in surveyed cantons for their cooperation and provision of valuable information and for allowing us to sample their animals.

And finally, I would like to thank my dear family and friends for their love, encouragement and support. You are the cause of my success and having you in my life is great happiness and privilege. I am forever indebted to my dear parents Sabina and Edhem for giving me opportunities and experiences that have made me who I am. In moments when I doubted myself, they selflessly encouraged me to explore new directions in life and seek my own destiny. Dragi moji, bez vaše ljubavi i podrške nikada ne bih bio to što jesam. Najiskrenije i punog srca, hvala.

Adis Softic,

Oslo, February 2019

Summary

The bovine industry represents the most important and the fastest growing branch of agriculture in Bosnia and Herzegovina (BH). Thus, this industry has experienced significant changes over the past twenty years regarding increased production and exports, as well as aligning its regulatory mechanisms with those in the European Union (EU). There are numerous challenges and limiting factors that affect the profitability and competitiveness of the cattle production in BH. Infectious causes/diseases of bovine reproduction are recognised not only as a limiting factor in the total production but also as a veterinary public health issue worldwide. However, the extent and epidemiological characteristics of reproductive disorders in cattle are largely unknown in BH. Hence, this PhD thesis aimed to fill some of the information gaps regarding the four infectious agents (*Brucella* spp., *Chlamydia abortus, Coxiella burnetii; Neospora caninum*) known to cause reproductive disorders in cattle and their links to management factors, as well as describing and evaluating the general reproductive indicators in commercial dairy farms in BH. This was achieved through three specific objectives which were our planned publications.

All studies were done in three cantons of the Federation of BH (FBH), i.e., the Una-Sana Canton, the Canton 10, and the Central-Bosnia Canton. The most prevalent agent found in our first cross-sectional study was *C. abortus*, while *C. burnetii* and *N. caninum* had a moderate distribution among cattle population in selected areas. Although brucellosis (*Brucella* spp.) was traditionally assumed as the primary cause of reproduction problems, the low animal- and herd seroprevalence found in our first study indicated that *Brucella* spp. does not seem to represent a problem in bovine reproductive health.

In our second study, we used a structural equation modelling (SEM) to establish a causal web of associations found in previously conducted multivariable mixed-effect logistic regression. This study showed that the presence of dogs and stray dogs in farm premises increased the odds of finding an animal- and herd level seropositivity to *C. abortus*, *C. burnetii* and *N. caninum*. Just *N. caninum* was found as the only infectious agent that was associated with reproduction problems such as abortions and stillbirths. Farm management practices and farm environment were associated with the animal- and herd seropositivity, as well as the occurrence of severe reproduction problems (abortion, stillbirth, retained foetal membrane and metritis).

Finally, in our third study, we identified, calculated and evaluated key reproductive performance in dairy farms in Una-Sana Canton. Our findings pointed to the lack of production-

and reproduction performance recording in BH. Moreover, managers/farmers of farms with an above-average number of animals were reluctant to record animal performance in their farms. The future challenge for veterinary authorities and farmers is to organise a comprehensive system of recording of animal production- and reproduction performances, which will contribute to the rapid integration and better competitiveness on the EU market.

Sammendrag (Summary in Norwegian)

Storfenæringa representerer den viktigste og raskest voksende grenen av landbruket i Bosnia-Hercegovina (BH). Denne næringa har gjennomgått betydelige endringer de siste tjue årene med hensyn til økt produksjon og eksport, samt en viss harmonisering med reguleringsmekanismer i EU. Det er mange utfordringer og begrensende faktorer som påvirker lønnsomheten og konkurranseevnen til storfeproduksjonen i BH. Smittsomme sjukdommer og andre reproduksjonsproblemer blir ikke bare anerkjent som en begrensende faktor i totalproduksjonen, men også som et dyrehelseproblem over hele verden. Imidlertid er omfanget og de epidemiologiske særtrekkene ved reproduksjonssykdommer hos storfe stort sett ukjent i BH. Denne doktorgradsavhandlingen har forsøkt å fylle noen av informasjonshullene angående fire viktige smittestoff (*Brucella* spp.*, Chlamydia abortus, Coxiella burnetii, Neospora caninum*) kjent for å forårsake reproduksjonslidelser hos storfe og også påvirker måten storfenæringa drives. I graden tos sikte på å beskrive og evaluere de generelle reproduksjons indikatorene i kommersielle melkebedrifter i BH. Dette ble undersøkt gjennom tre publikasjoner.

Alle studier ble gjort i tre kantoner av Føderasjonen BH (FBH), Una-Sana, Kanton 10, og Sentral-Bosnia. Det mest utbredte smittestoffet i vår første tverrsnittstudie var *C. abortus*, mens *C. burnetii* og *N. caninum* hadde en moderat spredning blant storfe i utvalgte områder. Selv om brucellose (*Brucella* spp.) tradisjonelt ble antatt å være den primære årsaken til reproduksjonsproblemer, viste den lave dyre- og flokkprevalensen som ble funnet i vår første studie at *Brucella* spp. ikke ser ut til å utgjøre et problem i reproduktiv helse hos storfe.

I vår andre studie brukte vi en spesiell statistisk modell (Structural Equation Model, SEM) for å etablere en årsaksmodell basert på flere multivariable logistiske regresjoner. Denne studien viste at tilstedeværelsen av hunder og løshunder i og omkring gårdene økte sjansen for å seropositivitet for *C. abortus*, *C. burnetii* og *N. caninum.* Bare *N. caninum* ble funnet som å være forbundet med reproduksjonsproblemer som abort og dødfødsler. Husdyrpraksis og gårdsmiljø var knyttet til denne seropositiviteten, som igjen var koblet til reproduksjonsproblemer (abort, dødfødsel, tilbakeholdt etterbyrd og metritt).

Til slutt, i vår tredje studie, beskrev vi viktige reproduksjonsprestasjoner i melkebedrifter i Una-Sana. Våre funn pekte på mangelen på produksjons- og reproduksjonsstrategier BH. Dessuten

var ledere / bønder på gårder med et over-gjennomsnittet antall dyr motvillige til å registrere informasjon om dyr på sine gårder.

Den fremtidige utfordringen for veterinærmyndigheter og bønder er å organisere et omfattende system for registrering av dyreproduksjon og reproduksjon, noe som vil bidra til rask integrering og bedre konkurranseevne på EU-markedet

Abbreviations

AFC	Age at first calving
AFI	Age at first insemination service
AI	Artificial insemination
BAM	Convertible mark (currency)
BD	Brcko District
BH	Bosnia and Herzegovina
BVD	Bovine viral diarrhoea
BVDV	Bovine viral diarrhoea virus
C10	Canton 10
CA	*Chlamydia abortus*
CB	*Coxiella burnetii*
CBC	Central Bosnia Canton
CCI	Calving-to-conception interval
CEFTA	Central European free trade agreement
CFI	Calving-to-first service interval
CI	Calving interval
ELISA	Enzyme linked immunosorbent assay
EU	European Union
FAMI	Federal Agro-Mediterranean Institute
FBH	Federation of Bosnia and Herzegovina (entity)
FSH	Follicle-stimulating hormone
GDP	Gross domestic product
GnRH	Gonadotropin-releasing hormone
GVA	Gross value added
LH	Luteinising hormone
NC	*Neospora caninum*
NSP	Number of services per pregnancy
PR	Pregnancy rate
RS	The Republic of Srpska (entity)
SEM	Structural equation model
SVO	State Veterinary Office of BH
USC	Una Sana Canton
VFS	Veterinary faculty in Sarajevo

List of papers

Paper I

The serostatus of *Brucella* spp., *Chlamydia abortus*, *Coxiella burnetii* and *Neospora caninum* in cattle in three cantons in Bosnia and Herzegovina

Authors: Softic, A.; Asmare K.; Granquist E.G.; Godfroid J.; Fejzic, N.; Skjerve E.

Status: Published in **BMC Veterinary Research** (2018) DOI:10.1186/s12917-018-1361-z

Paper II

A causal approach to understanding management factors, reproductive underperformance and reproductive infections in dairy cattle

Authors: Softic, A.; Asmare K.; Granquist E.G.; Godfroid J.; Fejzic, N.; Skjerve E.

Status: Submitted to **PLOS One Veterinary Epidemiology** (February 2019)

Paper III

Reproductive performance in a selected sample of dairy farms in Una-Sana Canton, Bosnia and Herzegovina

Authors: Softic, A.; Martin A.D.; Skjerve E.; Fejzic N.; Goletic T.; Kustura A.; Granquist E.G.

Status: Submitted to **Animal: The International Journal of Animal Biosciences** (February 2019)

Introduction

Bosnia and Herzegovina

General information

Bosnia and Herzegovina (BH) is situated in south-eastern Europe, i.e. in the western part of Balkan peninsula between 42° 33′ 00″ - 45° 16′ 30″ N and 15° 44′ 00″ – 19° 37′ 41″ E. The total state territory encompasses 51.209, 2 km^2. BH has a total of 1.538 km of the state boundaries with the Republic of Croatia in the north, west and south-west, Republic of Serbia in the east and Republic of Montenegro in the south-east. BH is a country with a complex governmental system. It is administratively composed of two entities, and one district, i.e. Federation of Bosnia and Herzegovina (FBH), Republic of Srpska (RS), and District Brcko (BD). Further, FBH is composed of 10 administrative units (cantons), and cantons are composed of municipalities. On the other side, RS is administratively composed of five regions, and regions are composed of municipalities.

***Figure 1**. Map of Bosnia and Herzegovina (E) and neighbouring countries (Source: Google maps)*

Climate

Given the geographical location, the climate of BH is influenced by the Pannonian lowland from the north, and the Adriatic Sea from the south. There are three basic climatic types: moderate continental type in the north, mountain type in mountainous and sub-mountainous regions and Mediterranean type in the south and south-west. Regions with moderate continental climate are characterised with four seasons, as well as moderate to warm summers and moderate to cold winters. The average air temperature recorded in the coldest month January ranges from -0.9°C to -0.2°C, while the average air temperature recorded in the warmest month July is between 18.7°C and 23°C (Anonymous, 2013c). Generally, a mountain climate is represented in regions with altitude ranging from 500 to >2000m. This climate type is characterised by mild and short summers and cold winters with abundant snowfall. Sub-mountainous (perymountain) subtype of mountain climate is represented in basins, and its average annual air temperature is between 8°C and 11°C. In regions over 1800 meters above sea level, the mountain climate has characteristics of alpine climate, with the average air temperatures of -3°C in January and <18°C in July. Southern and south-western parts of BH have a continental Mediterranean climate which is characterised by warm and bright summers and mild winters. Average air temperatures in January are ranged from 3°C to 5°C, while in July the average air temperature is >24°C (Anonymous, 2013c).

Demography

BH belongs to the group of small European countries with the total population slightly above 3.5 million people, as evidenced by the recent census in 2013. There is the negative difference between two consecutive censuses (approx. 4.4 million in 1991) (Anonymous, 1991), which represents a substantial decrease in total population of 19.3%. This reduction of almost a fifth of the population is a consequence of the war in BH (1992 - 1995), but also post-war social and economic transition and migrations. Forced and voluntary migration, the poor return of the refugees in the post-war period, as well as a recent outflow in EU countries, are factors that contribute to the population decline in BH (Cicic, 2017). As an indirect result of such dynamic, there is a change in age structure of the population which is accompanied by two parallel trends: an increase in the share of old people (>65 years) and the decline in the share of young people (0-14 years) (Emirhafizovic and Zolic, 2017). Thereby, the average age of the population has increased by 10 years (since 1991) and has reached 39.5 years. Furthermore, the unemployment rate in BH is nearly 25% and is among the highest in Europe (Anonymous, 2013a, Anonymous, 2018a). This is another factor that likely contributes to emigration and a constant decline in population.

Agriculture in BH

Historical aspect of agriculture in BH

BH was part of the former Yugoslavia, a federative country composed from six autonomous republics. Before the Second World War, BH was exclusively an agriculture-oriented country with a low degree of development in other sectors in comparison with its neighbours and countries in Western Europe. The agricultural sector was also strategically important for the reconstruction of the state economy in the post-war period. Industrial development based on the potential in raw material and mineral wealth of BH has favoured the development of heavy (metallurgy) and chemical industry. The employment in the industrial sector reached its peak in 1980's, and 58.4% of the population was employed in the industry in 1981 (Nurkovic, 2007). In rural areas there was a corresponding rapid population decrease as people moved to newly-industrialised and urban areas. The Yugoslavian socialist government system also introduced the concept of common ownership as a basis for social equality and justice, which had certain repercussions on the agriculture, in general. Agrarian reform, confiscation and nationalisation of the land led to the formation of a large number of small and medium-sized units, with no more than 10 ha per unit. Since the land units were mainly allocated to farmers for utilisation, but not for possession, there was a tendency of further fragmentation. Although there was an investment program that would tend to increase agricultural productivity and competitiveness, it was never fully achieved. On the contrary, former Yugoslavia experienced a deep economic crisis in the 1980's, and the already unstable agricultural sector was further weakened. A gradual recovery lasted until 1991 when the decomposition of Yugoslavia started, followed by the war in BH (1992-1995). The agriculture, among other sectors, suffered tremendous damage during the war, and the post-war period was mainly characterised by the change in state government system and socio-economic transition.

Economical aspect

Today, BH belongs to the group of mostly rural countries, i.e. 59.9% of the population live in rural areas (Anonymous, 2018e). However, there is no clear distinction between rural and urban areas, and hence, there are two facts that should be considered. According to the size, agricultural infrastructure and local services, there is the primary distinction between villages (local communities) and larger settlements. On the other side, there is a specific difference between the municipalities bordering the large cities and are economically influenced by them, and municipalities that are distant from the cities whose population works in public or private sector, and/or has some agricultural activities (Anonymous, 2013b). There are approximately 1.5 million households in BH, of which 31.4% are engaged in some agricultural activity. However, the number of commercial agricultural households is far smaller. A total of 15.6% of households with any agricultural activity also have placement of their products on the market, i.e. 4.9% of the total number of households are commercial agricultural estates.

Still, the sector is one of the most important sectors for employment in BH, given that 18.9% of the total population are engaged in agricultural or related activity (Anonymous, 2017b, Kovacevic, 2017). Long-term trends, however, point to the gradual decrease in the number of employees in agriculture. A possible reason for such negative trends can be agrarian overemployment given the relatively small contribution of this sector to the social standard, i.e. labour force leaves the rural areas and seek for employment in non-agricultural sectors. In addition, a recently intensified migration to the EU countries could negatively affect the actual number of agricultural employments. Furthermore, less than 1% of population with agricultural activity, is formally registered as employed in agriculture (Anonymous, 2017f).

The occurrence of extreme weather conditions in the period 2006-2016 (particularly drought in 2012 and the catastrophic flood in 2014) also contributed to an unstable share of agriculture in the state economy and to a decline in the agricultural employment (Anonymous, 2015c). The great importance of the agricultural sector in a total economy of BH is reflected in the share of the total gross value added (GVA), trade, total employment, as well as, the provision of food for the population. Although the agricultural growth has been recorded in the period 2006-2016 (from 1.6 billion BAM (convertible mark) in 2006 to 1.8 billion BAM in 2016), the share of agriculture in the total GVA decreased in the same period, due to the faster growth of non-agricultural sectors. Recently (2016), the total share of agriculture in the GVA structure was 6.4%.

Agricultural production

The potentials for agricultural production in BH, reflected in favourable climatic conditions, cultivable land, many autochthonous species and original products as well as tradition, are not fully utilised (Anonymous, 2017a). BH disposes a total of 2.2 million ha of agricultural land of which 1.6 million ha represents arable land in general sense, while the remaining 600,000 ha are pastures (Table 1) (Anonymous, 2017d, Anonymous, 2017e). However, the agricultural land is not used by the general social interest. Although the arable land covers approximately a million ha of the total agricultural land in BH, only 52% of its territory was used in 2016 (Anonymous, 2017a). A portion of unused arable land is covered with minefields and unexploded remnants of war, representing 1081 km^2 or 2.2% of the total territory of BH (Anonymous, 2018b).

Table 1. *The structure of cultivable land in BH, entities and BD in 2016 (*000ha) (Anonymous, 2017d, Anonymous, 2017e).*

Entity/District	**Agricultural land***	**Cultivable land (ha)***					**Pasture***
		All	Arable land	Orchards	Vineyard	Meadows	
FBH	1179	742	425	46	5	267	435
RS	1004	812	575	51	1	185	191
BD	36	35	30	4	0	1	1
Total	2219	1589	1030	101	6	453	627

The structure of the used arable land in BH has been relatively constant in the period 2006-2016. Cereals represent the greatest share in the total sown area coverage (58%), followed by roughage (26%). Fruit and vegetable cultivation takes place at 15% of the total sawn area, while the cultivation of industrial crops comprises only 1% (Anonymous, 2017f, Anonymous, 2017a). The total plant production in BH mainly follows the trend in the coverage of the sown area, with the exception of the production of industrial crops that are more represented.

Livestock sector

Although it took a smaller share in the agriculture production in comparison with plant production, i.e. only 37% in 2015, the livestock sector represents the most important and most potent agricultural sector in BH, especially with a certain potential in international trade and income generation. After the complete devastation in the 1990's, there are constant efforts and investments in the reconstruction of the livestock sector through strategic plans of rural development at the entity level, as well as harmonisation of the state legislation with the standards and legislation set by the EU.

The system for identification and control of animal movement, however, records the undulant trend with the gradual decrease in the number of all categories of domestic animals, except poultry, in the last ten years. The starting point in the reconstruction of the sector was to renew the livestock fund.

Table 2. *The number of livestock in BH (preliminary result in 2017) (*000) (Anonymous, 2017a).*

Cattle*		**Swine***		**Sheep and goats***		**Horses***		**Poultry***	
Total	Dairy cows and heifers	Total	Sows and gilts	Total	Ewes	Total	Mares	Total	Laying hens
445	272	548	76	1083	593	16	6	21.583	5037

The number of poultry is 4.5 times higher than that recorded in 1991, while the numbers of small ruminants (85%) and swine (91%) has been substantially restored. The number of cattle in BH has still not reached the one from the last census (approximately 63% of the number of cattle in 1991), while the current number of horses is dramatically lower (23%) (Anonymous, 1991, Anonymous, 2013a)

Cattle production and breeding

Cattle represents the most important subsector in the total agricultural production in BH, and cattle production has the longest tradition among all livestock keepers and producers. The geographical position, climate and favourable composition of the agricultural land, i.e. the dominant share of meadows and pastures (Table 1) represent a significant resource for the further development of this subsector. Furthermore, beef and milk production represent the nutritional basis for the population in BH, and this industry has the potential for the development of more market niches for international trade with neighbouring countries, linked to the Central European free trade agreement (CEFTA) and EU countries. Accordingly, ministries of agriculture in FBH and RS have recently adopted breeding programs aimed at intensifying and systematising the production at the farm and animal level. Also, those programs define breeding goals, selection program for certain breeds, as well as the conditions for the improvement in product quality (Anonymous, 2016a, Anonymous, 2018d, Anonymous, 2018c). Thus, there is the opportunity for the increase of the number of registered agricultural holdings with commercial dairy/beef production.

On the other side, there are many weaknesses and limiting factors that directly affect the sustainability and production in this subsector. There is an exceptional fragmentation of the agricultural land properties, i.e. the properties consist of many spatially dispersed, small-sized and irregularly shaped parcels. This situation greatly impedes the intensification of the production through increased costs of the transport and storage of the products (Odak et al., 2017). Given the unresolved issue of the land restitution which is one of the prerequisites for the access of BH in the EU, there is a process towards the land redistribution, and if it would stay unresolved, this problem could be even more important in the future.

The lack of the technical equipment, the low level of organisation and infrastructure and inadequate technological, market and managerial skills of farmers are recognised limiting factors in cattle rearing system in BH. All these factors contribute to the production shortfalls, either individually or jointly. Further, most producers are economically dependent on the placement of their products and the means of the direct budgetary support from the state and entity levels. Consequently, any production shortfall results in the profit loss, and possible delays in the payment of the government subsidies makes this production unsustainable.

The cattle industry has steadily expanded in BH over the last twenty years regarding the cattle population and production. After the conflict in BH in 1990's, the rebuilding of the cattle population was ensured through organised donation programs of high-producing dairy and beef breeds, and through governmental rural development programs primarily aimed at bringing back the displaced population. Afterwards, the cattle population has increased to the number when it could be maintained exclusively through the rearing of own replacement animals, i.e. without the need for further donations and import.

One of the critical weaknesses in the intensification of the cattle production in BH is the small number of animals per farm (3-10 animals). Hence, the self-sustainment of the cattle population is clearly compromised, and there is a constant decrease in the number of dairy cows and heifers in the last ten years, from approximately 356,000 in 2006 to 272,000 in 2017 (Figure 2). Mainly small-sized producers, facing numerous limiting factors, cannot follow strict EU regulations on food safety and product quality. Consequently, they abandon the production or reorient themselves to another agricultural activity (Anonymous, 2017f). This partially explains the decrease in the number of dairy cattle in BH. However, the influence of other factors remains unknown.

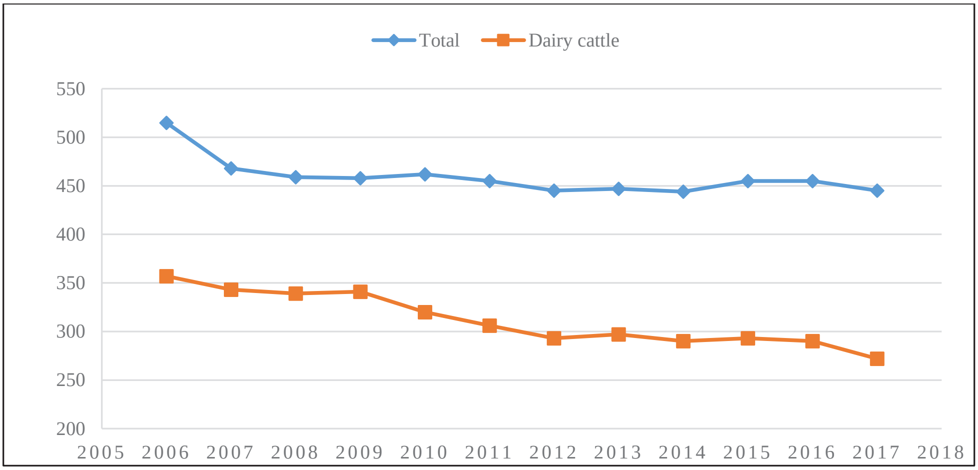


Figure 2. *The number of cattle in BH in the last ten years is fairly constant, while the number of dairy cattle is undulant and steadily decreasing*

Animal Nutrition is one of the fundamental factors in expressing phenotypic performance and genetic potential for the cattle production. Its impact on the physiological functioning of the organism is achieved primarily through the basic metabolism (maintaining needs), followed by the meeting all the additional productive and reproductive needs. Given the wide variety of plant species in BH, a large number of plants and their products is usually used in ordinary cattle diets. Also, the diet is adapted to the production type (dairy or beef cattle), reproductive status (pregnant/non-pregnant cows), and season (summer or winter scheme). The nutrition scheme for all groups of animals is usually composed of partial grazing in the period April-October, and feeding by forage such as cereals, industrial by-products and grain residuals, grass-cover mixture, grass and corn silage, hay, haylage, and the addition of concentrated nutrients, vitamin-mineral mixtures, premixes and dry-off supplements (supplements that support the natural decrease in milk production at the ned of a lactation cycle). There are certain differences in the choice and quantity of the given feed among producers, which are conditioned by the feed price, the purchasing power of the producers, as well as the type and volume of their production. Since most of the farmers have a mixed type of holding, i.e. crop-livestock, they mainly use own-produced feed for cattle. However, such production greatly depends on the weather condition, e.g. a long-lasting drought in 2012 and disastrous floods in 2014 resulted in a substantial production loss (Anonymous, 2015c).

Dairy production

Milk production is a strategic branch and the basis for the development of the entire agricultural sector in BH. Unlike other agricultural producers, milk producers are able to sell all the produced amount of milk, due to a stable milk market demand. However, the dairy production in BH has encountered serious problems in the period 2013-2015. The failure to meet the EU export criteria for milk and dairy products, followed by the loss of the most important market in the Republic of Croatia after its accession to the EU in July 2013, had an adverse effect on milk producers/processors and lead to the deficit in foreign trade. A positive shift was achieved after obtaining the official permit for the export of milk and dairy products to the EU market in 2016. This positive development has been continued by increasing the number of dairies (milk processors) licensed for the export and resulted in the final export permit for all types of milk and dairy products (lists A and B) in 2018. The raw milk production is mainly related to the production of cow's milk (97%), while the sheep (2%) and goat's (1%) milk production have a small share. The total milk production in BH reached 701 million litres in 2016 (Anonymous, 2017a). The positive trend in the total production is evident in the last three years, but the overall trend in milk production in the last ten years had certain variations. The leading product of the dairy industry is drinking milk intended for the domestic market and the export. Approximately 120 million litres of drinking milk is produced on an annual basis. The production of yogurt and other fermented milk beverages is a second-ranked dairy production in BH, with the largest production (approximately 40 million litres) obtained in 2016 (Anonymous, 2017a). The amount of produced cheese was approximately 6400 tons (data in 2016), and was 4% greater than in previous years (Anonymous, 2017a). The production of butter (540 tons in 2016) and other dairy products is represented to a lesser extent in BH (Anonymous, 2017a).

Beef production

The meat production in BH has the potential to become an important agricultural sector both for meeting the needs of the domestic market and as a prospective export good. However, the meat production and industry are faced with a number of restrictive factors and obstacles which resulted in the production drop and the imbalance between levels of production and market demand. There is an evident reduction in the number of breeding and beef cattle, and consequently, there is a negative trend in the number of slaughtered animals in the last ten years. In addition, the fattening costs are increased due to the lack of animal feed from the local land, as well as the high price of imported fodder. Such increase in the fattening costs has also lead to the price increase in the final product, i.e. animals for slaughtering. Hence, the representatives of the meat industry in BH have oriented on the import of livestock or meat mainly from the EU and CEFTA countries, instead of buying from domestic producers.

After several unfavourable years, the gradual recovery for the beef producers and meat industry started with the substantial increase in the export of meat and meat products to the Republic of Turkey in 2014. The total export reached 9 million kg in 2015, and this also influenced a gradual restoration of the slaughter capacities in BH (Anonymous, 2017a).

Organisation of veterinary services in BH

A prerequisite for an efficient livestock production is a coherent and structured veterinary service system to assist in dealing with a range of questions related to animal diseases and welfare. The veterinary service in BH is hierarchically organised at the state, entity, cantonal and municipal levels, in accordance with the state order. The State Veterinary Office (SVO) is the central veterinary organisation which is under the jurisdiction of the Council of Ministers of BH and Ministry of Foreign Trade and Economic Relations of BH. The role of the SVO is laid down by the Veterinary Law of BH ("Official Gazette of BH" 34/02) and consists in proposing regulations for control and prevention of infectious and parasitic animal diseases, prescribing conditions for the international trade of animals and products of animal origin and coordinating activities with other veterinary organisations in BH and international institutions. Further, veterinary service is organised at the entity/district level in form of sectors of the Ministries of agriculture, water management and forestry of FBH, RS and BD. Those sectors are responsible for the implementation of SVO's regulations, but also, have additional roles laid down by the Veterinary Law of FBH ("Official Gazette of BH" 46/00) and Veterinary Law of RS ("Official Gazette of RS" 42/08). Their role is reflected in ensuring the control and prevention of animal diseases and zoonoses, in improving the animal production and reproduction, as well as the veterinary public health. The cantonal ministries of agriculture, water management and forestry, i.e. their veterinary sectors are the competent authorities for implementing regulations passed at the federal level. The municipal veterinary services, i.e. ambulances, stations, hospitals or clinics are, on the other hand, the only executive bodies under the jurisdiction of all higher levels of the veterinary network in BH. They are engaged in routine field services, diseases prevention, treatments, and other animal and veterinary public health activities. All activities regarding the investigation, confirmation of the occurrence, control of diseases as well as food safety are undertaken at the state level by national analytical and diagnostic laboratories.

In all respects, the veterinary network in BH is extraordinarily complex. Such a veterinary organisation is not necessarily ineffective, especially considering ongoing efforts towards adaption of EU regulations. However, there are logistic, technical and financial limitations in all levels of the veterinary chain of commands. In addition, despite the presence of clearly defined actions, the inevitable overlap in responsibilities and competencies is still present. This could also have a negative impact on bovine reproduction. Figure 3 shows details of the organisation of the veterinary network in BH.

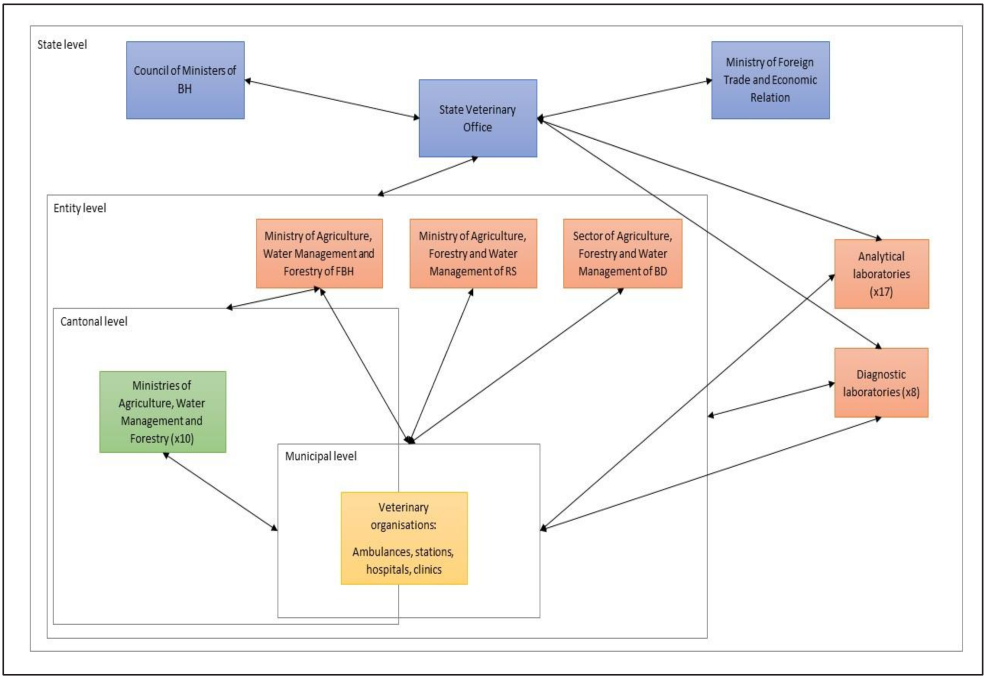


Figure 3. *The schematic depiction of the veterinary service network in BH*

Bovine reproduction

Normal reproductive physiology

Given the growing need for beef- and dairy products intended for human consumption, there is a permanent focus on the bovine reproductive traits and potency worldwide as a principal determinant in cattle production. There is a need to understand the potential influence on infectious- and non-infectious factors such as nutrition and management in the critical reproductive function of the cow.

The overall reproductive physiology can be divided into several episodes considering the time course of reproductive events. A first critical physiological milestone in cow reproductive life is puberty, which represents the transition from the anovular state to regular cyclic ovulations (Estill, 2015). The thinning of the sensitivity to oestradiol 17-β negative feedback may lead to the release of luteinizing hormone (LH) ovulatory surge. Such frequent LH pulses stimulate follicular maturation, leading to the first ovulation. Although the first ovulation is not synonymous with puberty, it is generally accepted that puberty in cattle occurs at the approximate age of 10 to 15 months (Estill, 2015). Besides the inevitable endocrinal changes, the average puberty age is primarily influenced by the heifer's body weight, as well as genetics, breed, nutrition and season (Robinson and Shelton, 1991). After initiating the normal cyclicity, heifers/cows continue to have oestrus cycles regularly, and are interrupted either by physiological (pregnancy) or pathological conditions (Peter et al., 2009).

The neuroendocrinal control of the oestrus cycle is determined by the feedback mechanisms of the hypothalamic-hypophyseal-gonadal axis (Chagas et al., 2007, Estill, 2015). Gonadotropin-releasing hormone (GnRH) reaches the adenohypophyseal gonadotrophs and stimulates synthesis and release of LH and follicle-stimulating hormone (FSH). Further, these hormones have a direct impact on the gonads stimulating growth and evolution to the dominant follicle (FSH), and its selection and ovulation (LH). In addition to these hormones, the crucial role in expressing the typical oestrus behaviour is linked to oestradiol, while progesterone stimulates the formation and maturation of the corpus luteum (CL). After the ovulation at Day 0 (oestrus), the circulating concentration of progesterone increases and remain elevated for the duration of the luteal phase of oestrus. If there is no pregnancy, the embryonal interferon-tau is not recognised, and the prostaglandin (PG)$F_{2\alpha}$ release leads to the luteolysis (Lonergan and Forde, 2014). An elevated circulating concentration of oestradiol marks the start of follicular phase characterised by the rapid growth and maturation of the follicles. Furthermore, elevated oestradiol leads to oestrus behaviour and preovulatory surge of GnRH, and thus the beginning of the new cycle. The cow is normally a mono-ovulatory animal, i.e. only one follicle will ovulate per oestrus cycle. The typical length of the oestrus cycle is 18 to 23 days (Sartori et al., 2004), although some authors suggest a wider range of 17 to 24 days, with the average at 21 days (Estill, 2015).

The postpartum is the period between parturition and the resumption of ovarian cycles. This is economically the most important period in cows' reproductive life, from the farmer's perspective. The late gestation is characterised by the elevated concentrations of oestradiol (placenta-derived) and progesterone (CL). Just before the parturition, there is a drop in the concentrations of these hormones and their gradual removal from the circulation. Consequently, the negative feedback of oestradiol is stopped, and there is depletion of LH and FSH stores in the adenohypophysis. Follicular growth rapidly resumes within 7 to 10 days postpartum (Crowe et al., 1998), while the ovulation resumption is delayed due to a GnRH-mediated lack of LH surge (Crowe et al., 2014). The timeline and mediating influences underlying the anovulatory period in postpartum are invariably related with suckling, and low body condition in cattle, as well as a metabolic burden and negative energy balance in dairy cows (Crowe et al., 2014, Estill, 2015).

Reproduction problems

Reproduction and reproductive management of cattle are expected to have one of the crucial roles in maintaining the current cattle population and in its prospective growth. Beef and dairy cattle farming operations are based on the successful production of the offspring, i.e. one calf per cow per year. The need for such operations is evident in the cow-calf system in BH, where the calves are the final product – slaughtered beef. On the other hand, the maintenance of such reproductive intensity (one calf per cow per year) in the dairy industry may not be completely necessary. Previous studies suggested that the shape of the lactation curve and deliberate increases in calving intervals may have a certain economic benefit (van Amburgh et al., 1997, Arbel et al., 2001). However, dairy farmers in BH recognised two major advantages in achieving one calf per cow per year. Namely, the increase in milk production in postpartum and a calf, regarded as a by-product in production, represent a substantial and constant income.

Generally, the reproductive performance in the dairy population has decreased worldwide over the past 50 years (Lucy, 2001). The reasons for such a decline are multifactorial and complex. However, increased production efficiency and milk yield per cow, genetic adjustments and changes in the herd and reproductive management can partially explain the observed decrease (Grohn and Rajala-Schultz, 2000, Lucy, 2001, Nebel and McGilliard, 1993). However, recent studies suggested that the declining trend has been slowed down (Philipsson, 2011), or that the link between increased production and decreased reproduction is statistically significant, but the effect size is practically small and modulated by the herd production level (Rearte et al., 2018).

BH is no exception in the global trend, and among the many factors responsible for this development are the reproductive disorders that are frequently observed and reported among the farmers and the veterinary service network. In our studies, a total of 62 municipal veterinary officers from 18 municipalities were engaged in the realisation of the field part of this PhD project. All veterinary officers were interviewed and also gave their written records regarding the occurrence and persistence of reproductive problems in the establishment of the normal cyclicity (anoestrus, silent oestrus, elevated days-to-conception intervals), the failure of fertilization and pregnancy wastage (repeat breeding cows, irregular cyclic activity), foetal and perinatal deaths (abortion, stillbirth, dystocia), and diseases in the postpartum (retained fetal membranes, metritis). This information constituted an important reference point for the PhD project. Reported reproductive disorders were likely the result of the synergy of pathophysiology, management or disease-related factors. The common classification in non-infectious and infectious causes of reproductive disorders were followed in this PhD project.

Non-infectious reproductive disorders

Given the metabolic and immune load that pregnancy represents for the cow's organism, there are many opportunities for the development of non-infectious reproductive disorders. A thorough diagnosis of non-infectious origin of the reproductive problem can be achieved only by excluding infectious agents (Hassig, 2007). Further, the non-infectious causes of reproductive disorders can be divided into endogenous and exogenous origin (Hassig, 2007). The **endogenous** origin of reproductive disorders is reflected in the occurrence of heritable and congenital defects which were recognised at an increasing rate worldwide, while exogenously originated reproductive disorders are related to nutritional or managerial factors (Whitlock and Coffman, 2015).

The relationship between bovine reproduction and nutrition is bidirectional, i.e., the reproductive status influences the nutritional requirements, but also assimilated nutrients affect the reproductive functions (Swecker Jr, 2015). The transition period (a turning point in the productive cycle of the cow from one lactation to another) represents extraordinary metabolic stress for cows, leading to the down-regulation of some energetic processes and general health. The occurrence of reproductive problems with uterine health, delayed conceptions and lower pregnancy rates in the transition period were repeatedly observed by farmers and veterinary officers included in this project. The relationship between metabolic stress and impaired fertility have been thoroughly reviewed (Leroy et al., 2008a, Leroy et al., 2008b). Another aspect of nutrition are toxicant materials originating from poisonous plants that are capable of causing reproductive problems such as infertility, early embryonic death, foetal developmental deformities (teratogens), abortions and stillbirths (Baughmann, 2015). Their deleterious effects are caused by acting alone or in interaction with other nutritional, environmental or infectious stressors (Evans, 2011).

Reproductive disorders in cattle are numerous, and their systematic investigation would provide ease of understanding the epidemiological and economic consequences, and hence there is a rationale of finding their actual causes. Given the fact that many of the reproductive disorders are presumably of infectious origin, the exclusion of infections helps in finding the actual cause which can be related to management or nutrition.Veterinary officers engaged in the realisation of this PhD project observed and reported the occurrence of some congenital birth defects or foetal abnormalities that should not be neglected as a reproductive problem in cattle in BH. Obvious defects such as skeletal malformation and soft tissue abnormalities were more likely to be recognised (Figure 4 a-b-c-d), whereas defects of foetal internal organs or abortions might be less obvious and easily missed.

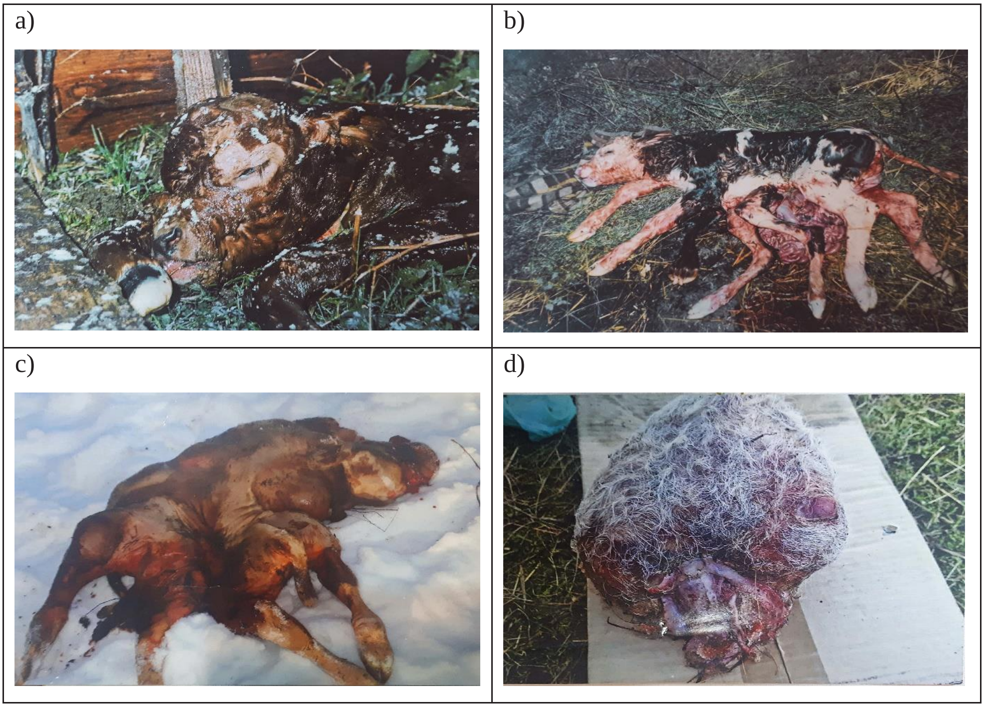


Figure 4. *Congenital defects in calves: (a) Neuropathic hydrocephalus and arthrogryposis of the distal joints in the distal part of forelimbs of a new-born calf. (b) Polymelia (seven legs) in a stillborn affected Simmental calf. (c) Craniothoracopagus conjoined twins with a head, two pelvises and six legs. (d) Acardiac twin foetus (amorphous globosus) with an umbilical cord. A hair-covered soft tissue globe (Photo courtesy by Edhem Softic, DVM)*

Failure in identification or reluctance to report the potentially heritable defects may allow further distribution of mutated genetics among cattle population. Physiological conditions such as twinning are associated with higher rates of stillbirth and abortion (three to seven times more than normal) (Andreu-Vazquez et al., 2011), and the occurrence of aborted or terminated twin pregnancies are documented by the veterinary officers in BH (Figure 5).

As elsewhere, the reduced reproductive performance in cattle in BH caused by naturally occurring toxicants or teratogens are seldom but also can have a considerable economic impact. In the light of reviewed information, however, reproductive problems in cattle in BH cannot be explained without considering nutrition as one of the crucial reproductive constraints.

a)

b)

Figure 5. *a) Abortion in the first trimester of pregnancy (Photo courtesy by Amel Murga, DVM). b) Twin abortion in the second trimester of pregnancy (Photo courtesy by Edhem Softic, DVM).*

Infectious causes of reproductive disorders

Infectious biological agents represent an important cause of reproductive problems in cattle worldwide (Yoo, 2010). The reasons for their high priority in the bovine industry are reflected in their direct negative impact on bovine (re)production, but also in their role in the (veterinary) public health as pathogens with potentially zoonotic character. There are four broad categories of pathogens that have been associated with infertility and abortions in cattle; bacteria, viruses, protozoa and fungi (Baumgartner, 2015). Bacteria are considered the most common infectious agents that are causing reproductive problems in cattle, with more than 25 different opportunistic or pathogenic species involved in abortions (Yaeger and Holler, 2007). Opportunistic bacteria originating from normal microflora of the mucosal surfaces or the environment are the cause of the vast majority of abortions. However, their significance to the herd is minimal (Yaeger and Holler, 2007). The list of the most important bacteria associated with bovine abortion includes *Brucella* spp., *Leptospira* spp., *Listeria monocytogenes*, *Campylobacter fetus* subsp. *veneralis*, *Histophilus somnus*, *Chlamydia* spp., *Salmonella* spp., *Yersinia* spp. (Baumgartner, 2015, Givens and Marley, 2008, Yoo, 2010). Viral infections are also associated with diminished reproductive performance and death in cattle. Their mechanistic background including abortion, stillbirths, deformed neonatal calves, but also the possibility of secondary bacterial or protozoal infections due to the compromised immune system of cows. The most important viruses linked to reproductive disorders in cattle are bovine viral diarrhoea virus (BVDV) (*Flaviviridae*), Bovine Herpesvirus-1, Infectious bovine rhinotracheitis (IBR) virus (*Herpesviriade*), Bluetongue virus infection (*Orbiviridae*), Akabane and Simbu serogroup, Rift Valley fever (*Bunyaviridae*) (Asmare et al., 2018, Givens and Marley, 2008, Kelling, 2007, Yoo, 2010). Ruminant protozoal agents causing abortions are widely distributed, and the list includes four important species, *Tritrichomonas foetus*, *Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* spp. (Abbitt and Rae, 2007, Dubey and Schares, 2011, Michi et al., 2016, Ondrak, 2016). Bovine infertility and abortions related to the mycotic pathogens are sporadic, with the variable incidence worldwide (Walker, 2007). The most commonly isolated fungus from the aborted material is *Aspergillus* spp., followed by the fungi from the order *Zygomycetes* (Givens and Marley, 2008). Given that most of these agents are saprophytic fungi, their natural habitat is in the moist organic environment, as well as improperly stored hay and silage. Taking into account the unfavourable climatic conditions in BH in the last decade, fungi should be considered as one of the possible causes of reproductive disorders.

Accordingly, the State Veterinary Office of BH has adopted the Program of control measures of animal infectious and parasitic diseases that is updated annually. The list of infectious causes that should be investigated in case of abortion (all animals) includes brucellosis, leptospirosis, genital campylobacteriosis, Q fever, listeriosis, and salmonellosis (Official Gazette of BH, issue no. 4/16) (Anonymous, 2016c). Chlamydiae as a possible cause of abortion in animals have been added to the program’s list in 2017 (Anonymous, 2017c). This program has found its application in the testing of brucellosis as a possible cause of abortion in cattle, alongside brucellosis vaccination in small ruminants. The magnitude of reproductive problems in cattle caused by *Brucella* spp., however, remains obscure in BH. The investigation of other agents, however, takes place less often. *N. caninum* is still not added in the list although it is considered as a direct causative agent of abortion in cattle worldwide. Therefore, the absence of official information on *N. caninum*, as well as the lack of scientific information on *C. abortus* and *C. burnetii* as causative agents of abortion in cattle warrants further investigation.

This PhD project focuses on a few of the above infectious and contagious agents of reproductive disorders in cattle including *Brucella* spp., *Chlamydia abortus*, *Coxiella burnetii* and *Neospora caninum*.

Brucella spp.

Brucellosis is an infectious bacterial disease of domestic, feral and wild animals caused by bacteria of the genus *Brucella*. Bovine brucellosis represents one of the economically most important contagious and infectious diseases due to its impact on bovine reproduction (Franc et al., 2018). The zoonotic potential of brucellosis is well-known, and it is the commonest zoonotic disease worldwide, with more than 500,000 new cases annually (Pappas et al., 2006). However, the disease is not sustainable in humans, and the source of infection always resides in animal reservoirs (Godfroid et al., 2005).

Brucellae are Gram-negative, non-sporulating, non-motile cocci, cocco-bacilli or short rods (Alton and Forsyth, 1996). The bacteria penetrate nasal or oral mucous membranes and survive in cells of the reticuloendothelial system (Baumgartner, 2015). After penetration, bacteria incite local or regional lymphadenitis followed by the bacteraemia which may persist for several months or be recurrent for years (Godfroid et al., 2004). The growth of bacteria in the host is due to their ability to avoid the killing mechanisms of macrophages, but also the possibility to replicate inside macrophages (Jones and Winter, 1992). The localisation of the infection in the endometrium of the gravid uterus is the result of the affinity of *Brucella* spp. for the placental trophoblasts (Schlafer and Miller, 2007), and erythritol (Godfroid et al., 2004) which occurs in the placenta and foetal fluids after the fifth month of gestation. The rate of intracellular replication of brucellae in trophoblasts is dependent on the stage of gestation, with the higher replication rates in late gestation, when trophoblast secrete steroid hormones (Samartino et al., 1994). Hormonal changes in infected placentas, i.e., the increase in the level of prostaglandin $F_{2\alpha}$, and the decrease in the level of progesterone likely contribute to the abortion (Carvalho et al., 2010). Although the exact mechanism by which the infection causes abortion remains unclear, the interference with foetal circulation due to placentitis has been suggested as a possible cause of foetal abortion (Hagius et al., 2015).

The principal sign of *Brucella* spp. infection in cattle is abortion, typically occurring at approximately five to seven months of gestation, while the birth of weak calves is sometimes encountered. Apart from abortion, the retention of placenta followed by metritis is a common symptom of the infection with brucellae (Godfroid et al., 2004). Pathological changes can be found in cotyledons in the form of sticky, odourless and brownish exudate, followed by multifocal yellowish-grey necrosis (Godfroid et al., 2004, Schlafer and Miller, 2007). The most important clinical sign of brucellosis in bulls is uni- or bilateral orchitis characterised by multifocal or diffuse necrosis of testicular parenchyma or epididymis (2007). Occasionally, hygromas of the carpal joints, as well as cervical bursitis are reported as symptoms of bovine brucellosis (de Macedo et al., 2018, Fensterbank, 1978).

The gold standard for diagnosis of brucellosis in cattle is the isolation of *Brucella* spp. from the aborted material, which may be difficult. Serology is the most widely used methodology to detect infected individuals, and is officially recommended (Anonymous, 2016b). The official diagnostic testing of brucellosis in ruminants (sheep, goats and cattle) in BH is done by two serological tests applied in series, i.e., the Rose Bengal Plate test (RBPT) and the Complement Fixation test (CFT), as recommended (Anonymous, 2016b).

Brucellosis in ruminants in BH has posed a significant problem for animal health and production, but also for public health. The control of animal brucellosis was oriented to the test-and-slaughter strategy, but it has not proved to be effective in BH, most probably due to the inconsistency in the implantation of the program in the field setting. In 2009, a mandatory vaccination measure (ocular Rev-1 vaccine) against brucellosis was established for all small ruminants older than three months, except for pregnant ewes (Official Gazette of BH, issue no. 43/09; 83/09) (Anonymous, 2009a). This measure was continued on an annual basis vaccinating all replacements and ewes that were not vaccinated earlier due to the pregnancy. The vaccination was planned to cease in 2017, but the plan was slightly extended (Seric-Haracic et al., 2018). The implementation of the national brucellosis mass vaccination program has resulted in the successful control of brucellosis in small ruminants with prevalence rates steadily decreasing (FAO, 2015). However, the existing operating program of vaccination has to be thoroughly evaluated to determine the actual brucellosis status among the animal population in BH. So far, the only isolated species form ruminants (sheep, goats and cattle) (Velic, 2012) and humans (Arapovic et al., 2018, Tappe et al., 2012, Velic, 2012) was *Brucella melitensis* biovar 3, while *B. abortus* has, to date, not been isolated. The infection and reproductive problems in cattle caused by *B. melitensis* have been documented in France (Verger et al., 1989) and Spain

(Alvarez et al., 2011), countries with the successfully implemented eradication of *B. abortus*. However, little effort has been put into the epidemiological mapping of disease outbreaks and phylogenetic studies on pathogens associated with livestock in BH. Accordingly, getting a better understanding of the role of brucellosis in reproductive problems in cattle in BH is one of the aims of this PhD project.

Chlamydia abortus

Chlamydiae are obligate intracellular, bacteria that cause a wide range of diseases in animals and humans (Storz and Kaltenboeck, 1993). The new order *Chlamydiales,* with the family *Chlamydiaceae* and genus *Chlamydia* was first introduced in 1945, and later reaffirmed (Page, 1966). A subdivision of the family Chlamydiaceae into two genera *Chlamydia* and *Chlamydophila* was proposed by Everett et al. (Everett et al., 1999). The revision of the genetically heterogeneous species resulted in the definition of nine species: *Chlamydia* (*C.*) *trachomatis*, *C. muridarum*, *C. suis,* as well as *Chlamydophila* (*Cp.*) *abortus, Cp. pecroum, Cp. cavie*, *Cp. felis*, and *Cp. psittaci*, respectively. This subdivision of the family into two genera has been disputed. Sachse et al. (2015) proposed, based on genetic analysis, the classification of all currently recognised species in a single genus, the genus *Chlamydia* (Sachse et al., 2015). The recent proposal is used in this PhD project.

Chlamydiae are able to infect a wide range of eukaryotic cells, but the multiplication takes place in the cytoplasm primarily of the epithelial cells (Dautry-Varsat et al., 2005). Their growth cycle consists of two developmental forms, the elementary body (EB) which is the infectious form, and the reticulate body (RB) that is the non-infectious form of the bacteria (Andersen, 2004). The developmental cycle begin with the attachment and penetration of the EB into the host epithelial cell, followed by the differentiation into bigger, more pleomorphic RB. The RB is metabolically active, replicating form, and the multiplication of the RB is done by binary fission using energy form the host cell. The cycle ends after 2 or 3 days when bacteria have differentiated back to the EB and are released in the extracellular medium (Andersen, 2004, Dautry-Varsat et al., 2005). The possible routes of transmission of bacteria including faecal contamination, while bacteria may be shed in vaginal, ocular and nasal discharges, as well as urine, semen and uterine fluid (Reinhold et al., 2011, Shewen, 1980).

Chlamydial infections in cattle are associated with problems in several organic systems, i.e., respiratory, ocular, central nervous, musculoskeletal, and reproductive (Andersen, 2004, Reinhold et al., 2011). The reproductive problems caused by chlamydiae are infertility (DeGraves et al., 2004, Wehrend et al., 2005), vaginitis and endometritis (Wittenbrink et al., 1993a, Wittenbrink et al., 1993b), chronic mastitis (Biesenkamp-Uhe et al., 2007), and abortion (Borel et al., 2006). Serological detection of the bacteria is generally only suitable for prevalence surveys. The standard method for detecting antibodies to chlamydiae in animals is CFT, using the preparation of *Chlamydiaceae*-specific lipopolysaccharide (Perez-Martinez et al., 1986). In addition, numerous ELISA methods have also been used in the serodiagnosis of chlamydial infections (Sachse et al., 2009). Infection of humans with chlamydiae (*C. psittaci*, *C. abortus*) can result in acute, and occasionally life-threatening disease (Walder et al., 2005). Chlamydial infection could be a potential occupational hazard for farmers and health workers in BH, however, no epidemiological data are currently available.

Coxiella burnetii

C. burnetii is an obligate intracellular, Gram-negative, rod bacterium that has been found worldwide, except New Zealand. The bacterium easily resists desiccation, pH change (acidophilic), ultraviolet radiation, and disinfectants (Kelly, 2004). The term Q fever (query fever) was proposed in 1937 to describe the febrile illness in abattoir workers in Brisbane, Australia (Derrick, 1937). It is well-accepted and has been maintained, although the term coxiellosis can be more appropriate for cases without fever (Agerholm, 2013).

The replication of bacteria is carried out within monocytes/macrophages, i.e., phagolysosomes of eukaryotic cells, by transverse binary fission (Kelly, 2004). There are two distinct morphological forms of the bacterium, a small cell variant (SCV) that is metabolically inactive and represents the extracellular form of the organism, and a large cell variant (LCV) that undergoes sporogenic differentiation (McCaul and Williams, 1981). At the end of the cycle, the LCV develop to the SCV that is released with the cell lysis. In addition, *C. burnetii* displays a phenomenon of antigenic phase variation that is a phase I (Ph-I) and phase II (Ph-II), mainly due to variations in the lipopolysaccharide of the organism (Kelly, 2004). Organisms in Ph-I are highly infectious and are found in naturally infected humans and animals (Maurin and Raoult, 1999), while the Ph-II is less infectious and obtained after serial passages in cell cultures or embryonated eggs (Amano and Williams, 1984). Ph-II antibodies are produced early after infection, while the increase in the Ph-I titre is substantially delayed (Bottcher et al., 2011).

The reservoirs of the bacterium are many wild and domestic mammals, birds, arthropods (ticks), and humans (Kelly, 2004). The bacterium is excreted in milk, urine, faeces, uterine discharge, as well as in amniotic fluid, placenta and foetal membranes of affected animals (Guatteo et al., 2007, Woldehiwet, 2004), while the most effective infection routes seem to be inhalation of desiccated excrements and direct contact with the infected animal material (Kelly, 2004). The infection with *C. burnetii* may cause abortion, stillbirth, premature or delivery of weak offspring in cattle and small ruminants (Arricau-Bouvery and Rodolakis, 2005). Invasion of the pregnant uterus in cows and bacterial localisation in the placenta may lead to the occurrence of abortion-premature deliver-stillbirth-weak offspring (APSW) complex, but this means that the epidemic of Q fever in the herd should only be suspected if the entire APSW complex occurs (Agerholm, 2013). The association of *C. burnetii* with other reproductive problems (infertility, retained placenta, metritis) is an object of ongoing scientific discussion, but has not been clearly demonstrated (Garcia-Ispierto et al., 2014). There are six genomic groups of *C. burnetii* defined (Russell-Lodrigue et al., 2009), and different genotypes of bacteria could explain why some studies have detected diminished reproductive performance, while others have not (Garcia-Ispierto et al., 2014). Vaccination against *C. burnetii* in non-infected cattle herds, as well as all presumably susceptible animals in infected ones, seems to be the only way to control Q fever among cattle population (Guatteo et al., 2008).

Epidemics of Q fever in humans and animals in BH was first reported in 1951 (Simovic et al., 1951). So far, the occurrence of human Q fever in BH has been mainly sporadic with occasional epidemics which also representing a serious public health problem (Beslagic et al., 2006, Puvacic et al., 2005). The most probable transmission route of infection for farmers was the direct contact with animals. On the other hand, windborne spread or contact with contaminated dairy products seem to be more likely responsible for infection in the general population in BH (McQuiston et al., 2003). Q fever is a reportable disease in cases of animal abortion. However, epidemiological data regarding Q fever in cattle are still lacking in BH.

Neospora caninum

N. caninum is an obligate intracellular, protozoan parasite of animals (Dubey, 2004). This parasite was first described in 1984 as a causative agent of encephalomyelitis in dogs in Norway (Bjerkas et al., 1984), and new a genus and species were proposed in 1988 (Dubey et al., 1988). *N. caninum* is a coccidian parasite structurally and biologically related to *Toxoplasma gondii*, with the main difference in the definitive host of the parasite. Domestic and wild canids are both an intermediate and a definitive host, while a wide range of animals such as cattle, sheep, goats, horses, deer, chickens serve as intermediate hosts (Dubey, 2004).

The heteroxenous life cycle including the definitive and intermediate hosts is characterised by the three known infectious stages: tachyzoites, tissue cysts (bradyzoites), and oocysts (sporozoites) (Dubey et al., 2007). Asexual development consists of tachyzoites and tissue cysts and occurs in intermediate hosts, while the sexual reproduction (all three stages) occurs within a dog as a definitive host. Tachyzoites are located intracellularly in many types of cells, and their replication is taking place by endodygeny (Dubey, 2004). Tissue cysts with the enclosed bradyzoites can be found primarily in the central nervous system, but may also be found in extra-neural tissues such as muscles (Peters et al., 2001). The oocysts, as the environmentally resistant and unsporulated stage in the life cycle of *N. caninum*, are excreted in the faeces of definitive hosts. The shizogonic and gametogenic stages that precede the formation of oocysts in the intestines of dogs have not yet been observed (Dubey et al., 2004). The sporulation of oocysts outside the host usually occurs within 24 hours (Lindsay et al., 1999, McAllister et al., 1998). Sporulated oocysts contain two sporocysts, with four sporozoites in each (Dubey, 2004).

The possible transmission routes of the parasite among cattle are horizontal (postnatal) after the ingestion of sporulated oocysts, and vertical (congenital, transplacental) from the infected dam to her offspring (Dubey et al., 2006). Also, transplacental transmission can have two forms: exogenous from the pregnant dam that was infected horizontally via ingestion of sporulated oocysts, and endogenous from the persistently infected dam after the recrudescence of the infection in pregnancy (Trees and Williams, 2005). Theoretically, the parasite can be excreted in milk or uterine discharge of infected cows, but these transmission routes seem to be of little importance for maintaining the infection (Davison et al., 2001). The main clinical sign of bovine neosporosis is abortion in the period between three and eight months of gestation (Dubey, 2004, Dubey et al., 2006), and this agent is a major cause of abortion worldwide (Almeria et al., 2017). Abortions may have sporadic, endemic and epidemic patterns, while a small proportion of cows may have repeated abortions due to neosporosis (Schares et al., 2002, Wouda et al., 1999). The localisation of the parasite in the placenta may compromise the foetal survival directly, or by releasing maternal prostaglandins that cause luteolysis and abortion. Furthermore, foetal damage probably occurs due to the parasitic infestation and multiplication, and insufficient nutrition, secondary to the damaged placenta (Almeria et al., 2017, Dubey et al., 2006). Clinical signs of neosporosis in other livestock species mainly include abortions, while neosporosis in dogs may be manifest itself as a disease of a central nervous system including disseminated meningo-encephalomyelitis, polymiositis, and hind limb paralysis (Dubey, 2004, Peters et al., 2001).

The diagnosis of bovine neosporosis includes clinical and epidemiological investigation followed by different serological methods such as indirect fluorescent antibody test (IFAT), immune-blotting (IB), agglutination tests and ELISA (Bjorkman and Uggla, 1999). Post-mortem examination includes histo-pathological, immunohistochemical and molecular methods (McAllister, 2016, Wouda, 2000). A number of strategies have been employed to control neosporosis in cattle herds including the improvement of farm biosecurity, test and cull programs, artificial insemination of seropositive dairy cows using bull semen (Dubey et al., 2007, Estill and Scully, 2015). There is accumulating evidence that some havlogen-adjuvanted, killed vaccine yield protection against bovine abortions (Romero et al., 2004, Williams and Trees, 2006) with the overall efficacy of 46%. However, at present, a commercial vaccine is not available (Dubey et al., 2007). Antibodies to *N. caninum* have been reported in humans (Lobato et al., 2006, Tranas et al., 1999) but, the parasite has not been demonstrated in human tissue. In this respect, the zoonotic potential of *N. caninum* remains unclear. Epidemiological and clinical data regarding bovine neosporosis lack in BH, and this disease is not on the list of reportable disease in case of bovine abortion that is prescribed by State veterinary authorities (Anonymous, 2017c).

Knowledge gaps

Reproductive disorders may pose a severe problem for the entire bovine industry in BH. Although diminished reproductive performance at the farm or individual levels are very well recognised and reported by farmers and veterinarians, the scientific information regarding clinical features, frequency and scale of reproductive shortfalls are lacking in BH. Therefore, the lack of information regarding the reproductive status and different reproductive disorders has prompted this study.

The main knowledge gaps when the study was initiated included:

- The occurrence of various pathogens potentially causing reproductive problems in cattle in BH was largely unknown. The occurrence of *Brucella* infection in cattle was of special concern, following the vaccination campaign against *B. melitensis* in small ruminants.
- Factors influencing the levels of reproductive infectious agents were largely unknown.
- The role of non-infectious (environmental and management) factors in the occurrence of reproductive disorders in cattle in BH was largely unknown.
- Key indicators of reproduction performance in cattle were mainly non-documented.

Aims and objectives

The overall aim of this PhD project study was to fill some of the knowledge gaps regarding the four infectious agents (*Brucella* spp., *C. abortus*, *C. burnetii*, *N. caninum*) known to cause reproductive disorders in cattle and their links to management factors, as well as describing and evaluating the general reproductive indicators in commercial dairy farms in BH.

The overall aims of the study were followed up through the three specific objectives described below.

Specific objectives

1. To assess the epidemiological pattern of *Brucella* spp., *C. abortus, C. burnetii* and *N. caninum* among the cattle population in BH (Paper I).
2. To investigate the causal associations between farm managerial traits and serostatus of these pathogens, as well as reproductive disorders (Paper II)
3. To describe the key reproductive performances in a set of dairy cattle farms in BH (Paper III).

Materials and Methods

Study areas

All studies of this PhD project were carried out in BH, i.e., in three cantons of the FBH. These are: the Una-Sana Canton (USC) with an area of 4 125 km^2 (Anonymous, 2015c), the Canton 10 (C10) with an area of 4 934 km^2 (Anonymous, 2015a), and the Central-Bosnia Canton (CBC) with an area of 3 189 km^2 (Anonymous, 2015b). These cantons represent a total of 23.9% of the total area of BH. The selection of these cantons was based on the presence of cattle farms of interest, as well as the logistic requirements of the planned specific studies. A total of 94 villages and local communities from 19 municipalities were selected for a study visit. Seven municipalities were visited in the USC: Bihac, Bosanska Krupa, Bosanski Petrovac, Cazin, Kljuc, Sanski Most and Velika Kladusa. Another six municipalities were visited in the CBC: Bugojno, Donji Vakuf, Gornji Vakuf, Jajce, Novi Travnik and Travnik, while five municipalities were visited in C10: Drvar, Glamoc, Kupres, Livno and Tomislavgrad. Figure 6 shows a map of study municipalities and their location in BH. The map was generated using QGIS software (QGIS Development Team, 2016). The detailed map of farm locations is given in Paper II. The study areas are characterised by the temperate continental climate characterised by four seasons, as well as moderate to warm summers, and mild to moderate winters. Additionally, areas with an altitude higher than 1000 meters above sea level are characterised by sub-mountainous and mountainous subtype of the temperate continental climate (Anonymous, 2013c).

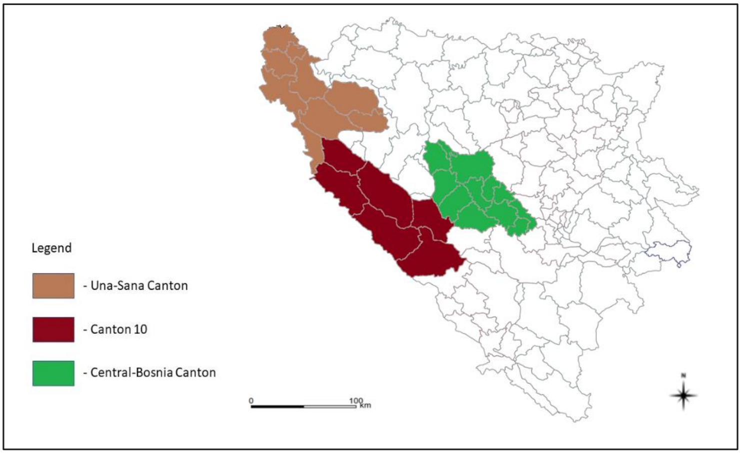


Figure 6. *Map of BH presenting the study areas for this PhD thesis and their geographic location. 1 - USC; 2 - C10 and 3 - CBC (Source: Paper I)*

Study population

Papers I and II

The target population was all dairy cattle herds from the selected areas, while the source population consisted of all dairy farms that were enrolled in an annual surveillance and disease control programme focused on bovine brucellosis and enzootic leucosis ("Official Gazette BH", issue number 34/02) (Anonymous, 2002). Herds/farms were randomly selected from the available lists made in collaboration with the cantonal veterinary departments and local veterinary organisations. Given the overall aim of the project, all animals older than 12 months within each farm were chosen for participation. The breed structure of the visited farms consisted of Simmental, Montafone, Holstein-Friesian, Red Angus breeds and their crosses.

Paper III

The target population was all dairy cows at the commercial dairy farms in the Una-Sana Canton. Since there was not a complete list of all dairy farms in the Una-Sana Canton, dairy farms form the available municipal lists of cooperatives with dairies were chosen for participation in this study. In addition to available lists, the selection of farms was based on a subset of dairy farms assembled retrospectively from papers I and II.

Study design and implementation

The research process that was followed in the planning and implementation of the research of this PhD thesis consisted of three stages. The process began by examining the epidemiological pattern of reproductive pathogens among cattle population in BH, followed by estimating the associations between reproductive pathogens, reproductive problems and farm managerial traits. The final stage of this thesis was a description of key reproductive performance in a smaller set of dairy farms. The overall design and approach to the study implementation are shown in Figure 7.

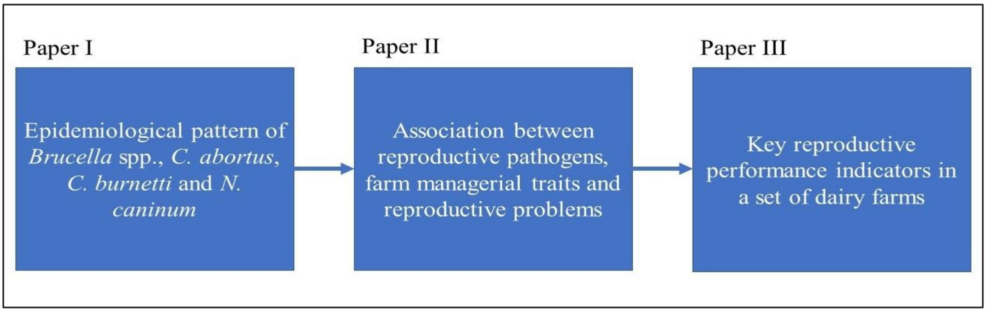


Figure 7. *A graphical depiction of study design and implementation for this PhD thesis*.

Papers I and II

These congruent studies were carried out using a cross-sectional design that included herd selection and visits, blood sampling, and administering interview-based semi-structured questionnaires. The minimum sample size of 60 farms per canton (n=199) was determined based upon the detectable herd level presence of seropositive animals at 5%, 95% level of confidence. The calculation of minimum sample size was done using the Epi Tools, an online epidemiological calculator (AusVet, 2014). According to the number of animals per herd, herd sizes were stratified into three groups: small (1-10 animals), medium (11-30 animals), and large (>30 animals). Finally, selected farms were unequally distributed among study areas due to demographic features of selected cantons (population density). There were a total of 67 (33.5%) farms in the USC, 37 (17.8%) farms in C10, and 96 (48.7%) farms in CBC. The two-level sampling approach resulted in the collection of 1970 individual serum samples of animals from the selected herds. The questionnaire consisted of 40 questions divided into three groups: socio-demographic, management and reproductive performance. Rating questions regarding perceived primary constraints of the production were also asked. Both studies were completed in the period January-August 2015.

Paper III

The final study in this PhD project was designed as a follow-up study aimed at describing individual animal reproductive performance in a subset of previously selected dairy farms in the USC. All written reproductive data were collected as calendar dates using a data collection form created as an individual animal timeline. Subsequently, key reproductive indicators were calculated from the collected data. Twenty-two commercial dairy farmers agreed for the participation of this study, of which five were excluded due to unreliable or unapproachable written data. Farm visits and data collection were completed in the period November-December 2016.

More detailed information regarding study design and implementation are available in the methodological sections of specific papers.

Laboratory methods

Sampling procedure

The blood sampling of animals was performed after the verbal consent of the owner/farm manager of previously selected farms. All samples were collected from the tail vein (*v. coccygea*) of animals (minimum 5 ml), using sterile needles and plain vacutainer tubes. Further, samples were kept at room temperature (approx. 22˚C) overnight to obtain sera. Exceptionally, some samples were centrifuged at 3000 x *g* for 5 minutes. Then, sera were pipetted into cryovials and stored at -20˚C until tested serologically.

Serological testing – description and principles

Brucella spp.

In the preparation before storage, all sera were screened for antibodies against *Brucella* spp. using the Rose Bengal Plate Test (RBPT). Tests were performed according to the standard protocol recommended by the OIE (Anonymous, 2016b). Sera with the positive reaction to RBPT were further tested using the complement fixation test (CFT) as a confirmatory test recommended by the OIE (Anonymous, 2016b). Positive and negative controls referred to brucellosis positive and negative national sera. Complement and hemolysin were obtained from IDEXX (Porquier, Montpellier, France), while the sheep blood originated from animals owned by the Veterinary Faculty in Sarajevo.

The presence of antibodies to *Brucella* spp. was also determined using the enzyme-linked immunosorbent assay (ELISA): CHEKIT Brucellose serum test kit (IDEXX Switzerland AG). Microtiter plates with ninety-six wells were precoated with the inactivated antigen of the agent. Sera dilutions were incubated in the wells of those plates, allowing binding and formation of antibody-antigen complexes. The unbound material was removed from the wells by washing. Further, a peroxidase-labelled anti-ruminant IgG conjugate was added in order to bind with antibody-antigen complexes. After the washing of the unbound conjugate, the 3, 3’, 5, 5’ tetramethylbenzidine (TMB) containing substrate was added. The analytical procedure was finalised with adding the stop solution. The degree of colour was measured at the wavelength of 450nm and was directly proportional to the number of antibodies specific for *Brucella* spp. Reactors that have shown positive results on the serial testing [(RBPT + CFT) + ELISA] were declared as *Brucella* positive and herds with those reactors were classified as infected.

Chlamydia abortus and *Coxiella burnetii*

For *C. abortus*, the serological screening was conducted using the ELISA Chlamydiosis total antibody test Kit (IDEXX Switzerland AG), and for *C. burnetii* the ELISA Q fever antibody test kit (IDEXX Laboratories, Inc.). The methodological principle was the same for these pathogens. Microtiter plates (96 wells) were precoated with inactivated antigens. Dilutions of controls (1:400) and sera (1:400) were incubated in specified wells. Unbound material was removed by a washing solution. Then, a peroxidase-labelled anti-ruminant IgG conjugate was added, which has bound to the antibody-antigen complexes. Once again, the washing solution was used for removing the unbound conjugate, and the TMB substrate was added to the wells. The degree of colour was measured at the wavelength of 450nm. Both ELISAs were rapid, simple, sensitive and specific methods for detecting antibodies against *C. abortus* or *C. burnetii*.

Neospora caninum

The presence of antibodies to *N. caninum* in bovine sera was determined using the ELISA *Neospora* X2 antibody test kit (IDEXX Laboratories, Inc.). *Neospora* antigens were coated on ninety-six well microtiter plates. Upon incubation of sera in coated wells, antibodies against *N. caninum* formed complexes with coated antigens. After washing away the unbound material, an anti-bovine, horseradish peroxidase-labelled conjugate was added which bound to antibody-antigen complexes. The final step of the assay included washing away of the unbound conjugate and adding an enzyme (hydrogen peroxide) substrate and chromogen (TMB). Subsequent colour development was proportional to the number of antibodies presented in sera and was measured at the wavelength of 620-650 nm.

The interpretation of all performed ELISAs is available in the specific paper (paper I), while test procedures are available in manufacturer's guides (IDEXX) (IDEXX, 2015a, IDEXX, 2015b, IDEXX, 2015d, IDEXX, 2015c). All laboratory analyses carried out in the accredited Laboratory for virology and serology (BAS EN ISO/IEC 17025:2006) of the VFS of the University of Sarajevo.

Data management and analysis

Paper I

A database was established in Microsoft Excel® 2013. After checking, data were transferred to the statistical programme Stata 14/SE (StataCorp., 2015) for further statistical analysis.

Statistical analysis was performed at the individual animal and herd levels. The proportion of seropositive animals was estimated using the command syntax *survey data analysis*, with the herd named as the primary sampling unit and the inverse sampling fraction of the herd as weight. Logistic regression (survey logistic model) reported as odds ratios was used to estimate the association between seropositivity and risk factors (canton, age, and breed) at individual animal level. The overall true individual prevalence was calculated for tests with known sensitivity/specificity using the AusVet Rogan-Gladen estimator (AusVet, 2015). The observed overlaps between seropositivities of *C. abortus*, *N. caninum* and *C. burnetii* were presented using a Venn diagram (*pvenn2*), while the correlation between overlaps was measured using tabulation and Goodman and Kruskal's gamma-statistics.

Afterwards, data were collapsed to the herd level defining herds as positive or negative. This procedure was based on test specificity. According to the manufacturer's data, ELISAs used for investigation of *C. abortus* and *C. burnetii* have demonstrated a 100% test specificity (IDEXX, 2015b, IDEXX, 2015d). Consequently, the herd was classified as positive if at least one reactor (seropositive animal) was within the herd. On the other side, the specificity of the ELISA for *N. caninum* was 99.2% (IDEXX, 2015c), and single reactor was considered to be sufficient to classify a herd as positive if there were no more than 7 animals (>95% herd specificity); two reactors for 7 to 45 animals per herd, and three reactors for 45 - 100 animals per herd. Herd level seroprevalences across cantons were calculated using the simple *proportion* command.

Paper II

Data were entered into a Microsoft Excel® 2016 spreadsheet before inspected and merged with data from Paper I. After cleaning data and initial analyses in Microsoft Excel®, using Pivot tables, data were transferred to the statistical software Stata 15/SE (StataCorp., 2017) for further analyses. Descriptive analyses and tabulation were done for each canton, and statistical differences between cantons were assessed using a chi-square test for categorical variables and the Kruskal-Wallis test for continuous variables.

After descriptive analyses, management-related factors were screened for their influence on the occurrence of reproductive problems and seropositivity to reproductive infectious agents. As all outcomes were dichotomous (coded as present/absent or seropositive/seronegative herd), a mixed-effect univariable logistic regression was used to screen management factors for potential statistical influence. An adjustment was made for variable “herd size” by including its natural logarithm (ln (herd size)) as an offset in the logistic model for the disease outcomes because the “herd size” effect on the occurrence of reproductive problems was expected. This adjustment was not done for the infection outcomes, as this was based upon a more systematic sampling in the herds. Factors with a p-value less than or equal to 0.20 from univariable analyses were used as candidate variables for subsequent multivariable modelling.

The multivariable mixed-effect logistic models were established using the *melogit* procedure in Stata with “canton” as a random effect. Postestimation procedures were used for all multivariable logistic models using the Hosmer-Lemeshow goodness-of-fit test and the area under the receiver operating curve (ROC) (Dohoo et al., 2014).

The final multivariable models were then merged into a Structural Equation Model (SEM) using the *gsem* procedure in Stata (StataCorp., 2017). The SEM models allowed confirmatory data analyses to be undertaken, utilising all options built into Stata’s multilevel mixed-effect models. The initial model was constructed using the graphical *sembuilder* interface and then refined using the Stata command-language syntax. The causal model was specified graphically using the online software for directed acyclical graphs (DAGitty, 2017).

Assumed farm production constraints were analysed graphically using Pivot functions in Microsoft Excel® 2016 and visualised through a radar graph in Microsoft Excel® 2016. Graphs were split according to herd size groups (quartiles).

Paper III

Databases were established in a Microsoft Excel® 2016 and initially analysed using filter functions and pivot analyses. Afterwards, data were transferred to Stata 15/SE (StataCorp., 2017) for descriptive analyses and graphical presentation of reproductive indicators. Calculated reproductive indicators included; age at first service (including artificial insemination and natural breeding), age at first calving, the pregnancy rate at first service, number of inseminations per pregnancy and its variations, calving to first service interval, calving to conception interval, calving interval and its variations, and overall pregnancy rate. More details regarding inclusion criteria and formulas for calculating reproductive indices are given in the specific paper.

Means, medians, and 5 to 95 percentile ranges were calculated for continuous variables. Frequencies were calculated for dichotomous and categorical variables. Kruskal Wallis test was used for assessing the statistical difference between continuous dependent (reproductive measures) and categorical independent (farms) variables. Data were graphically presented using box plots, bar graphs and histograms.

Ethics statement and consent for participation

All studies in this PhD project were conducted in accordance with the Law on Animal Protection and Welfare of BH ("Official Gazette BH" issue number 25/09) (Anonymous, 2009b). Also, studies were submitted to and approved by the Ethics Committee of the VFS of the University of Sarajevo.

All selected farmers/owner provided their verbal consent to participate in this PhD project. Sampling was performed after acquiring the permission from study participants. Questionnaires were conducted anonymously, using property codes as an identity. All property codes and information regarding farm location (village, municipality, and canton) are deleted from databases before their eventual publication or distribution.

Results - summary of papers

Paper I

The serostatus of *Brucella* spp., *Chlamydia abortus*, *Coxiella burnetii* and *Neospora caninum* in cattle in three cantons in Bosnia and Herzegovina

The objective of the first study in this PhD project was to describe the epidemiological pattern and serological status of reproductive pathogens *Brucella* spp., *C. abortus*, *C. burnetii* and *N. caninum*. A cross-sectional study was designed to document the status of these pathogens in dairy cattle in Bosnia and Herzegovina. Blood samples and relevant individual data were collected from 1970 animals in 199 farms. ELISAs were used for screening the animal seropositivity to four selected reproductive pathogens. The overall seroprevalences were estimated both at herd and individual level for each pathogen as shown in Table 3.

Table 3. *The overall herd and individual level proportions of seropositive animals (95% CI) of Brucella spp., C. abortus, C. burnetii and N. caninum*

	***Brucella* spp.**	***C. abortus***	***C. burnetii***	***N. caninum***
Individual level	0.2% (0.1 - 0.5)	52.1% (41.2 - 62.7)	8.8% (5.3 - 14.2)	9.2% (6.0 - 12.3)
Herd level	1.5% (0.5 - 4.6)	87.9% (82.6 - 91.8)	19.6% (14.6 - 25.8)	35.2% (28.8 - 42.1)

The estimation of true prevalence was based on assays' properties, and the only difference between apparent and true prevalence observed was for *C. abortus*; 56% (44.8 – 68.2). The presence of mixed-infections was observed for *C. abortus*, *C. burnetii* and *N. caninum*, both at individual- and herd level. A slight correlation (Goodman and Kruskal's gamma = 0.35) in agents overlaps was found only for *C. abortus* and *C. burnetii*. The performed logistic regression indicated that cows older than six years were more likely to be seropositive to *C. burnetii* (OR: 4.88; CI: 2.20 – 10.85) compared to younger cows. Simmental (OR: 2.02; CI: 1.11 – 3-58), Montafone (OR: 1.49; CI: 0.77 – 2.86), and Red Angus (OR: 5.05; CI: 2.10 – 12.18) breeds were more likely to be seropositive to *C. abortus* in comparison with their crossbreeds. Also, Montafone (OR: 2.99; CI: 0.85 – 10.51), and Red Angus (OR: 3.82; CI: 2.27 – 6.44) breeds were more likely to be seropositive to *C. burnetii* compared to crossbreeds.

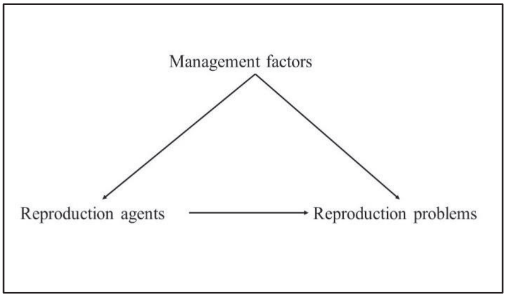
This study demonstrated a high level of antibodies to *C. abortus*, *C. burnetii* and *N. caninum* and highlighted the need for targeted control of infectious causes of reproductive disorders in dairy cattle. Given the low seroprevalence, *Brucella* spp. were not likely associated with reproductive health of cattle in studied areas. Also, this may suggest that *B. abortus* is not present in dairy herds in BH, but that *B. melitensis* has spilled-over from the small ruminant reservoir to cattle.

Paper II

A causal approach to understanding management factors, reproductive underperformance and reproductive infections in dairy cattle

The objective of this study was to investigate causal associations between farms' management practices and reproductive problems (abortion, stillbirth, retention of placenta, metritis) as well as reproductive diseases (*C. abortus, C. burnetii, and N. caninum*). Causal associations between reproductive diseases and outcomes were also investigated in this study (Figure 8). A secondary objective was to examine farmers' main constraints in the production process.

Figure 8. *The schematic depiction of the potential causal association investigated in Paper 2*



The study was carried out in the north-western (Una-Sana), western (Canton 10) and central part (Central Bosnia Canton) of Bosnia and Herzegovina. A total of 201 farms were selected for participation, while the final dataset included 197 farms (described in Paper I). The univariable logistic regression indicated a borderline association ($P \leq 0.20$) of 29 management factors (candidate variables) with reproductive diseases (agents), reproductive problems or both groups of outcomes, as well as the association between groups of outcomes.

Multivariable mixed-effect logistic regression was undertaken for all the outcomes, leading to seven multivariable models. All models showed that all reproductive outcomes were associated with some of the management factors. The final multivariable models were merged into a structural equation model (SEM), and a causal model was specified graphically. Herds that experienced abortions (OR: 4.3; CI: 2.2 – 8.4) and stillbirth (OR: 6.7; CI: 2.8 – 15.6) were associated with *N. caninum* herd seropositivity. Also, herds that experienced retention of placenta were strongly associated with the occurrence of metritis (OR: 10.1; CI: 3.9 – 26.3). *C. burnetii* and *C. abortus* herd seropositivities were mainly associated with environmental factors and contact with potential intermediate hosts. The use of SEM demonstrated the value of identifying some of the potential causal arrows from the multivariable regression models that need to be deleted.

The study demonstrated that management practices on dairy farms in BH contributed to the occurrence of severe reproductive problems and reproductive infections. *N. caninum* seemed to be an infectious agent that contributed substantially to the reproductive underperformance. Further, using causal modelling for understanding complex relationships between management factors and reproductive outcomes was highlighted in this study.

Paper III

Reproductive performance in a selected sample of dairy farms in Una-Sana Canton, Bosnia and Herzegovina

The purpose of this follow-up study was to describe individual reproductive performance in a subset of dairy herds in the USC. Twenty-four managers of dairy farms were approached, and twenty-two of them (92%) agreed to participate in this study. Five out of twenty-two dairy farms (23%) were excluded due to unreliable data recordings or unapproachable written data, leaving 17 farms in the final dataset. In individual terms, 57% (310/544) of individual data recordings were excluded from the study due to the unapproachable or memorising data. Finally, recordings for 234 animals from 17 dairy farms were confirmed eligible and included in the study.

The median age at first insemination was 493 days (5-95% percentile range 429-840), while the age at first calving was 802 days (708-1168). The median pregnancy rate at first insemination was 40% (17-62), while the cumulative pregnancy rate calculated at day-60, day-80, day-100 and day-120 shown that approximately 64% of all pregnancies happened before day-120. The calculated inter-service intervals demonstrated that approximately 69% of the repeat breeding animals came back to the oestrus in the period 18 to 24 days, which is an indication of very good oestrus detection in selected dairy farms. The mean number of services per pregnancy was

2.61 (1-12) counting for one oestrus cycle. The median calving-to-first-insemination interval was 62.5 days (16-408). The calving-to-conception interval was 101 day (36-506). Finally, the median calving interval was 385 days (329-773). The results indicated the existence of slight reproductive problems in selected dairy farms. However, the lack of records for many animals or complete farms is a clear indication of a current low quality in the cattle farming system in BH.

Discussion

General discussion

The bovine industry, as the most important agricultural sector in BH, is experiencing considerable commercial and regulatory changes, especially taking into account the country's EU integration process. However, there are many weaknesses and potential obstacles that need to be overcome in order to align the sector policy with the EU standards. Hence, there is a need for a scientific assessment of the reproduction performance in cattle in BH as a starting point from which to operate with all upcoming and necessary changes and to implement evidence-based programmes and solutions. The effective reproductive performance will remain the central part of cattle (re)production systems regarding their sustainability and profitability (Esslemont et al., 2001, Stott et al., 1999). The lack of data on overall bovine reproductive performance in BH, as one of the crucial factors in the bovine industry worldwide, highlights the importance of this study. Thus, this PhD project studied the reproductive aspects of the bovine industry in BH aiming to give insights into the epidemiology of reproductive infectious agents and their links to management factors and reproductive problems, as well as to describe the current reproductive performance in the cattle population. The aim was achieved through three specific objectives that are presented as papers I, II and III. In the following, our results are discussed paper by paper.

Paper I

Given the previously reported presence of brucellosis among human and production animals in BH (Obradović and Velić, 2010), assessing the epidemiological status of *Brucella* infection in cattle was the thesis' starting point. Given the very low seroprevalence of anti-*Brucella* antibodies, our research team hypothesised that this suggested a spill-over of *B. melitensis* from small ruminant reservoir, more than *B. abortus* infection. This low seroprevalence in cattle could be partially explained by the implementation of the national brucellosis control programme and vaccination is small ruminants that started in 2009 (Anonymous, 2009a). These low anti-*Brucella* antibody levels are insufficient to explain the whole magnitude of reproductive disorders in the bovine industry. Other infectious agents, i.e., *C. abortus*, *C. burnetii* and *N. caninum* were included in this study because of their worldwide distribution and relevance in causing reproductive problems (Agerholm, 2013, Dubey et al., 2007, Reinhold et al., 2011). Also, epidemiologic data regarding these pathogens and their potential importance could contribute to a better understanding of the overall reproductive problems in cattle in BH.

With this background, a seroepidemiological study was designed to investigate all four reproductive pathogens. The study findings demonstrated that cattle in the studied areas were highly exposed to *C. abortus*, moderately exposed to *C. burnetii* and *N. caninum*, but rarely exposed to *Brucella* spp.

This study was the first to give insight into the presence of chlamydial infection among cattle populations in BH. Other studies reported a relatively high seroprevalence of *C. abortus* in sheep (Krkalic et al., 2016), but also the presence of the infection in goats in BH (Krkalic et al., 2015). The high animal- and herd level seroprevalence of *C. abortus* in cattle in BH is not surprising as chlamydial infections are ubiquitous in cattle (Kaltenboeck et al., 2005). Reported data suggest a high seroprevalence of chlamydial infection in cattle herds worldwide, with the seropositivity ranging between 45 and 100% (Jee et al., 2004, Petit et al., 2008, Reinhold et al., 2008, Wang et al., 2001). Given the high prevalence worldwide, the interpretation of the significance of serological findings in bovine reproduction in BH is difficult, since the clinical bovine chlamydial infection is usually not obvious (Reinhold et al., 2011). However, the ubiquitous distribution of chlamydiae and their ability to infect the reproductive (Biesenkamp-Uhe et al., 2007, DeGraves et al., 2004), respiratory and intestinal tracts (Andersen, 2004) might have a measurable impact on reproductive animal and herd health in BH. In addition to this, there was a possibility of mixed chlamydial infections within one individual (host) as previously reported (Pantchev et al., 2009, Pantchev et al., 2010). This is important for two reasons: a) the actual seroprevalence of *C. abortus* found in this study could be serologically overestimated due to the antigenic cross-reactivity with other chlamydial species, and b) interactions and synergies between the co-infecting chlamydial agents can have a cumulative impact on bovine reproductive health in BH. Calves are free of chlamydiae at birth, but infection may come within two weeks of life (Jee et al., 2004). Further, the shedding of bacteria in adult animals is intermittent, indicating persistent and recurring infection in the host (Jee et al., 2004, Reinhold et al., 2008). This study included only animals older than 12 months as they started their reproduction life, and sero-reactors were equitably distributed among all age groups of animals. However, calves could be the potential reservoir of infection since the farmers in BH mostly rear own replacement animals and they usually stay in the herd for life. Hence, a study describing the chlamydial infection in calves in BH is needed to fulfil this information gap. Our study found an association between breed (Simmental, Montafone and Red Angus) and seropositivity to *C. abortus*. However, no obvious reason for the apparent association could be identified. On the other hand, the difference in seroprevalences between Red Angus (high seroprevalence) and other breeds can be partially explained by the farm management. The animals were kept in overpopulated free-stall, with a high contact rate between animals as well as with a potentially contaminated food and environment that could be the transmission routes for infection.

This study reported serological evidence for the presence of *C. burnetii* at the individual animal and herd levels in cattle in the studied areas of BH. Similarly, previous studies confirmed the worldwide distribution of *C. burnetii* among cattle over the five continents. These studies reported either the individual level prevalence ranging 4 - 82% (Biberstein et al., 1974, Htwe et al., 1992, McCaughey et al., 2010, Schelling et al., 2003) or herd level prevalence ranging 10 - 100% (Biberstein et al., 1974, McCaughey et al., 2010, Moffat et al., 1970, Scolamacchia et al., 2010). Also, the apparent prevalence values at animal and herd levels are found to be slightly higher in cattle in comparison with small ruminants (Guatteo et al., 2011). Put together, reported public health issues (Beslagic et al., 2006, Puvacic et al., 2005, Zvizdić et al., 2002) and moderately exposure of cattle to *C. burnetii* found in this study make Q fever a disease of interest for policy makers and the food industry, especially for the expanding dairy industry. Given the cattle rearing tradition and practices among farmers, the prevalence level found in the studied areas is likely to be representative for the entire country. Also, findings from this study indicate that Q fever (*C. burnetii*) should probably be kept in mind when veterinarians encounter fever of unknown cause in animals and examining aborted foetuses or other reproductive problems. Since this study reported seroprevalence on animal and herd levels, the within-herd prevalence study would probably offer a more detailed insight into optimisation and implementation of control programs against Q fever, as previously suggested (Guatteo et al., 2011). Also, the study aimed to describe shedding prevalence is necessary to estimate the potential routes of transmission of the infection between animals, herds and potential risk of infection for humans.

This study reported a relatively frequent exposure of cattle to *N. caninum* at animal and herd levels. Neosporosis is a worldwide distributed infection in cattle. Previous studies reporting the presence of this infection at animal and herd levels are thoroughly reviewed (Dubey and Schares, 2011, Dubey et al., 2007). The results of our study indicate that the seroprevalence of *N. caninum* in cattle at the animal level in BH is slightly lower in comparison with the global median seroprevalences in dairy (16.1%; range 3.8 – 89.2) and beef (11.5%; range 2.5 – 81.7) cattle (Reichel et al., 2013). *N. caninum* is not included in the monitoring of infectious reproductive problems in cattle in BH, and the results of this study point to the importance of this agent and the rationale for it to be included in national monitoring programmes. The detection of antibodies against *N. caninum* in bovine sera using ELISA is optimal for identification of infected animals and herds (Ortega-Mora et al., 2006, von Blumroder et al., 2004), and has been the basis for developing control programmes against bovine neosporosis in different countries, although a general strategy to control neosporosis is not applicable due to regional differences in the epidemiology of the disease (Dubey et al., 2007). Thus, this study may serve as a starting point in the prospective control of bovine neosporosis in the studied areas of BH, as infected animals can potentially introduce the parasite to naïve animals/herds or in areas where the disease does not exist. The neosporosis-induced reproductive failures represent the major economic loss resulting from direct loss involved in abortions (Larson et al., 2004), indirect costs of diagnosis (Ortega-Mora et al., 2006), loss of milk yield (Thurmond and Hietala, 1997), professional help, and culling cost of aborted cows. Hence, a thorough cost-benefit analysis is needed before starting a prospective control programme for neosporosis in BH.

The animal- and herd level seroprevalences of *Brucella* spp. in cattle was found to be very low in this study. This finding is consistent with the FAO regional workshop on brucellosis control (FAO, 2015) which reported the reduction of brucellosis' seroprevalence in cattle in BH. There is, to date, no brucellosis control programme implemented in cattle in BH. This trend may therefore be explained by the successful vaccination strategy applied in small ruminants against *B. melitensis* infection, which could be imply a reduced source of infection for cattle. Proving the absence of *B. abortus* is not possible. The fact that the only isolated *Brucella* species in ruminants and humans in BH is *B. melitensis* biovar 3 is of limited importance here (Arapovic et al., 2018, Tappe et al., 2012, Velic, 2012),. Accordingly, this study indicates that a successful vaccination programme in small ruminants may result in controlling spill-over infection to cattle without the need to vaccinate cattle (Godfroid et al., 2013, Godfroid et al., 2014). Finally, *Brucella* spp. does not seem to represent a reproductive problem in cattle in studied areas of BH. However, given the agent's undeniable role in abortions (Alton and Forsyth, 1996, Godfroid et al., 2004), its endemic nature in the Mediterranean (Balkan) area (Godfroid et al., 2005, Pappas et al., 2006), and zoonotic potential (Pappas et al., 2006), these bacteria should remain on the priority list for testing of infectious causes of reproductive disorders in cattle in BH.

This study discovered a substantial number of mixed-infections (animals seropositive to two or more agents). The statistical correlation was found only for the combination *C. abortus* - *C.*

burnetii, which might indicate the existence of common managerial factors that contribute to the mixed-infection with these agents. These factors were investigated in the subsequent study of this thesis (Paper II). Another study reported that *N. caninum* associated abortions were more likely to occur in herds seropositive to *C. burnetii* than in seronegative herds (Hassig and Gottstein, 2002). In this particular case, it is likely that the observed abortions are due to *N. caninum* rather than *C. burnetii*. However, in the current circumstances in BH, it is essential to have a thorough diagnostic examination of aborted materials in order to exclude one agent in mixed-infected animals. Another important aspect of mixed infections is their synergism in inducing reproductive problems in cattle. There is evidence of such synergism between *N. caninum* and BVDV in inducing abortion or stillbirth in dairy cattle (Bjorkman et al., 2000). Although there is some evidence of BVDV persistently infected animals in dairy farms in studied areas (unpublished data), further studies investigating the epidemiology of BVD as well as its synergism with *N. caninum* is needed in BH.

Paper II

The first study in this PhD project (Paper I) pointed to potential threats to the profitability of bovine industry due to the existence of investigated reproductive infectious agents (diseases) in cattle population in BH, but also to public health issues due to the zoonotic potential of agents, except *N. caninum*. On the other side, the occurrence of severe reproductive problems in cattle such as abortions, stillbirth, retained placenta, and metritis were previously observed at the visited farms. Two questions that needed to be answered were: i) Are management exposures (animals' environment and husbandry system) associated with the occurrence of reproductive infectious agents or reproductive problems, and ii) are reproductive infectious agents and problems interconnected? Thus, the second study (Paper II) aimed to identify exposure factors and to evaluate their association with various outcomes of interest (reproduction agents/diseases and reproduction problems). Details of the questionnaire used in this study and case definitions are presented in paper II.

This study described a regional difference in management factors (exposures) that were related to farm features (barn area, flooring type, presence of calving and calf pens), animals (herd size, number of cows and heifers, breed), cohabitation with other domestic animals (small ruminants, horses, poultry, dogs), and the occurrence of wild animals (wild birds, rodents, game). Interestingly, management factors of farms located in CBC differed from the same factors in farms located in other cantons, i.e., USC and C10. Indeed, these differences can be partially conditioned by the geographical location of farms in CBC. That canton is characterised by the dense type of villages, situated in the hilly/mountainous areas surrounding the neighbouring town (Anonymous, 2015b). Such socio-geographical limitation is one of the likely explanations for significantly smaller areas of the barn and consequently smaller herd size in the CBC. However, feeding and reproductive management were the same for all farms, i.e., no substantial differences observed between the studied areas.

All independent (explanatory) variables, i.e., socio-demographic characteristics, information about farm management and information about reproductive performance in cattle, were tested for association with all outcomes (reproduction agents and reproduction problems) using univariable logistic regression (Dohoo et al., 2014). This strategy was chosen as a pre-selection to reduce the total number of candidate variables. A total of 29 of explanatory variables that have shown a moderate association with the outcomes ($p \leq 0.20$) were considered as candidate variables for subsequent multivariable logistic regression. Given the fact that this study had seven outcomes (three reproduction agent/diseases and four reproduction problems), a total of seven multivariable models were established (details in Paper II). These models were treated as component-cause models, and have brought insight into the combination of exposure variables that likely contribute to the outcomes of interest. Some of the variables were present concomitantly in two or more multivariable models, as factors that likely contribute to the occurrence of more than one outcome of interest, e.g. the presence of dogs' at the visited farm as a factor that was associated with the occurrence of *N. caninum*, *C. abortus* and abortions in the same farm. However, the interpretation of variables as potential risk factors for the occurrence of one or more outcomes was ambiguous (e.g. the role of dogs' presence in the case of abortion) or without a real biological linkage. On the other hand, there was a need to conceptualise how concomitantly present factors can combine to cause more than one outcome of interest, either as a proximal or indirect cause.

Therefore, all statistically significant associations found in multivariable logistic regression models were summarized using directed acyclic graphs (DAGitty, 2017). As with any regression analysis, the causal interpretation was based on a biologic underlying rationale, i.e., linking the explanatory variables and outcomes chronologically as previously proposed (Dohoo et al., 2014). Afterwards, all associations from seven multivariable models were merged into a path analysis using the structural equation model (SEM). Structural equation modelling encompasses a broad array of linear and generalised linear models including path analysis, confirmatory factor analysis, latent growth models, multiple indicators – multiple causes models, and item response theory (StataCorp., 2017). This statistical framework is also developed to model complex relationships between directly and indirectly observed variables (Stein et al., 2012). However, in the view on the statistical conclusions drawn from seven multivariable regression models and study limitations (see Methodological consideration), the use on indirectly observed (latent) variables was not statistically justified. Thus, in this study the SEM was implemented as a confirmatory causal path analysis, using a maximum likelihood approach for estimating all paths into the one generalised multivariate model (Rabe-Hesketh et al., 2004). The results of this study indicated that farms' managerial practices and features were directly associated with the occurrence of severe reproductive problems, but also with the herd seropositivity to one or more reproductive infectious agents. Apart from direct associations, management factors could potentially influence the occurrence of reproductive problems, favouring the occurrence and existence of reproductive infectious agents that are likely to cause reproductive problems in cattle herds. The benefit of SEM could be seen in the reduction of the number of explanatory and non-biologically related variables that are found in the multivariable logistic regression.

According to the results of this study, the dog population (proprietary and free-roaming) is one of the most important management factors in cattle reproduction epidemiology in BH. It was found to be associated with the herd seropositivity to *C. abortus*, *C. burnetii* and *N. caninum*. Traditionally, most farmers keep dogs as guardians, shepherds or pets. Accordingly, those dogs have restricted movement within the estate, or as they follow the livestock. On the other hand, there is a certain number of abounded, free-roaming dogs that are commonly found near the farm facilities, searching for food. Their uncontrolled movement between farms and their potentially contaminated excrements (defecation) could be another extraordinary infection burden for cattle in the visited areas. Since dogs are definitive hosts of *N. caninum*, the association between the presence of dogs and seropositivity in cattle is not surprising and has previously been reported in other epidemiological studies (Corbellini et al., 2006, Mainar-Jaime et al., 1999, Pare et al., 1998). The transmission of the parasite from dogs to cattle in the studied areas could be achieved through direct contact, or by the faecal-oral way, i.e., defecation by farm dogs on feeding area or stored feeds, but also through contaminated water (Figure 9).

Figure 9. *Possible transmission routes of N. caninum: Litter of puppies is located in the area of the barn. Direct contact between cattle and dogs is achieved on the daily basis, but also indirectly through the cows' feeding area and the same water source (concrete water trough). Source: own photo*

Another important aspect in this causal path is a potential infection risk for free-roaming dogs by eating material originated from infected cattle, as disposal of such material is usually located at places available for consumption by stray-dogs. Findings of this study highlight the need to prevent cattle accessing feedstuff and water contaminated by dog (proprietary and free-roaming) faeces. It is recommended to physically separate dogs and cattle in the housing area and the feed storage area (Dubey et al., 2007). Also, organic material (foetal membranes or other tissue) from cattle should be disposed of safely with no access for dogs. The control of the number of stray-dogs seems to be of great importance in reducing the risk of cattle infection with the parasite. There is a mass neuter campaign for stray dogs in BH with >40.000 neutered stray dogs in the period 2012-2018 (DogsTrust, 2018). After neutering, dogs are kept in asylums for certain time where they should, among other things, be treated with anti-parasitic drugs. However, this campaign takes place mostly in urban areas, while such campaign in rural areas is still not fully available due to logistic and administrative issues.

The causal association of dogs with seropositivity to *C. abortus* and *C. burnetii* is not as evident as it is in case of *N. caninum*. Dogs could serve as mechanical carriers of agents to cattle, or can be a reservoir of *C. burnetii* infection to other animals and humans as previously reported

(Lyoo et al., 2017, Shapiro et al., 2016). However, well-organised control of dog population could probably contribute to the reducing of their potential “carrier role” for cattle in BH.

This study discovered *N. caninum* as the only reproductive agent that was associated with some reproductive problems (abortion, stillbirth) in cattle in studied areas. Given that *N. caninum* herd seropositivity was associated only with the presence of dogs and the occurrence of stray dogs in visited farms (lack of fit in the multivariable model), there is a possibility that some cases might have arisen from mechanisms not included in the model. There are several possible explanations of this phenomenon. Hosmer-Lemeshow goodness-of-fit test is based on grouping data using percentiles or fixed values of estimated probability. Once the data are grouped, the number of groups should not be <6 (Dohoo et al., 2014). Our data, however, were grouped in 4 groups and this could be a reason why the Hosmer-Lemeshow goodness-of-fit test showed a significant result (indication of lack of fit). In addition to the statistical test used to calculate the goodness-of-fit, the variables that represent farm dogs and stray dogs are binary (present/absent). Information regarding dogs’ age, numbers, type of feeding, and time since they are present in the farm or observed in farm facilities, could have some additional explanatory power (Dubey et al., 2007). Although these variables are expected to be highly correlated, there is a need for an epidemiologic study that will take into account these aspects of dogs’ presence in cattle farms in BH. In addition, abortions were associated with the occurrence of rodents and hedgehogs, but these associations disappeared in the SEM. However, rodents are described as potential intermediate hosts for *N. caninum* (Hughes et al., 2006, Jenkins et al., 2007), but also as a source of infection for dogs. The potential association of rodents with the *N. caninum* in this study could be masked with their observed association with abortions in cattle.

Further, this study found the temporal associations dogs – *N. caninum* – abortion/stillbirth that indicate the potential exogenous transplacental (vertical) transmission of the parasite. Pregnant cows likely participate in the transmission by ingestion of food or water contaminated by sporulated oocysts originated from infected dog faeces. However, caution is needed in the interpretation of this finding. Although the animal- and herd level seropositivity to *N. caninum* was recognised as an abortion risk (Garcia-Vazquez et al., 2005, Hassig and Gottstein, 2002, Hobson et al., 2005, Pare et al., 1998), it does not prove that the infection is an actual cause of the abortion (Jenkins et al., 2002). However, since *N. caninum* is the only causative agent that was found to be associated with abortions, and given the fact that this agent is not on the list of recommended agents that should be routinely tested in cases of bovine abortions (Anonymous, 2017c), our study strongly suggest that *N. caninum* is indeed an important cause of abortions in cows in BH. This suggests that establishing control measures against bovine neosporosis should be one of the priorities for governmental and veterinary organisations in BH. Based on previous studies (Haddad et al., 2005, Reichel and Ellis, 2002), the standard biosecurity measures are the first step in the prevention of infection in *N. caninum*-free farms. In addition, the reduction of the number of seropositive animals, and consequently, decreasing the vertical transmission seem to be effective control measures for already infected herds. Another important aspect for controlling the infection in BH is the control of populations of dogs (owned and stray dogs) as definitive hosts of the parasite. Accordingly, studies aiming at the fast detection of the infection (bulk milk testing), and further investigation of transmission routes identified in this study are recommended in the organisation of the control measures of neosporosis in BH.

The first study of this PhD project (Paper I) reported a high seroprevalence of *C. abortus* at the animal- and farm levels in cattle in the studied areas. The study results indicated that *C. abortus* seropositivity was associated with the occurrence (presence) of wild birds, dogs and rodents. The reasonable (potential) biological link can be found between *C. abortus* seropositivity and the presence of wild birds. The presence of *C. abortus* was previously confirmed in cloacal swabs and faecal samples of pigeons (Sachse et al., 2012). In this study, the most commonly observed birds were pigeons, sparrows and swallows. Pigeons and sparrows are non-migratory birds in BH, i.e., their breeding and nesting are taking place at the relatively close area, regardless of the season. Swallows, on the other hand, are migratory birds, but they are breeding and nesting in the spring/summer on the northern hemisphere. The nesting places for all observed birds were commonly located in the barns, as a place with optimal environmental temperature and a constant source of food (Figure 10). Hence, their movements in searching for food in- and between farms could probably spread the infection to cattle. Given the ubiquitous distribution of chlamydiae in the environment, dogs and rodents could serve as potential "mechanical" carriers of the bacteria. Our study was not able to discover any association between *C. abortus* and reproductive problems. The high level of antibodies was a challenge here, as the power to find any effects associated with the bovine reproduction was limited in our study approach. If most herds are infected, chlamydiae may represent a baseline condition for inducing reproduction problems, and also by interacting with other agents. The chronic and recurrent chlamydial infection might also have a quantifiable impact on the occurrence of subclinical mastitis (Biesenkamp-Uhe et al., 2007) or pulmonary problems in calves (Reinhold et al., 2011), and hence, more research oriented on this specific problems is needed in BH. According to our study findings, the control measures for this infection should include the basic biosecurity measures and control of potential carriers. Given that dogs and rodents (optionally) were associated with the infection with *N. caninum*, the same control measures directed to dogs could be of benefit in controlling this infection.

Figure 10. *A swallow nestlings observed in the roof of the visited farm in Una-Sana Canton. (Own photo)*

Unlike other outcomes of interest, the animal- and herd level seropositivity to *C. burnetii* was associated with a number of management factors, which could contribute in the spread of infection within- and between farms. One of the most important predisposing factors for seropositivity to *C. burnetii* was the presence of separate calving pens where cows were grouped and prepared for parturition. This strong association can be partially explained by the shedding pattern linked to parturition, i.e., they shed bacteria through birth products (e.g. placenta) (Kruszewska and Tylewska-Wierzbanowska, 1997) or vaginal discharge, urine and faeces (Arricau-Bouvery and Rodolakis, 2005, Guatteo et al., 2008). Additionally, the period around parturition is found to be a period-at-risk for small ruminants, as they shed a high bacterial load at that time (Berri et al., 2002). However, the presence of separate calving pen is reported only by farms that are bigger than average size in this study (10 animals), and the association of this management feature with the infection with *C. burnetii* could be likely overestimated. Contact between herds was possible by using the common pastures areas in densely populated areas where distances between farms are small, and this activity could probably contribute to the spread of infection from infected to naïve herds.

Permanently employed workers at farms were recognised as a protective factor for the seropositivity to *C. burnetii*. Permanently employed workers are found predominately on small farms, and they were usually family members who implement the same hygienic principle and ways of feeding or milking the animals. Consequently, this finding might indicate that the presence of permanently employed workers excludes the presence of seasonally employed workers. Seasonally employed workers could have different hygienic habits and routines and could potentially influence the spread of infection within the herd, by feeding, cleaning or milking activities. In addition to working activities, seasonally employed workers that are potentially infected with *C. burnetii* could contribute to the zoonotic spread of the infection form humans to animals if they are employed at more farms during one season. The control of previously described predisposing factors by the implementation of basic biosecurity measures could be of great importance in the control of this infection in BH. Also, other studies aiming at finding shedding animals (or humans) and excluding them from the production could be crucial in the planning of the control of this infection, as similarly proposed in Paper I.

Normal separation and expulsion of the placenta is associated with the placentome maturation, endocrinal and immunological changes in the peripartal period, its structural composition, as well as vascular and contractile changes post-partum (Peter, 2015). Consequently, pathological changes in any of these factors are described mechanisms for the retention of the placenta. There is no obvious biological link between the placental retention and silage feeding, but this association was noticed in this study. This could possibly indicate an aetiological hypothesis of the nutrition-induced placental retention. More research is needed to investigate this association. This study reported an association between two reproductive problems (outcomes) i.e., retained foetal membranes and metritis. Similarly, other studies have found that retention of the placenta is often implicated as predisposing factor for uterine infections (Gröhn et al., 1990).

Paper III

The results of the previous two studies (Paper I and II) highlighted the presence of reproductive infectious agents and reproductive problems in cattle population in BH, as well as their interconnectedness and association with management factors in farms in selected study areas. In order to obtain a more complete information of bovine reproduction, there was a need to describe the current overall reproductive performance in cattle. Hence, the study aimed at identifying and describing the individual animal reproductive performance in a subset of selected farms in Una-Sana Canton. While such data are available in massive amounts in many countries with a developed bovine industry, this was not the case for BH. Consequently, we had to take a more laborious approach and collect data from individual animals and herds.

Internationally, many reproductive measures and indices are used for the monitoring of reproductive performance in dairy cattle (Fetrow et al., 2007). These measures are usually used to evaluate the overall fertility status of animals or herds, and to point to the areas that need to be improved. In this study, the choice of reproductive indicators was based on the availability of data. Available data included: age at first insemination (heifers only); age at first calving (primiparous cows only); pregnancy proportion at first insemination (heifers only); the cumulative pregnancy rate; number of services per pregnancy, inter-service intervals, calving-to-first-insemination interval, calving-to-conception-interval, and calving interval. Several difficulties were experienced in the calculation of some indicators because they could be calculated only for animals that either had two consecutive calvings or have been re-inseminated and/or confirmed as being pregnant. The detailed information regarding selection criteria can be found in Paper III. This strategy could introduce a selection bias and, possibly, under- or overestimate herds' actual reproductive status.

The results of this study, i.e., the calculated reproductive measures indicated an undulant trend in reproductive performance among selected dairy farms in the Una-Sana Canton of BH. This is particularly the case in the rearing of dairy replacement (heifers) since both AFI and AFC were higher than the recommended international standards (Le Cozler et al., 2008). Therefore, there is a need for the optimisation of reproductive programmes and strategies for heifers in dairy farms, as well as adjusting their breeding goals. However, in addition to management, the general bovine fertility is multifactorial, i.e., many factors such as environment (Windig et al., 2005), nutrition (Butler, 2000), and health status (Fourichon et al., 2000) influence reproductive performance. So, the immediate implementation of recommended breeding goals with no information regarding all potentially contributing factors could possibly have a counter-effect on general reproductive health in cattle in BH. Hence, the gradual adapting of breeding programmes primarily at the farm/herd level, then at the regional and national levels impose as the optimal dynamics for necessary changes. Also, results of the previous study in this PhD thesis (Paper I) pointed to the need for further epidemiological investigation of offspring and replacement animals as a possible reservoir of some reproductive infection in the farm. Therefore, a systematic approach to the rearing of replacement animals would bring multiple benefits for the bovine industry in BH.

Further, this study highlighted the need for a regular and comprehensive performance recording system primarily in dairy farms in the Una-Sana Canton, but also in the entire country. Such a system is necessary in order to investigate, record and establish the baseline animal performance levels in cattle production systems (dairy and beef). Consequently, farmers/veterinarians would have the necessary animal- and herd level information for taking actions to improve the productivity and health of their animals and increase the overall profit of their farms. Another important benefit of introducing this system in BH would be the possibility to merge with previously established systems for animal identification and registration, and animal traceability (FAMI, 2018, SVO, 2018). Such an integrated and comprehensive recording system would enable not only professionals but also farmers to participate in data collection, and over time also contribute to the potentially stronger integration in the EU markets in the future.

Re-emergence of brucellosis in BH?

The data collection and analysis for the first study of this PhD project (Paper I) was done in 2015. At that time, the mass vaccination programme against brucellosis in small ruminants had already lasted for six years (enrolled in 2009). This study reported a very low seroprevalence of *Brucella* spp. at the animal- and herd levels. Given the fact that *Brucella melitensis* biovar 3 (Velic, 2012) is the only *Brucella* species isolated in BH, the low seroprevalence in cattle could be partially explained by the successful implementation of the vaccine in small ruminants. This is consistent with the spill-over of *B. melitensis* from small ruminants to cattle as the predominant brucellosis problem. Importantly, the peak of human brucellosis (778 confirmed cases) was reported in 2008 (Obradović and Velić, 2010), just before the vaccination of small ruminants started. After the mass vaccination started, the number of new human cases of brucellosis was substantially reduced. This strongly suggest that mainly *B. melitensis* is of zoonotic concern in BH. Other studies from countries implementing the Rev1 vaccination reported good results regarding the reduction of human brucellosis (Blasco, 2010, Minas et al., 2004, Zinsstag et al., 2005). The vaccination was planned to cease in 2017 entirely, i.e., two years after obtaining the data for Paper I.

The animal and human health organisations in BH were expecting to see the results of a vaccination programme against brucellosis in BH implemented until 2017. A recent study evaluated the effects of the implementation of small ruminants vaccination programme on flock prevalence (Seric-Haracic et al., 2018). This modelling study reported that the vaccination programme reflected a slow decrease of flock prevalence in all of the initial average flock prevalence (2%; 5% or 10%), but also those decreasing trends (slopes) were influenced by the initial disease prevalence. Also, the ability of the national surveillance system to recognise and remove the infected animals and/or flock was found to be significantly reduced during the vaccine implementation period. In conclusion, it was reported that brucellosis of small ruminants will remain an important issue for (veterinary) public health in BH (Seric-Haracic et al., 2018). Nowadays, disease information form studied areas (USC, CBC) indicate the increasing trends of brucellosis in small ruminants and humans (unpublished data). Therefore, there is a need for an integrated (one health) approach in order to better understand the current situation of brucellosis in BH and to be able to advocate any further control measures (Godfroid et al., 2013).

Methodological consideration

Details of the methodologies are given in each paper (I-III). This section will provide additional information regarding the methodology, explain the choices of statistical tests and highlight limitations of the studies included in this PhD thesis.

Paper I and II

These cross-sectional studies included three cantons of FBH. The choice of this region was based on their logistic convenience, i.e., geographical location, and the proximity of veterinary institutions that were suitable for storing and analysing collected samples. In addition, the total number of cattle in these cantons represent one-third of the dairy cattle population in BH (Anonymous, 2017a). Although the reproductive management does not differ significantly between farms, the choice of other cantons might bring a slightly different epidemiological pattern of reproductive pathogens. However, we do not believe that this would alter our overall conclusion.

The minimum sample size of 60 farms per canton (n=180) was determined based upon the detectable herd level presence of 5% seropositive animals, and 95% level of confidence. The choice of sampling frame from the target population was a challenge due to the lack of complete herds' lists and absence of organised national database. Hence, data collected for this study might have missed some relevant information about the investigated reproductive infectious agents and reproductive problems.

The collection of animal- and herd level data using a semi-structured questionnaire was done through personal visits of each selected farm. The data included owners', veterinarians' and personal observation, and this reduced substantially the amount of missing data. However, farmers' answers were largely dependent of farmers' memory and attitudes, and therefore, the occurrence (frequency) of some of the factors found to be associated with the reproductive infectious agents and problems could be over- or underestimated. Some answers were not foreseen with multiple-choice questions. However, those answers were recorded and processed later.

The reproductive problems (e.g. abortions) were classified as occurred/not occurred. However, abortions that occurred during the first trimester of pregnancy could hardly be recognised or remembered. Hence, there was a possibility of misclassification bias. Also, the occurrence of metritis could also be easy to miss, or could be overestimated because some farmers reported preventive treatment of metritis in the postpartum period. This challenge was partially overcome by including veterinary history or written records in the definition of this reproductive problem.

These studies have sought to respond to research questions aiming at fulfilling knowledge gaps. Our main aim was to address the almost total lack of research evidence on reproductive infectious agents (*Brucella* spp., *C. abortus*, *C. burnetii*, *N. caninum*), as well as reproductive problems in cattle population in BH. We have done so by directly observing the seroprevalence of these pathogens and the frequency of these problems, with special attention to the management practices that contribute to the causal web of associations between them. Accordingly, the major contribution of these studies is that they provide epidemiological data on the investigated pathogens /problems. Also, we recommend some of the potential measures that need to be undertaken to control these problems in the bovine industry in BH.

Paper III

This was a follow-up study for a subset of previously selected dairy farms in the USC. The choice of farms was based on their commercial milk production (cooperatives with dairies). Similar to paper I and II, there was not a complete list of all dairy cooperatives in the USC. Hence, the sampling frame consisted of dairy farms from the available municipal lists of cooperatives with dairies. This could possibly introduce a selection bias, which was partially prevented because selected farms were assembled retrospectively from the previous studies of this PhD project.

The database preparation was done through visits of all previously selected farms, and collecting farms' handwritten papers, diaries and daily tables. Animal timeline data included: birth date, date of first inseminations in a reproductive cycle, type of insemination (AI or natural), repeated inseminations and their types, date of calving, calving outcome (normal, elevated, abortion, stillbirth, twins). For heifers, dates of first insemination and calving were recorded as AFI and AFC. Taking into account all the above, there was a possibility for omissions in the recording of some of the points in the timeline. Farms' records did not include data on animals that were culled due to reproductive reasons. In addition, selected farms with incomplete or missing written records were excluded from the study. Since the excluded farms were predominately farms with the above-average number of animals, the reproduction measures calculated in a study including these farms, could possibly be different with greater variations. Hence, caution is needed when interpreting reproductive performance indicators, because they may indicate a better reproductive situation than actually exists in BH.

The results of this study are presented as herd and study population averages. However, caution is needed in the interpretation and link of some herd mean values (e.g. AFI, CFI). This is described as the ecological fallacy which would entail ascribing the group mean to individuals (Robinson, 2009). On the other hand, there were animals whose reproduction indicators substantially deviated from farm average. The potential link of the performance of these animals with the group average (farms or study population) would probably lead to the exceptional fallacy in the interpretation of the results.

Our main aim was to identify and describe key reproductive performance indicators in a subset of dairy farms selected for participation in previous studies. We have done so through the calculation of these indicators from collected farms' written data. Our study highlights that the individual animal reproductive life and farms' reproduction management should be improved in the selected area of BH. Also, our study suggests that the establishing of animal performance recording system in BH is strongly recommended.

Conclusisions and recommendations

The present PhD project aimed to fill some of the information gaps regarding four infectious reproduction agents (*Brucella* spp., *C. abortus*, *C. burnetii* and *N. caninum*) in cattle population and their link with the management, as well as to identify and describe key reproductive performance indicators in dairy farms in BH.

We described the presence and equitable distribution of these pathogens among the cattle population in selected areas of BH. Our studies highlight that some of these infections remain undetected and this calls for an improved monitoring system of reproductive problems/diseases that occur in cattle population in BH. Based on the findings of this PhD study, we suggest adding *N. caninum* to the programme of control measures for infectious and parasitic diseases of livestock in BH. Also, there is a need for performing a cost/benefit analysis to verify our recommendation. Management practices and farm environment were found to be associated with the seropositivity of a specific infectious agent, but also with the occurrence of reproduction problems in cattle farms. Hence, there is a need for evaluation of existing managerial practices and adjusting/updating them to the conditions described in this thesis. This means the engagement and joint actions of farmers and professional staff in all levels of the veterinary network in BH, through the implementation of basic biosecurity measures, the control of the potential biological reservoirs ("carriers") of infections and the continuous education programmes. Identified and calculated reproductive indicators point to improving the reproduction management. Accordingly, this is a call for an organised, timely and structured recording of reproductive events enabling the calculation and interpretation of reproductive indicators.

There is a real need for the establishment of the national animal performance recording system, as one of the first challenges linked to the intensification and profitability of the dairy industry in BH.

The following specific conclusions are based on the specific objectives previously mentioned (see section Aims and objectives)

- The relatively high animal- and herd levels seroprevalence of some of the investigated infectious agents partially confirm our hypothesis that the magnitude of reproduction problems can be partially explained by the high prevalence of some infectious reproductive agents among cattle population in BH.

- Given its low-level seroprevalence, *Brucella* spp. does not seem to represent a reproduction problem in cattle in the studied areas. This favourable situation in cattle follows the implementation of a vaccination program in small ruminants, which might result in controlling *B. melitensis* spill-over infection to cattle. However, the vaccination is planned to cease entirely in 2017/2018, so there is a possibility that *Brucella* spp. will remain an important public health issue, and accordingly, should not be neglected as the infection cause of reproduction problems in cattle.
- *N. caninum* is found to be associated with abortion and stillbirth in cattle farms of north-western, eastern and central BH. The infection dynamic of neosporosis in cattle seems to be influenced by the presence/occurrence of dogs/stray dogs that are the definitive hosts of this parasite.
- Dogs/stray dogs are found to be associated with animal- and herd levels seropositivities of *C. abortus*, *C. burnetii* and *N. caninum*. Hence, the population of dogs/stray dogs have to be controlled, i.e., their access to the farm premises has to be restricted or completely prohibited.
- The high animal- and herd level seroprevalence of *C. abortus* is a future challenge for veterinary authorities, as the power to find any effects associated with the bovine reproduction was limited in our study approach. Most farms were found seropositive (infected), so chlamydiae may represent a baseline condition for inducing reproduction problems specifically, or by interacting with other infectious agents.
- The animal- and herd levels seropositivity of *C. burnetii* was more associated to the farms' reproduction management, in comparison to other infectious agents. Hence, farmers should pay particular attention to animals in peripartal period and avoid any contact between parturient animals and other animals in order to prevent potential transmission of the agent.
- There is a need to introduce basic but strict biosecurity measures by enhancing the technical and logistic capacity. The farmers' awareness of the importance of such measures should be increased through governmental and professional advice and services.
- We were able to identify and evaluate key reproductive performance indicators for dairy farms in the selected region, but the validity of this particular study is somehow limited due to the lack of reproductive records.
- Animal (re)productive performance recording is currently not an officially organised activity in BH and is based on individual handwritten farm records. Moreover,

managers/farmers of farms with an above-average number of animals are urged to introduce the recording of animal performance in their farms.

- Another future challenge for veterinary authorities in BH is to organise a comprehensive system and national cattle database, which will contribute to the rapid integration and better competitiveness on the EU market.

Future perspectives

This thesis provides some answers to previously recognised research questions related to the knowledge gaps regarding bovine reproduction in BH. However, some aspects of our research questions could not be adequately addressed. It raises some additional questions that need to be answered, and more research will, in fact, be necessary to refine and further elaborate on our findings. We provided scientific information on epidemiological characteristics and distribution of reproduction infectious agents and reproduction problems in cattle population of BH, as well as information on factors that contribute to their occurrence and/or persistence and possible transmission. Since our sampling plan did not include animals younger than 12 months which may be the natural reservoir of all investigated infections, our research should be extended to the epidemiological investigation of all age groups of animals, but also an investigation of other areas in BH. In addition, there is a need for investigating the animal level shedding prevalence which is crucial to estimate the risk of transmission of pathogens, as well as to identify the actual transmission routes. Another important future extension of our research may be the seroepidemiological and molecular study of *N. caninum* in a population of dogs/stray dogs. Such a study would bring more detailed insight into the actual epidemiological situation of this parasite and would help in the estimation of the infection burden for cattle. Also, transmission routes could be better identified.

Infectious causes of reproductive disorders in cattle are numerous, and this thesis could not cover all of them. Other infectious agents including Bluetongue virus, bovine herpesvirus-1, bovine viral diarrhoea virus, IBR, Schmallenberg virus, *Campylobacter fetus* subsp. *veneralis, Haemophilus somnus*, *Leptospira* spp., *Listeria monocytogenes*, and *Trichomonas foetus* that are globally known to have a negative impact on bovine reproductive health. The scientific information regarding these infectious agents is largely unknown. Therefore, the status and associated impacts of these infectious agents should be investigated in cattle population in BH.

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Scientific papers I – III

Paper I

Softic et al. BMC Veterinary Research (2018) 14:40
DOI 10.1186/s12917-018-1361-z

BMC Veterinary Research

RESEARCH ARTICLE Open Access

The serostatus of *Brucella* spp., *Chlamydia abortus*, *Coxiella burnetii* and *Neospora caninum* in cattle in three cantons in Bosnia and Herzegovina

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Abstract

Background: Dairy production in Bosnia and Herzegovina exhibits limited productivity, which may partly, be explained by extensive reproductive problems of non-infectious and infectious origin. *Brucella* spp., *Chlamydia abortus*, *Coxiella burnetii* and *Neospora caninum* are common infectious causes of decreased reproductive outcomes in cattle worldwide. Little is, however, known about the disease status of herds with reduced reproductive performances. A cross-sectional study was designed to document the status of these pathogens in dairy cattle in Bosnia and Herzegovina. A total of 1970 serum samples were collected from cattle in farms located in three cantons (regions). Enzyme linked immunosorbent assays were used to screen for seropositivity against four selected pathogens.

Results: The overall seroprevalence was estimated at both the herd level and at individual level for each pathogen. At the individual animal level, the prevalence for *C. abortus, C. burnetii, N. caninum* and *Brucella* spp. was 52.1% (95% CI: 41.2–62.7) , 8.8% (95% CI: 5.3–14.2), 9.2% (95% CI: 6.0–12.3 and 0.2% (95% CI: 0.1–0.5), respectively. The corresponding estimates for herd level were 87.9% (95% CI: 82.6–91.8), 19.6% (95% CI: 14.6–25.8), 35.2% (95% CI: 28.8–42.1), and 1.5% (95% CI: 0.5–4.6). A substantial overlap was observed in the presence of *N. caninum*, *C. abortus* and *C. burnetii* at individual and herd level.

Conclusion: Our study demonstrated a high level of antibodies to *Chlamydia abortus*. Considering the association of this agent with reproductive disorders in cattle, future studies should be directed to the epidemiological traits of this infection. Additionally, the relatively high levels of exposure to *C. burnetii* and *N. caninum* found in this study highlights the need for targeted control of infectious causes of reproductive disorders in dairy cattle of the studied areas. Given the low seroprevalence, *Brucella* spp. does not seem to represent a problem in the reproductive health of cattle in the studied areas.

Keywords: *C. abortus*, *C. Burnetii*, *N. Caninum*, *Brucella* spp., Cattle, Bosnia and Herzegovina

Background

The cattle industry is faced with a number of challenges affecting its further development. Reproductive disorders represent one of these challenges across the world, and may be due to intrinsic or extrinsic factors imposed on the herd and individual animals such as genotypic traits, feeding, contaminants and toxins in feeds or other environmental factors [1]. The incidences of reproductive diseases in cattle are reported to be increasing over the years [1]. Infectious agents are known to cause infertility, early embryonic death, protracted calving seasons, abortion and stillbirth [2, 3]. These infectious agents could seriously damage general agriculture of countries in transition, such as Bosnia and Herzegovina.

Agriculture has been an indispensable part of the gross economy in Bosnia and Herzegovina (BH) over the last decade. Growing market demands and convergence to standards set by the European Union forces animal husbandry practices to shift from extensive to semi-intensive production system, along with improved biosecurity and

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focus on herd health status [4]. In the wake of structural changes, the enhancement of reproductive performance of livestock is a necessity in contributing to increased production [5].

However, little is known about the epidemiology of various infectious agents in the cattle populations of BH. Several viral, parasitic or bacterial pathogens are known to be associated with reproductive failure in cattle, including bovine viral diarrhoea virus (BVDV), infectious bovine rhinotracheitis (IBR) virus, *Brucella abortus*, occasionally *B. melitensis*, *Neospora caninum*, *Coxiella burnetii*, *Campylobacter fetus venerealis* or *C. fetus fetus*, *Leptospira* spp., *Tritrichomonas foetus*, *Chlamydia abortus* [6–10]. Some of these infectious agents are ubiquitous in cattle populations, and their occurrence is associated with biosecurity measures in farms. However, in-herd disease control measures are rarely effective, since viruses and bacteria may be shed continuously through faecal, vaginal, urine, seminal, ocular, and nasal discharges. Vertical transmission also occurs frequently with BVDV, *Brucella* spp., *Campylobacter* spp. and *N. caninum* [7–10].

Control of reproductive disorders in cattle relies upon systematic and coordinated efforts of the country's Veterinary Services at the national and regional levels and requires financial resources. Trade and transportation of animals are contributing factors in the spread of diseases. Hence, controlling herd biosecurity and adopting artificial insemination may aid in the prevention of contagious reproductive diseases. Until now, there has been very limited information on the occurrence of infectious reproductive problems in BH. In this study, the authors focused on four of the assumed important agents: *Brucella* spp., *C. abortus*, *C. burnetii*, and *N. caninum.* The selection was based upon the relative importance of these agents in the occurrence of reproductive problems in cattle worldwide. Additionally, the limited information about the importance of *Brucella* spp. and *C. burnetii* and the uncertainty about the presence of *C. abortus* and *N. caninum* in cattle in BH contributed to the selection.

Chlamydiae are obligate intracellular, Gram-negative bacteria that cause a wide range of diseases in animals and humans [11]. Some of the chlamydiae are ubiquitous in cattle populations. Infections of cattle with *Chlamydia abortus*, *C. pecorum, C. psittaci and Chlamydia suis* have been associated with reproductive disorders including abortion, endometritis, repeat breeding, vaginitis, birth of weak calves and perinatal mortality. Chlamydial diseases are frequently asymptomatic in nature, and clinical expressions among individual cattle are often noticed as a non-specific loss in reproduction. Infection with chlamydiae has also been associated with bovine sub-clinical mastitis. Also, clinical manifestation in calves are recorded as pneumonia and weight loss [3, 11–13].

C. burnetii is a rickettsial pathogen which causes Q-fever in cattle [14]. The infection is generally asymptomatic but can lead to abortions, premature offspring, stillbirths and delivery of weak offspring [6]. Worldwide, the apparent prevalence is slightly higher in cattle than in small ruminants [15].

Brucellosis (*Brucella* spp. infection) in cattle may result in abortion after the fifth month of pregnancy or delivery of weak calves. In addition, retained fetal membrane and metritis often occurs [2, 12, 16]. Cows with puerperal metritis resulting from retained placenta are prone to uterine diseases such as clinical metritis, clinical endometritis and subclinical endometritis, which lead to failure of conceiving. When a successful eradication of *B. abortus* has been implemented in cattle, sporadic infection of cattle with *B. melitensis* have been documented in Spain [17] and France [18], countries that are not free of brucellosis in small ruminants. *B. melitensis* biovar 3 is the only *Brucella* spp. isolated from cattle, small ruminants and humans in BH [19, 20], and *B. abortus* has never been isolated. However, little effort has been put into epidemiological mapping of disease outbreaks and phylogenetic studies on pathogens associated with livestock in BH. The implementation of the national brucellosis mass vaccination program in sheep and goats since July 2009 (with ocular Rev-1 vaccine) has resulted in the successful control of brucellosis in small ruminants with prevalence rates steadily decreasing [21].

N. caninum is a protozoan parasite of cattle. Dogs are definitive hosts while cattle are intermediate hosts. Transmission is by two ways: highly effective transplacental (vertical) or post-natal (horizontal) via the oocysts ingestion from feces of dogs. [22, 23]. Cows of any age may abort from 3 month of gestation to term, but most abortions occur at 5–6 month of gestation [22, 24].

To better understand the epidemiological pattern of these infections, a cross-sectional serological study was designed to describe the epidemiological characteristics of *Brucella* spp., *C. abortus*, *C. burnetii* and *N. caninum* in BH.

Methods

Study area

The study was conducted between January and August 2015 in three regions/cantons of the Federation of Bosnia and Herzegovina (FBH). These are Una-Sana Canton with an area of 4125 km^2, Canton 10 with an area of 4934 km^2 and Central Bosnia Canton with an area of 3189 km^2. These cantons represent 23.9% of the area of BH (Fig. 1). In general, the study area is characterised by the temperate continental climate. [25]. This

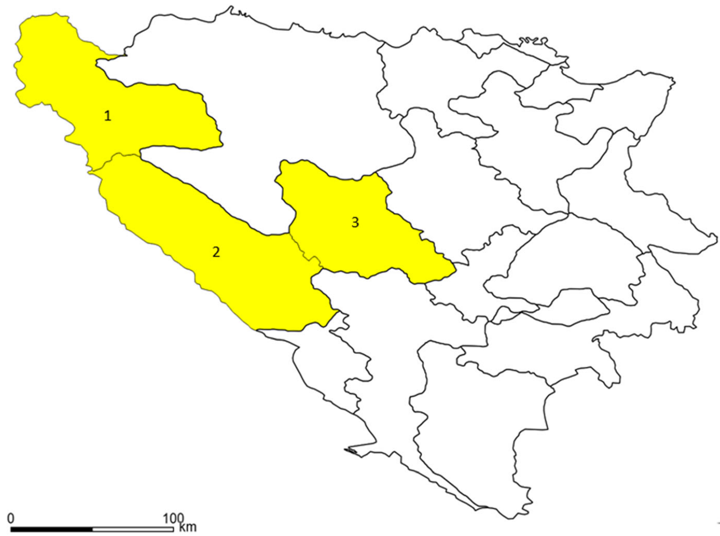

Fig. 1 Map of Bosnia and Herzegovina shows the study area consisted of: 1 – Una Sana Canton; 2 – Canton 10; 3 – Central Bosnia Canton

climate in northern and central parts of BH is characterised by four seasons, as well as moderate to warm summers, and mild to moderate winters. Additionally, areas with an altitude higher than 1000 m above sea level are characterised by sub-mountainous and mountainous subtype of the temperate continental climate.

The selection of cantons was guided by convenience for visits, sampling plan as well as the proximity of the veterinary institution where the samples were prepared for testing. The number of dairy cattle in the selected cantons represents 33.9% of the total number of dairy cattle in BH (Table 1) [26].

Study design and sampling of herds

The study was a cross-sectional study that included blood sampling of cattle and interviews of farmers based on a semi-structured questionnaire. The minimum sample size of 60 farms from selected regions of the country ($n = 180$) was determined based upon the detectable herd-level presence of seropositive animals at 5%, 95% level of confidence [27]. The herd sizes were stratified into small (1–10 animals), medium (11–30 animals) and large (> 30 animals). The sample size was slightly increased to 202 herds. From these herds, three were excluded due to missing data, so the final dataset included 199 herds.

Table 1 Number of cattle in selected regions of BH, given as number of animals and percentage of the total dairy cattle population of BH. [26]

Canton	No. of dairy cows (%)	No. of heifers (%)
Una - Sana	20,881 (18.5%)	3692 (15.2%)
Canton 10	9967 (8.8%)	2145 (8.8%)
Central Bosnia	7460 (6.6%)	1783 (7.4%)
Total	38,308 (33.9%)	7620 (31.4%)

Study animals

The target population was all dairy cattle herds from the selected cantons, which are composed of Simmental, Montafone, Holstein-Friesian, Red Angus breeds and their crosses. The source population was all dairy herds in the selected regions that participated in an annual surveillance and disease control programme focused on bovine brucellosis and enzootic leucosis ("Official Gazette BH", issue number 34/02). In collaboration with the cantonal veterinary department, a sampling frame of known herds was prepared, and herds were randomly selected based on the available list. The sampling was carried out in a two-level approach, selecting first individual herds and then sampling all animals older than 12 months within each herd. A slightly modified type of sampling was performed in Central Bosnia canton, as animals older than 12 months were previously sampled as a part of the same annual surveillance and disease control program and then herd selection was done randomly based on the available list. The difference between sampling scheme is mainly that the minimum number of animals sampled in each herd was lower in the Central Bosnia Canton. A total of 1970 serum samples were collected from the selected cattle herds. Questionnaire-based interviews were conducted in all farms whose samples are included in this study. In addition to the dairy herds, a small number of beef herds were also sampled.

This study was submitted to and approved by the Ethics Committee of the Veterinary faculty in Sarajevo.

Sample collection

Blood samples (5 ml) were collected from the tail vein (*v. coccygea*) of each animal, using sterile needles and plain vacutainer tubes. The samples were allowed to stand overnight at room temperature to obtain the serum. Optionally, the samples were centrifuged at 3000 x g for 5 min. Serum was pipetted into cryovials and stored at − 20 °C in the Veterinary Institute of Bihac. Samples were then transported to the Veterinary Faculty in Sarajevo (Department for Infectious Diseases) on ice packs and stored at − 20 °C until tested.

Laboratory tests

All sera, in their preparation prior to storage, were screened for antibodies against *Brucella* spp. using the Rose Bengal Plate Test (RBPT). Tests were performed according to the standard protocol recommended by the OIE [28]. Positive sera were further tested using the complement fixation test (CFT) as a confirmatory test by standard protocol [28]. Brucellosis positive and negative national control sera were always included during the testing. Complement and hemolysin were obtained from IDEXX/ Porquier, Montpellier, France. Sheep blood was obtained from animals from the farm of the Veterinary Faculty in Sarajevo. The presence of antibodies to *Brucella* was also determined using the IDEXX Chekit Brucellose serum AB test (IDEXX, Switzerland) and interpretation was based on a serum to the positive ratio (S/P%) where < 80% was considered negative and ≥80% positive, according to the manufacturer's protocol.

The presence of antibodies to *N. caninum* was determined using the IDEXX *Neospora* X2 Ab test kit (IDEXX, Switzerland). A serum with absorbance value (S/P) with a cut-off level of ≥0.50 was considered to be *Neospora* positive. For *C. abortus*, antibody screening was conducted using the IDEXX Chlamydiosis Total Ab test kit (IDEXX, Switzerland). Interpretation of the results was based on S/P% where < 30% was considered negative, ≥30% to < 40% suspect and ≥40% positive. For Q fever (*Coxiella burnetii*) antibody screening was conducted using the IDEEX Q fever antibody test kit and interpretation was based on S/P% where < 30% was considered negative, ≥30% to < 40% suspect and ≥40% positive. Suspect findings for *C. abortus* and Q fever were recorded. The tests were not repeated for suspect results. The test protocol and interpretation of all ELISA tests were performed according to the manufacturer's instruction (IDEXX). Animals positive to the Rose Bengal Plate Test (RBPT), Complement fixation test (CFT) and Enzyme Linked Immunosorbent Assay (ELISA) test were classified as seropositive to *Brucella.* Animals positive to ELISA tests, according to the manufacturer's instruction, were classified as being seropositive to *C. abortus*, *C. burnetii* and *N. caninum*. Vaccination against these agents has never been implemented in BH and seropositivity was considered to be due to natural infections.

Data management and statistical analysis

A database was established in Microsoft Excel® 2013. The raw dataset used in this study is attached in Additional file 1). After cleaning and checking, data were transferred to Stata SE/14 for Windows (Stata Corp., College Station TX) for further statistical analysis. (Questionnaire used in this study please find in the Additional file 2).

Statistical analysis was performed at the individual animal and herd level. The proportion of seropositive animals was estimated using survey data analysis [29], with the herd named as the primary sampling unit and the inverse sampling fraction of the herd as weight. Estimates were also calculated for each canton, age, and breed. The association between seropositivity and these risk factors were calculated using a survey logistic model on individual data, adjusted for the study design. Herd level seroprevalences across cantons were calculated using the simple proportion command in Stata. The overall true individual prevalence was calculated where sensitivity/ specificity of the test was available using the Rogan-Gladen estimator [27].

Finally, a Venn diagram [29] was produced to show the overlap between *C. abortus*, *N. caninum* and *C. burnetii*, and the Goodman and Kruskal's gamma-statistics was used as a measure of correlation between them.

Data were then collapsed to herd level and herds defined as positive or negative based on test specificity, using the AusVet Epitool. According to the manufacturer's data, ELISA tests used for investigation of *C. abortus* and *C. burnetii* have demonstrated 100% test specificity (manufacturer's data). Consequently, herds with at least one reactor / seropositive animal were classified as been infected. However, the ELISA test used for investigation of *N. caninum* has demonstrated a 99.2% test specificity. Taking test properties into consideration, a single reactor was sufficient to classify a herd size up to 7 animals as positive (> 95% herd specificity), two reactors for 7 to 45 animals and three reactors for 45–100 animals. Reactors that have shown positive results on the parallel testing (RBPT + CFT + ELISA) were declared as *Brucella* positive and herds where those reactors were observed were classified as infected.

Results

In this study, the most frequently detected seropositivity at the individual animal level was against *C. abortus,* with an overall seroprevalence of 52.1%, The seroprevalence of *N. caninum* was 9.2%, for *C. burnetii* 8.8%. The lowest seroprevalence of 0.2% (95% CI: 0.1–0.5) was observed for *Brucella* spp. (data not shown in the table). The *Coxiella burnetii* and *Neospora caninum* tests were assumed to have a sensitivity and specificity close to 1 by the manufacturers. Thus, the estimates did not change substantially when calculating the true prevalence. However, the estimate of the true prevalence of *Chlamydia abortus* was adjusted to 56.6% (95% CI: 44.8–68.2) based on the test properties. Table 2 displays the distribution and individual level seroprevalence across regions, while Table 3 shows the statistical analyses of the same data, adjusted for regional differences.

Cows older than six years were associated with a higher seropositivity for *C. burnetii*. Further, there were breed predispositions in Red Angus (OR 5.05; $p < 0.001$), Simmental (OR 2.02; $p = 0.016$) and Montafone (OR 1.49; $p = 0.029$) for *C. abortus* compared to cross breed. In addition, there were breed predispositions in Red Angus (OR 3.82; p < 0.001) and Montafone (OR 2.99; $p = 0.09$) for *C. burnetii* compared to cross breed. There was a substantial number of multi-seropositive individuals for *N. caninum*, *C. abortus* and *C. burnetii* (Fig. 2a). The observed overlap was, however random for *N. caninum* vs. *C. abortus* and *C. burnetii*, (gamma close to 0) while a higher correlation was found for *C. abortus* and *C. burnetii* (gamma = 0.35).

Table 4 shows the results for the herd level data. Regional differences at the herd level were found, except for *N. caninum*. Most herds in all three cantons were seropositive for *C. abortus*. In addition, the proportion of seropositive herds in Central Bosnia Canton (4.1%) was notably different in comparison with Una-Sana Canton (27.7%) and Canton 10 (45.9%), for *C. burnetii*. Regional differences at the herd level were found using chi-square test ($p < 0.001$). A total of 4.6% (95% CI: 1.5–13.5) *Brucella* spp. reactors positive herds were found, but only in Una-Sana Canton (data not shown in the table).

Figure 2b shows the herd-level overlap for *N. caninum*, *C. abortus* and *C. burnetii* using the Venn diagram. As for individual data, the observed overlap was more random for *N. caninum*, but a higher correlation (gamma = 0.73) was found for *C. abortus* and *C. burnetii.*

Discussion

This study demonstrates that cattle in three regions of BH were frequently seropositive to *C. abortus*, less frequently to *C. burnetii* and *N. caninum* and rarely to *Brucella* spp. Recent studies on the seroprevalence of *C. abortus* in sheep in BH reported an overall seroprevalence of 43.3% at the individual and 84.2% at the herd level [30]. In addition, *Chlamydia* infection in goats has been previously reported in the southern part of BH [31]. Our study represents the first insight into the presence of *C. abortus* infection of cattle in BH. Moreover, several studies have reported substantial variation in the seroprevalence of *C. abortus* infection in cattle worldwide. In a study on cattle from Turkey [32], a *C. abortus* seroprevalence of 8.3% was reported at the individual animal level, in cows with histories of abortion and 26.9% at the herd level. In Poland, a total of 19.3% out of 1333 bovine sera tested positive for *C. abortus* and *C. psittaci* [33]. Generally, anti-*C. abortus* antibodies were

Table 2 Individual animal seroprevalence of *Neospora caninum*, *Chlamydia abortus*, and *Coxiella burnetii* presented over canton, age and breed. (95% CI)

Variables	Category	n=	*Neospora caninum*	*Chlamydia abortus*	*Coxiella burnetii*
All	Total	1970	9.2% (6.0–12.3)	52.1% (41.2–62.7)	8.8% (5.3–14.2)
Canton	Una-Sana	778	11.4% (7.7–16.6)	47.5% (39.5–55.6)	3.2% (2.0–5.2)
	Canton 10	820	8.4% (5.2–13.1)	65.4% (56.6–73.3)	15.3% (10.8–21.2)
	Central-Bosnia	372	8.0% (3.3–18.4)	35.5% (26.2–46.1)	4.3% (1.9–9.3)
Age	Heifers	186	14.8% (9.4–22.5)	62.3% (51.8–71.7)	6.2% (3.2–11.6)
	Cows < 6 years	1725	8.9% (6.2–12.6)	51.4% (39.6–63.0)	8.6% (5.3–13.5)
	Cows > 6 years	59	4.8% (1.4–15.4)	50.8% (37.1–64.3)	22.0% (8.6–45.7)
Breed	Cross breed	483	9.0% (4.0–18.8)	38.7% (27.9–50.7)	5.9% (4.0–8.7)
	Holstein-Friesian	76	12.0% (4.8–26.9)	51.8% (36.5–66.8)	6.8% (2.2–19.5)
	Simmental	1111	9.0% (6.0–13.4)	56.2% (44.5–67.3)	8.3% (3.2–19.6)
	Montafone	138	15.2% (6.9–30.2)	49.5% (37.6–61.4)	15.6% (5.3–38.0)
	Red Angus	162	6.0% (2.2–15.6)	75.9% (60.8–86.5)	18.6% (14.9–23.0)

Table 3 Distribution of *Neospora Caninum*, *Chlamydia abortus* and *Coxiella burnetii* in relation to the age and breed using survey logistic regression on individual animal data– adjusted for study design. Results shown as Odds Ratio (95% CI); *p*-value)

Variable	Level	*Neospora caninum*	*Chlamydia abortus*	*Coxiella burnetii*
Age	Heifers	1.00 (–); –	1.00 (–); –	1.00 (–); –
	Cows < 6 years	0.59 (0.33–1.06); $p = 0.075$	0.60 (0.31–1.16); $p = 0.13$	1.47 (0.89–2.42); $p = 0.13$
	Cows > 6 years	0.29 (0.08–1.08); $p = 0.065$	0.67 (0.32–1.40); $p = 0.29$	4.88 (2.20–10.85); $\mathbf{p < 0.001}$
Breed	Cross breed	1.00 (–); –	1.00 (–); –	1.00 (–); –
	Holstein - Friesian	1.39 (0.36–5.41); $p = 0.63$	1.72 (0.80–3.72); $p = 0.17$	1.20 (0.34–4.24); $p = 0.78$
	Simmental	0.99 (0.41–2.41); $p = 0.99$	2.02 (1.14–3.58); $\mathbf{p = 0.016}$	1.44 (0.63–3.26); $p = 0.39$
	Montafone	1.74 (0.52–5.83); $p = 0.37$	1.49 (0.77–2.86); $\mathbf{p = 0.029}$	2.99 (0.85–10.51); $\mathbf{p = 0.09}$
	Red Angus	0.65 (0.16–2.59); $p = 0.54$	5.05 (2.10–12.18); $\mathbf{p < 0.001}$	3.82 (2.27–6.44); $\mathbf{p < 0.001}$

found more frequently in our study than in the above-mentioned studies. The seroprevalence of *C. abortus* did not vary significantly across the regions and age groups. Contrary to our finding, a study from Jordan reported significant regional differences in addition to differences between age groups [34]. In the current study, Red Angus was the breed with the highest *C. abortus* seroprevalence noted. This might be explained by the differences in farm management between dairy and beef cattle in BH. Red Angus cows were kept for several months in the overpopulated stables, which increased the contact between animals and exposing animals to contaminated feed and the infectious environments. The seroprevalence of *C. abortus* may be overestimated in terms of antigenic cross-reactivity between *Chlamydia* species, which may also operate as co-infections in the same herd or in the same animal [35]. Therefore, planned follow-up studies using molecular tests will presumably improve our knowledge regarding chlamydial infection in cattle. The vaccination control programme against *C. abortus* infection in cattle are not currently being implemented in BH. Also, *C. abortus* is not listed as a causative agent in the annually updated reporting strategy for severe reproductive failures in cattle ("Official Gazette of BH" issue number 4/16). Moreover, our field experiences have shown that the implementation of biosecurity measures are not common practice among farmers. Hence, the high level of antibodies to *C. abortus* found in this study, indicates the need for further epidemiological investigations.

In this study, the overall seroprevalences of *N. caninum* on individual and herd level were 9.2% and 35.2%, respectively. The occurrence was widespread, with no evident difference between regions, age groups, and animal breeds. A recent study [20] reported the presence of anti-*N. caninum* antibodies in ruminants in BH, with 16 (8.7%) of 184 positive samples in the period of 2005–2009. In Croatia, the seroprevalence of *N. caninum* antibodies was reported at 5.8% [36]. In Serbia, it was reported [37] that individual and herd seroprevalences were 4.6% and 27%, respectively. Another study from the northern part of Serbia showed an overall individual seroprevalence of 15.4% using ELISA and indirect fluorescent antibody test (IFAT) [38]. The difference of

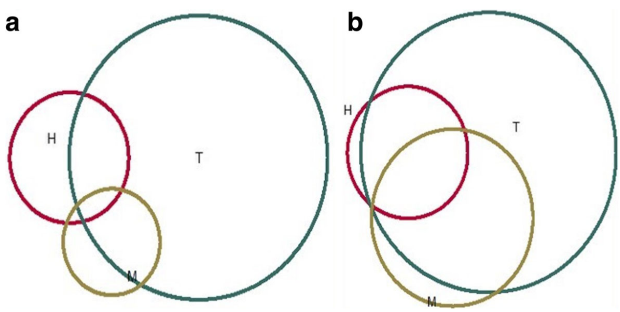

Fig. 2 Observed overlap in seroprevalences of *Neospora caninum*, *Coxiella burnetii* and *Chlamydia abortus* at individual animal level (**a**) and herd level data (**b**). H – *Neospora caninum*; M – *Coxiella burnetii*; T – *Chlamydia abortus*; Blue square represents an investigated population

Table 4 Overall and cantonal proportions (95% CI) of *Neospora caninum*, *Chlamydia abortus*, and *Coxiella burnetii* seropositive herds ($n = 199$)

Canton	Herds	*Neospora caninum*	*Chlamydia abortus*	*Coxiella burnetii*
Una-Sana	65	40.0% (28.7–52.4)	96.9% (88.3–99.2)	27.7% (18.1–39.9)
Canton 10	37	40.5% (25.9–57.1)	97.3% (82.6–99.6)	45.9% (30.5–62.2)
Central-Bosnia	97	29.9% (21.6–39.8)	78.4% (68.9–85.5)	4.1% (1.5–10.6)
Total	199	35.2% (28.8–42.1)	87.9% (82.6–91.8)	19.6% (14.6–25.8)

estimates between our study and studies from neighboring countries may be explained by the differences in study designs, the diagnostic test used, sample size and management related factors. The overall picture, however, is that *N. caninum* is frequently found and represents a potential cause of reproductive failures in BH, thus being subject to future attention in Balkan countries.

Q fever is not a part of the Directive for control of infectious animal diseases in BH ("Official Gazette" No 34/02) and information about this pathogen in cattle populations is very scarce on a national level. Previous studies reported the occurrence and spread of Q fever in the human populations of BH [39, 40]. Our study found an overall seroprevalence for *C. burnetii* at the individual and herd level of 8.8% and 19.6%, respectively. A recent study from Croatia [41] reported that 2.7% of cattle tissue samples were positive for the presence of *C. burnetii* DNA. In the same study, 13 novel *C. burnetii* genotypes unique for Croatia were reported. In Albania, the reported seroprevalence [42] of Q fever was 7.9% in cattle. The circulation of *C. burnetii* among cattle population indicates the need for its control in BH.

The overall individual seroprevalence of *Brucella* spp. in cattle in our study was 0.2%. In 2008, while a test-and-slaughter control strategy was still being used in cattle and small ruminants, a seroprevalence of 4.6% was found, mainly in sheep and brucellosis in humans was also reported [43]. Authorities in BH then changed the control strategy, and mass vaccination of small ruminants with the ocular Rev-1 vaccine was implemented in 2009. Importantly, as reported in the last FAO regional workshop on brucellosis control in Central Asia and Eastern Europe, the prevalence of brucellosis in cattle has been reduced in BH, due to the vaccination strategy [21]. Our study validates this trend and underlines that anti-*Brucella* antibodies detected in cattle in BH could be possible due to a spill-over of *B. melitensis* from the small ruminant reservoir. Although proving the absence of *B. abortus* is impossible, cattle being the spill-over host of *B. melitensis* originating in the small ruminant reservoir could be considered as a possible explanation for the low prevalence of anti-Brucella antibodies detected in cattle. It is important to re-emphasize that the favorable situation in cattle follows the implementation of a successful vaccination program in small ruminants in BH, as it has been suggested in different countries in the region [21]. Such findings suggest that in mixed small ruminants-cattle herds, a successful vaccination program in small ruminants may result in controlling *B. melitensis* spill-over infection to cattle without the need to vaccinate cattle [44, 45].

Our results indicate that *N. caninum, C. abortus,* and *C. burnetii* are frequently present in cattle herds in BH. This may be of clinical importance for reproductive disorders and may restrain the production performance of cattle herds. Importantly, brucellosis in cattle resulting from the spill-over of *B. melitensis* is most probably controlled in BH as a result of implementation of a successful mass vaccination program in small ruminants. The very few positive animals, found in this study could be possible linked to a spill-over of *B. melitensis* from sheep and goats. Currently, brucellosis is not contributing significantly to reproductive disorders in cattle in BH. Today, small ruminants are not tested, as they are under vaccination program. There is an annual program for brucellosis in cattle. A rise in the prevalence of anti-*Brucella* antibodies in cattle may indicate a problem in the control program in small ruminants. *N. caninum* is frequently found in cattle herds of BH which may be, probably linked to an un-controlled population of dogs. For *C. burnetii* and *C. abortus,* the high levels found in this study call for attention as possible constraints to the cattle reproduction and breeding in BH. Continued studies will investigate the potential impact of these above-mentioned agents on reproductive performance in cattle in BH. Also, we found a high correlation (gamma = 0.73) between *C. abortus* and *C. burnetii*, while the observed overlap between other agents were more random. This may be partially explained as the existence of common factors that contribute to the occurrence of these agents. Nevertheless, this association should be investigated further.

Conclusions

The study demonstrates a high level of antibodies to *Chlamydia abortus* in BH cattle herds, but substantial levels of antibodies to *Coxiella burnetii* and *Nesopora* *caninum* were also found. The findings illustrate a situation where these agents may be influencing reproductive performance in the cattle population. Currently, *Brucella* spp. does not seem to represent a reproductive problem in cattle in the studied regions.

Additional files

Additional file 1: Softic_BMC_raw_dataset; The raw dataset used in this study. (XLSX 147 kb)

Additional file 2: Softic_BMC_questionnaire; The questionnaire used in this study. (DOC 102 kb)

Abbreviations

BH: Bosnia and Herzegovina; BVDV: Bovine viral diarrhoea virus; ELISA: Enzyme linked immunosorbent assay; FBH: Federation of Bosnia and Herzegovina; OR: Odds ratio

Acknowledgements

The authors warmly thank to all study participants, veterinarians and personnel of the Veterinary Institute in Bihac and Veterinary faculty in Sarajevo, Department for Infectious Diseases, who helped in the realization of this study.

Funding

The study has been kindly supported by the Norwegian quota program and the Norwegian University of Life Sciences and the Veterinary Faculty of University of Sarajevo, BH.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its Additional files].

Authors' contributions

Conceived and designed the study: AS, ES, EEG, KA, JG, NF. Questionnaire designed: AS, ES, EEG, KA. Performed sampling and laboratory testing: AS. Analysed the data: AS, ES, KA, EGG. Wrote the manuscript: AS, ES, EEG, KA, JG, NF. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was conducted in accordance with the Law on Animal Protection and Welfare of BH ("Official Gazette BH" issue number 316/09). Samples were collected after acquiring the permission from study participants. All farmers/owners who participated in this provided their verbal consent to participate in this study. This study was submitted to and approved by the Ethics Committee of the Veterinary faculty in Sarajevo.

Consent for publication

Not applicable

Competing interests

The authors declared that they have no competing interests.

Publisher's Note

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Received: 12 June 2017 Accepted: 24 January 2018
Published online: 02 February 2018

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Paper II

A causal approach to understanding management factors, reproductive underperformance and reproductive infections in dairy cattle

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Abstract

The objective of this study was to investigate causal associations between cattle farms' management practices and reproductive disorders (abortion, stillbirth, retention of placenta, metritis). Besides, direct causal associations between farms' management and reproductive infections (*Chlamydia abortus, Coxiella burnetii,* and *Neospora caninum*), reproductive disorders and infections were also investigated in this study. As a secondary objective, constraints that affect the production in cattle farms were examined. The study was carried out in the north-western (Una-Sana), western (Canton 10) and central part (Central Bosnia Canton) of Bosnia and Herzegovina. A total of 201 farms were selected for participation. A semi-structured questionnaire-based interview was conducted among farmers/managers from January 1st to August 31st, 2015. The 40 questions were divided into three groups: socio-demographic, management, and information on reproductive performances in cattle. Supplementary questions were asked about the perceived primary constraints of the production.

A multivariable mixed-effects logistic regression was used to screen management factors for potential statistical influence. All investigated outcomes were associated with farms' management. The final multivariable models were merged into a Structural Equation Model (SEM). The causal model was then specified graphically. The SEM model showed that herds that experienced abortions (OR=4.3) and stillbirth (OR=6.7) were associated with *N. caninum* seropositivity. Also, herds that experienced retention of placenta were strongly associated with the occurrence of metritis (OR=10.1). *C. abortus* and *C. burnetii* herd seropositivities were mainly associated with environmental factors and contact with potential intermediate hosts.

Our study demonstrated that management practices on dairy farms in Bosnia and Herzegovina contributed to the occurrence of severe reproductive outcomes and reproductive infections. *N. caninum* seems to be an infectious agent that substantially contributed to the reproductive underperformance. Further we demonstrate the need for using causal models in understanding complex relationships.

Keyword**s:** Cattle; Management; Reproductive infections; Reproductive underperformance

Introduction

Agriculture is one of the most vital sectors of economy for the countries in Western Balkan. Most of them have substantial share in the total state gross value added (GVA) ranging from 7.6% in Bosnia and Herzegovina (BH) to 20.1% in Albania. [1]. The situation in the agricultural sector is gradually improving in most of Western Balkan countries, but there are still some critical steps in reforming the agricultural policy and its harmonising with the EU standards. Agriculture is especially important for the economic development of BH [2]. The government of BH has committed to raising the little share (7.6%) of agriculture in the gross domestic product (GDP) through the focused strategic approach to the sector [2]. To promote this development, a new legislation was drafted to enforce compliance with standards set by the European Union (EU) for agricultural commodities. Livestock production is one of the focus areas, where the agriculture economy is expected to grow. Production of milk and dairy products increased in the period 2010-2015, with the production of approximately 701 million litres of milk in 2015 [3]. Also, authorization to the export of dairy products to the EU (in August 2016) represents an opportunity for further intensification of this production. This opportunity makes milk one of the principal export commodities of the country. To cope with the new requirement in the dairy production, infectious and non-infectious causes of the low reproductive performance of cattle become a priority to improve production performances.

Performance indicators are essential tools for monitoring overall reproductive performance in cattle. Some indicators are specific and easily recognised, such as abortion, retained placenta, metritis, and stillbirth. Problems in the postpartum period are usually well recognised by the farmers. Although a reporting system for serious reproductive failures ("Official Gazette of BH" issue number 4/16) is updated annually, it has not been fully implemented and is not made available to the farmers as a decision tool. The subtler effects of reproduction underperformance such as prolonged calving intervals, repeat breeder cows and losses due to early embryonic death require more systematic recording. These reproductive problems are usually calculated from the recorded reproductive events including calving date, insemination dates, and pregnancy outcome [4]. Fertility measures calculated from reproductive events can be divided into two categories as fertility scores and interval traits [5].

Losses due to infectious diseases are typically reflected in impaired (re)productivity and lack of market access. In a previous paper, a relatively high occurrence of exposure to infectious agents in cattle; *Chlamydia abortus*, *Coxiella burnetii*, and *Neospora caninum*, in selected cantons of BH [6] was demonstrated. Each of these pathogens can lead to clinical disease and have effects on bovine reproduction [7-9]. Notably, control measures against brucellosis in small ruminants seem to have more or less eliminated *Brucella melitensis* spill-over to cattle in the selected study regions, and there is no clear indication that *Brucella abortus* is present in cattle in BH [6]. Other infectious agents, like Bovine Viral Diarrhoea Virus (BVDV), may also be directly associated with reproductive failures [10, 11] and indirectly because transiently or persistently infected herds would likely lead to some immunosuppression of cattle [12]. Unfortunately, there are still knowledge gaps regarding the importance of BVDV infection in BH.

While we have documented high levels of certain reproduction pathogens in three cantons in BH, the importance of these agents remains unclear. Reproductive performances are also influenced by a multitude of management factors [13]. Cattle farming in BH still faces some limiting factors, and it is mainly taking place on small farms with an average area below 2 hectares and a high level of land fragmentation. Furthermore, there is a limited number of animals per farm (1-10) with approximately 75% of the farms having one or two cows [14]. Poor housing conditions followed by poor management linked to the inadequate education of the farmers may also contribute substantially to low productivity and losses in animal production. Management may also directly influence the occurrence of infectious agents. In this case, management will have an indirect effect on reproduction through increasing exposure to infectious agents. To be able to suggest sound interventions for improving reproductive performance, there is a need to evaluate which factors are dominant and their causal relationships with reproduction performance. As an improved production will also typically imply larger herds, new factors may also become more critical as, e.g., the dynamics of infections, that depend on herd size.

Although improvement of the cattle sector in BH partially depends on the views and motivation of the farmers/ owners, the scientific data from farms often do not include the perspective of the farmer/ owner. Importantly, their perceived main constraints on production will complement the data collected through epidemiological studies.

The primary objective of the present study was to describe the importance of management practices and to identify the causal interaction between management practices and selected infectious diseases (*C. abortus*, *C. burnetii*, and *N. caninum*) on reproductive underperformance and specific reproduction disorders. The secondary objective was to identify perceived constraints that affect production in the cattle farms in the study area.

Materials and methods

Study area and study design

The study was carried out in the north-western (Una-Sana Canton), western (Canton 10) and central (Central Bosnia Canton) parts of BH, which covers a 23.9% of the total BH territory. Based on a detectable herd-level prevalence of 5% and 95% level of confidence, a minimum sample size of 60 farms (total n=180) was selected in each region to achieve the detection of the agent. In the period from January 1st to August 31st, 2015, a total of 201 cattle farms were visited, blood samples were collected, and a questionnaire-based interview was conducted. Four farms were excluded due to insufficient farm records, leaving 197 farms in the final dataset for statistical analyses (Fig 1). A complete farm sampling frame was unavailable, and farm selection was made using the available list of farms at the municipal level, within each region. In each farm, all female animals older than 12 months were selected for sampling. The breed composition among chosen farms was different, including; Simmental, Montafon, Holstein-Friesian, Red Angus, and their crosses. This study provided a total of 1970 individual blood samples.

Serological tests

Sera were analysed for antibodies against *C. abortus*, *C. burnetii*, *N. caninum*. The presence of antibodies against these pathogens was determined using the commercial Enzyme-Linked Immunosorbent Assays (IDEXX, Switzerland). Vaccination against *C. abortus*, *C. burnetii*, and *N. caninum* has not been implemented in BH, and hence, seropositive animals were considered to be naturally infected. Details of this serological survey are given in a former companion publication [6]. In the current study, we used the herd classification (presence or absence) for these agents based on prior publication.

Reproductive outcomes (questionnaire)

The questionnaire was developed to collect information about management factors and reproductive underperformance in selected farms. Subsequently, the questionnaire was pre-tested in a small number of farms (n=5). After the pre-testing, the questionnaire was administered through a visit and an in-person interview with the farmer, made by the first author. The 40 questions were divided into three groups: socio-demographic information, management information, and information about reproductive performances in cattle. This questionnaire was also used in the previous paper [6], and may be found under this paper (https://bmcvetres.biomedcentral.com/articles/10.1186/s12917-018-1361-z). Supplementary rating questions were asked about the perceived primary constraints of the production. The pre-defined scale was based on Likert five-point scale (from "never" to "almost always") [15].

Variables

The primary reproduction outcomes defined in the present study were abortion, stillbirth, retention of placenta and metritis. Abortion was defined as any termination of the pregnancy after its confirmation by transrectal palpation or no return to oestrus. Transrectal palpation was done between 40 and 70 days after artificial insemination or natural breeding. In case of natural breeding, the date of such event was recorded by the farmer/owner. Stillbirth was recorded as a part of perinatal mortality. Perinatal mortality is defined as a death of the calf before, during or within 48h of calving at full term [16]. Retained placenta was considered to be any retention at 12 to 24h postpartum, while metritis was regarded as any condition with abnormal vaginal discharge, which was documented and treated by the veterinarian. We were not able to extract figures on the frequency of reproductive outcomes in each farm and had to rely upon information about farms' and veterinarians written records in the period of five years.

The infectious outcomes defined in the current study were *C. abortus*, *C. burnetii* and *N. caninum* serostatus in the selected farms, based on results of our previous study [6]. Also, seropositivity to the listed reproductive infections was considered as an exposure factor for the occurrence of the aforementioned reproductive outcomes. Management-related factors were defined as potential predictors for both reproductive outcomes and reproductive diseases. A detailed description of all management-related factors investigated in this study is given in Tables 1-5. Further, the full list of management factors established as candidate variables is given in Table 6.

Databases and statistical analyses

The questionnaire data were entered into a Microsoft Excel® spreadsheet before inspected and merged with data from the previously reported serological survey (Softic et al., 2018). After cleaning data and initial analyses in Microsoft Excel®, using Pivot tables, data were transferred to Stata (Stata/SE 15 for Windows, StataCorp, College Station, TX, USA) for further statistical analyses.

Descriptive analyses and tabulation were done for each canton, and potential differences between cantons were assessed using a chi-square test for categorical variables and the Kruskal-Wallis test for continuous variables. After descriptive analyses, management-related factors were screened for their influence on abortion, stillbirths, retained placenta and metritis, as well as, infectious agents. As all reproduction and infection outcomes were coded as present/ absent (seropositive/ seronegative herd), a mixed-effects univariable logistic regression was used to screen management factors for potential statistical influence. Herd size effect on the occurrence of severe reproductive outcomes was expected, e.g. larger herds were more likely to report an abortion, stillbirth, metritis or retention of the placenta. Hence, an adjustment was made for herd size by including its natural logarithm (ln (herd size)) as an offset in the logistic model for the disease outcomes. This was not done for the infection outcomes, as this was based upon a more systematic sampling in the herds. Factors with a p-value less than or equal to 0.20 from univariable analyses were used as candidate variables for subsequent multivariable modelling.

The multivariable mixed-effects logistic models were established using the melogit procedure in Stata with canton as a random effect. Models were built using a backward selection procedure [15] with a set p-value less than or equal to 0.05 (5%) of the Likelihood Ratio test as exclusion criteria. After modelling all the management variables, the effect of herd infection status (*N. caninum*, *C. burnetii*, and *C. abortus*) were tested, by including these in the model and subsequently reducing the model using backward selection again. Notably, herd size variable was not adjusted and added as an offset in these models. Finally, all multivariable logistic models were assessed using the Hosmer-Lemeshow goodness-of-fit test and the area under the ROC curve (AUC) assessment on models based upon the *logistic* procedure [15].

The final multivariable models were then merged into a Structural Equation Model (SEM) using the *gsem* procedure in Stata. The structural equation modelling is a statistical framework used to model complex relationship between directly and indirectly observed variables [17]. In veterinary research, it is used as a primary statistical option [18] or as an additional technique based on previous statistical inference [19]. The SEM models, as available in the *sem* or *gsem* command in Stata, allows a confirmatory data analyses to be undertaken in the form of a path analysis, utilising all options built into Stata's multilevel mixed-effects models. The initial model was constructed using the graphical *sembuilder* interface and then refined using the Stata command-language syntax. The model was built using the same strategy as the logistic model, but with a causal model specified. Additionally, the causal model was then specified graphically using the online software for Directed Acyclical Graphs (DAGitty) (Fig 2) [20]. As SEM allows an assessment of direct and indirect effects, we were able to reduce the standard logistic models further into more realistic causal models.

Assumed farm production constraints were analysed graphically using pivot functions in Microsoft Excel® and visualised through a radar graph in Microsoft Excel®. Graphs were split according to herd size groups (quartiles), the variable found to determine the owners' approaches to production constraints.

Results

The study participants were distributed across three cantons of BH. The data were collected in a total of 19 municipalities and 94 local communities. There were a total of 66 (33.5%) farms in Una-Sana Canton, 35 (17.8%) farms in Canton 10 and 96 (48.7%) farms in Central Bosnia Canton. For the majority of farmers, cattle raising was not the only source of income but combined with other agricultural activities or non-agricultural employment. Altogether, 90% of selected farms had permanently employed workers, mainly family members, while a total of 10% farms used seasonally hired personnel.

Cattle in BH are usually housed during the winter period (December-April). During the rest of the year, cattle are allowed to graze on a daily basis and returned to the barn in the evening. Grasslands that cattle use for grazing surround the farm are displaced by a different distance from the farm. Farm facilities are usually located in the rural area, next to the family house.

Housing and management

The distribution of farms by housing characteristics in three cantons in BH is shown in Table 1. In most of the visited farms (193/197, 98%), animals were tethered in the barns on wooden or concrete flooring.

A quarter of all farms used a combination of these two types of flooring. A total area of the barn's facilities ranged from 20 to 4000 square meters, depending on herd size. Consequently, the laying area ranged from 1.5 to 5 m^2 per animal. The majority (85%) of the farms had separate pens for calves, while a small number of the farms had separate calving pens. Substantial differences in farm management between cantons were observed. They are reflected in the farm size, number of animals per farm, and the breed composition. Also, there was a substantial difference in the presence of dogs and poultry on the farms in three cantons. On the other hand, there was no difference in feeding management between cantons.

In Table 2, basic information about herd sizes and management strategies are presented. The overall median number of herd size in the selected farms was six animals (range from 1 to 584). Despite the small number of cattle per farm, the majority (67.5%) of the farms used their stock as a source of replacement, while a limited number of the farms (5.1%) exclusively purchased animals for replacement. The breed composition of cattle in the selected farms was heterogeneous, with the Simmental as the most common breed observed in 76% of the farms. Distribution of farms by the presence of other domestic animals is shown in Table 3.

A total of 28.4% of the farms were exclusively cattle farms, while other farms practised combined keeping cattle with small ruminants (sheep and goats), poultry or horses. Also, more than half of the farms had companion animals, (dogs and cats). A total of 54.3% farmers have noticed stray dogs in and around their premises, especially in suburban areas. In 46.2% of farms, game (roe deer, wild boars, foxes) were observed within farms facilities or on grasslands. Stray dogs were included in the questionnaire as variable of interest as they are definitive host of *N. caninum* [8], and could directly and indirectly be included in the persistence of that agent in farms, while game can serve as a mechanical carriers of the studied infectious agents. A total of 48.7% of farmers reported herd-to-herd contact, mainly in the more densely populated villages close to cities. Rodents were observed in 46.7% of farms, and were more often found on larger farms that had a feed storage. Non-migratory wild birds were observed in a total of 74.1% of farms, with slightly difference between visited regions.

Depending on the season, roughage was based on grazing, feeding by cereals, industrial by-products and hay. Predominantly larger farms, i.e. farms with the greater number of animals (37.6%) have also used corn and grass silage. Water supply was ad-libitum usually coming from public or private sources (Table 4).

Reproductive outcomes

The median age of animals at first artificial insemination was 15 months (range 11-24). Approximately 59% of farms used artificial insemination as a method of choice, while 41% used combined, natural breeding and artificial insemination. Almost all the visited farms have experienced some reproductive problems. Distribution of farms by the occurrence of reproductive problems is shown in Table 5. Reproductive problems such as abortion, stillbirth, metritis, and retained placenta were observed with substantial differences between cantons.

Less observable disorders such as anoestrus, silent oestrus, and repeat breeder cows were recorded, but information on these was incomplete and are not included in the paper.

All potential factors listed in Tables 1-5 were tested in the univariable logistic regression. Table 6 displays the candidate variables showing a moderate association ($p \leq 0.20$) with severe reproductive outcomes and infectious agents that can lead to the reproductive failures.

Table 7 shows the multivariable models for candidate variables that were associated with abortion, stillborn calves, retention of the placenta and metritis, as well as with the occurrence of *N. caninum*, *C. burnetii*, and *C. abortus* seropositive herds.

Table 8 and Fig 2 show how the models were transformed into a causally oriented SEM structure. As shown, the models were reduced in complexity due to the possibilities inherent in the SEM model to distinguish between direct and indirect causes. *N. caninum* was the only crucial infectious source for reproduction outcomes, while for some outcomes, environmental factors were important. Fig 2 illustrates our suggested causal structure, based on the results given in Table 8.

Production constraints

Independent of herd size, the lack of market access due to production underperformance, and low purchasing prices of bovine products were the most common production constraints of all cattle farms in three cantons in BH (Fig 3).

Discussion

Substantial differences in farm management between cantons were observed. These differences are mainly reflected in the herd size and structure. Farms located in the Una-Sana Canton and Canton 10 were mostly medium-sized to large, while farms located in the Central-Bosnia Canton were mostly small farms. Consequently, the number of animals per farm, and the breed composition were found significantly different. Also, the presence of poultry, companion animals (dogs and cats), wild birds, and rodents on the farms in three cantons were found significantly different. There were no differences between feeding practices between cantons.

In this study, the SEM was used as a confirmatory approach for assessing potentially unobserved direct associations between outcomes and predictors. Based on the results from the multivariable logistic regression, four different causal complexes were identified. In the following, we discuss the findings based on the SEM model in Table 8/ Fig 2.

Abortion – Stillbirth – *N. caninum* herd seropositivity complex

One or more cases of abortion were observed in a total of 46.2% of the visited farms. Herds that were seropositive to *N. caninum* were more likely to report abortions compared to the herds that were seronegative to *N. caninum*. Several studies have observed an association between *N. caninum* seroprevalence in cattle herds and increased risk of abortion [21-23]. This is explained by an increased abortion risk because of vertical (latently infected dams) and horizontal transmission of *N. caninum*.

Results from the multivariable logistic regression showed that farms with dogs reported more abortions in cattle compared to farms with no dogs in their facilities. This may partially be explained by the *N. caninum* life cycle. As a definite host of the parasite, dogs participate in horizontal transmission through contamination of fodder by dog faeces [8]. Also, dogs might be an efficient vector of horizontal transmission due to permanent contact with cattle. Similar to our findings, other studies identified an association between farm dogs and bovine abortion as well as between the presence of dogs and the presence of *N. caninum* seropositive animals [21, 24]. Additionally, the occurrence of rodents or hedgehogs in and around farm facilities was associated with the abortions in cattle. The presence of these potential intermediate hosts of *N. caninum* might suggest that these animals could be important sources of infection for carnivores [8]. However, after SEM modelling these predictors disappeared as potential risk factors for abortion, leaving the seropositivity to *N. caninum* as the single explanatory variable.

A total of 25.3% of the visited farms reported stillbirth as a severe reproductive outcome. Herds that were seropositive to *N. caninum* were more likely (OR=6.5) to report stillbirth compared with the seronegative herds. Asmare et al. (2013) also found an association between abortion and/or stillbirth and individual animal level *N. caninum* seropositivity in Ethiopia [25]. Brickell et al. [26] reported an increased risk of perinatal mortality in *N. caninum* seropositive animals. The farms where the new-born calves were kept in groups reported more stillbirths than the farms where the new-born calves were kept individually. Additionally, farms with wooden flooring type had more (OR=2.5) stillborn calves compared to the farms with other flooring types. This could partially be explained by the calving management and calving assistance. There was an increased number of stillborn calves reported in the municipality of Livno (Canton 10) in 2015, which may partly be explained by inappropriate and unprofessional calving assistance, applied by farmers (Amel Murga, DVM, personal communication, April 18, 2015).

Antibodies against *N. caninum* were found in a total of 40.6% of the visited farms. Cattle in farms with the presence of dogs were more often (OR=3.0) seropositive compared to cattle in farms with no dogs. Similarly, farms that observed free-roaming dogs in and around their farm facilities had more seropositive (OR=4.1), compared to farms where no free-roaming dogs were observed. This finding is not surprising, as dogs are definitive hosts of *N. caninum.* Also, the farmer usually keeps dogs for many years, and it is often more than one dog per farm. In most epidemiological studies related to *N. caninum*, the presence of the farm dogs was found a risk factor for seropositivity in cattle [21-25]. However, caution is needed in the interpretation of these results. The Hosmer-Lemeshow goodness-of-fit test for the multivariable logistic regression model showed a lack of fit. It may be partially explained by the low number of groups in which the data were divided (n=4), while the recommendation is a minimum of 6 groups [15]. Another possible explanation could be that some cases of *N. caninum* herd seropositivity may result from exposure to risk factors and variables not included in this study. In summary, the SEM model allowed us to simplify and present a more appropriate causal model for this complex.

Retention of placenta – Metritis complex

A total of 54.3% of the selected herds experienced retention of the placenta. Herds that fed predominately by silage had reported More retained placenta (OR=3.1) compared to herds that fed with a combined feed ration. This might be partially explained by nutritional demands in periparturient animals. Nutritional deficiencies, and consequently negative energy balance lead to metabolic disorders which represent a risk factor for the appearance of the retained placenta [27, 28]. Also, contact between herds seemed to be a substantial factor contributing to the appearance of retained placenta, possibly due to the transmission of infectious agents which can result in problems in the puerperium [7]. Additionally, such contact between animals in the field could plausibly mediate a part of the effects of uterine disease through social stress [29]. Herds that reported retained placenta had more (OR=9.4) metritis cases. The feature of the retained placenta as a predisposing factor for metritis is well documented [30, 31]. Manual removal of the retained placenta to prevent the source of infection is the dominant treatment among local veterinarians in the study area (Personal communication, January-August 2015). However, manual removal of an attached placenta may cause damage to the endometrium and could lead to bacterial invasion [32]. The retention-metritis complex, as shown by the SEM model, was associated with management factors, and we could not identify any infectious link here.

C. abortus

Farms that reported the presence of birds (pigeons, sparrows, swallows) had more (OR=11.0) animals seropositive to *C. abortus* compared to farms that did not report the presence of birds in their farm premises. Sachse et al. [33] found a few positive pigeons to *C. abortus* in their study of *C. psittaci*. Migration of these birds and their contact with dairy cattle or farm equipment could probably contribute to transmission of the agent, and consequently seropositivity in dairy cattle. Herds on the farms that noticed rodents (mice and rats) had more *C. abortus* (OR=4.0) compared to herds on the farms with no rodents in their farm facilities. Contrary to our findings, Sun et al. (2015) reported that management factors and presence of *Muridae* in farms were not associated with seropositivity of cattle to *C. abortus* [34]. Also, there was an association between dogs' presence on the farm and seropositivity of dairy cattle. This complex was mainly linked to contact with other animals or environmental sources.

C. burnetii

Seropositivity to *C. burnetii* was associated with many farm management factors, contact between herds in the field, and presence of stray dogs in and around farm facilities. A possible explanation for this association is reflected in the indication that dogs (companion or feral) can be a potential reservoir species and source of infection for animals and humans [35]. Additionally, stray dogs as a population of homeless, abandoned dogs could be a particularly important source of infection for other animals and humans. Shapiro et al. (2016) observed that stray dogs from Aboriginal communities were more likely to be seropositive to *C. burnetii* than other population of dogs included in their study [36].

Herds that have contact with other herds on the common pastures had more seropositive to *C. burnetii* compared to isolated herds (OR=2.8). Contact with infected herds, contaminated equipment or biological material or other intermediate host seemed to be a possible explanation for this finding. Additionally, partial grazing system could favour seropositivity to *C. burnetii*. After re-housing in the winter period, seropositivity could be partially explained by exposure to aerosols or contaminated material [37].

Our suggested final causal model, depicted in Fig 2, joins a statistical model based upon SEM and a biological understanding of the reproductive diseases in question. The SEM model allowed us to conclude that there is a primary indirect effect of contact with dogs through *Neospora* infection, and we could also show the strict causality between placental retention and metritis, as the SEM models allow mediating effects in the model. A standard logistic model would not do this, as we would have to choose between correlated intervening variables. We did not assess model fits directly in SEM, as this was done in the original logistic models. The primary purpose of the SEM model was to reduce the complexity of the multivariable models and use SEM as a confirmatory technique to identify potential causal pathways.

The question of using appropriate statistical methods is a crucial part of epidemiological research. The development towards using mathematically more complex model has been fast, and today multivariable, multilevel regression has become a standard in analysing data from veterinary epidemiological studies, where hierarchical clustering often occurs. However, causal modelling has been used less. Most modern literature on causality and directed acyclical graphs, as used in this paper, is based upon the seminal book *Causality* by Judea Pearl [38]. In this book he argues well for using causal models including DAGs and either SEM (as used in this paper) or Bayesian Networks in causal inferences. Notably, these models are more efficient that traditional regression to deal with central topics as confounders and colliders, and also the complex problem of mediating variables. We support the idea that causal modelling is necessary in veterinary epidemiology, a science where we aim at suggesting interventions in populations and not only measure associations.

Data quality

Recording of reproductive disorders is still at insufficient levels in BH. This was also reflected in our study, partially explained by the lack of an organised national cattle database. Farmers have limited skills in recognising a reproductive problem, and they seem to only on exceptional occasions seek professional assistance for contributing to data in the poor recording system. Reproductive outcomes such as abortion, stillbirth, retained placenta, and metritis are perceived as relatively severe, and the farmers can recognise the disease. Additionally, these outcomes affect (re)production, which also can motivate for consultancy. Subtler reproductive outcomes such as anoestrus and silent oestrus require more data and were not included in this study. Unfortunately, farms do typically not keep proper farm records, so we were not able to assess the frequency of these outcomes. Furthermore, farmers could overestimate or downscale the importance of specific factors investigated in this study. This was partially prevented by analysis of perceived production constraints.

Production constraints

As the principal production constraint in this study, the respondents recognised inadequate and limited market access. This view is based on economic losses in their output. Such financial losses are categorised as direct, caused by relatively small and volatile purchasing prices of bovine products, and indirect, as a consequence of production underperformance. Furthermore, milk price volatility might be explained by criteria on milk quality ("Official Gazette BH" issue number 2205/2016). Changes in the composition of milk are leading to changes in its price. Also, milk price volatility could persist, following the permit for milk export to the EU and adapt to the external market laws. On the other hand, production underperformance could be partially explained by the occurrence of reproductive disorders, which result in direct loss of the offspring and indirect losses with reduced production in the recovering period.

External validity of the findings

Although Western Balkans are at different stages of negotiations with the EU, apart from Croatia, they are faced with similar challenges. Mainly, their livestock farming is characterised by small and fragmented farms, with a small number of animals per farm. Such farm structure is inefficient and non-competitive either in export or in the domestic market. So far, a small number of farms have been commercialised and adapted to the requirements of the EU market. Also, traditional and outdated (extensive and semi-intensive) cattle rearing is still present in some areas of Western Balkans and represent an obstacle that is necessary to overcome. Given that most of these countries were members of the former Yugoslavia, the tradition of dairy farming and its management is more or less the same across them. One of the most important strategic points for improving dairy production is effective and timely dairy cattle reproduction. Previous studies have reported the presence of some reproductive agents in Croatia [39, 40], Serbia [41, 42] and Albania [43, 44]. Our findings bring a useful insight and can partially contribute to understanding the causality of management factors associated with reproductive underperformance and reproductive infections not only in BH but also in the Western Balkans.

Conclusions

This study has demonstrated that management practices contributed to the occurrence of severe reproductive outcomes and reproductive infections. *N. caninum* seems to be an infectious agent that substantially contributed to the reproductive underperformance, particularly in inducing abortions and perinatal mortality. Also, there was a causal relationship between retention of placenta and metritis, which seems to be associated with poor managerial decisions. Our results illustrate the benefits of applying the SEM approach in elucidating potentially causal pathways in analysing complex epidemiological data.

Acknowledgement

The authors express sincere gratitude to all study participants, veterinarians and contributors who helped in the development of this study.

Author contributions

Conceptualization: AS, ES, KA

Data curation: AS

Formal Analysis: AS, ES

Funding acquisition: NF, ES

Investigation: AS

Methodology: AS, KA, EGG, ES, JG

Project administration: AS, NF, ES

Supervision: ES

Validation: EEG, ES, JG, NF

Writing – original draft: AS, ES

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Table 1. Distribution of 197 study farms by housing characteristics in three cantons in Bosnia and Herzegovina, given as median (range) for continuous or proportion (95% CI) for categorical variables.

Variable	Region			Overall	p-value
	Una-Sana Canton (n=66)	Canton 10 (n=35)	Central-Bosnia Canton (n=96)		
Area of the barn	100 (20-1600)	100 (20-4000)	40 (15-600)	70	<0.001
Flooring type - wood	28.8% (19.1-41.0)	57.1% (40.2-72.5)	84.4% (75.6-90.4)	60.9% (53.9-67.5)	<0.001
Flooring type - concrete	92.4% (82.9-96.9)	77.1% (60.1-88.3)	40.6% (31.2-50.8)	64.5% (57.5-70.9)	<0.001
Presence of calving pen	1.5% (0.2-10.2)	20% (9.7-36.8)	2.1% (0.5-8.1)	5.1% (2.7-9.2)	<0.001
Presence of calf pen	93.9% (84.8-97.7)	94.3% (79.4-98.6)	75% (65.3-82.7)	84.8% (79.0-89.2)	0.001

Table 2. Distribution of 197 study farms by a number of cattle and breed composition in three cantons in Bosnia and Herzegovina, given as median (range) for continuous or proportion (95% CI) for categorical variables.

Variable	Region			Overall	p-value
	Una-Sana Canton (n=66)	Canton 10 (n=35)	Central-Bosnia Canton (n=96)		
No. of cattle					
Herd size	10 (2-101)	11 (4-584)	3.5 (1-150)	6	<0.001
No. of cows per farm	8 (2 - 62)	10 (3-350)	3 (0-112)	5	<0.001
No. of heifers per farm	2 (0-30)	2 (0-234)	0 (0-38)	1	<0.001
No. Of calves per farm	1 (0-25)	1 (0-65)	0 (0-94)	1	0.003
Breed composition					
Cross breed	51.5% (39.4-63.4)	54.3% (37.6-70.1)	69.8% (59.8-78.2)	60.9% (53.9-67.5)	0.043
Holstein-Friesian breed	21.2% (12.9-32.9)	17.1% (7.8-33.7)	8.3% (4.2-15.9)	14.2% (10.0-19.9)	0.06
Simmental breed	95.5% (86.6-98.5)	57.1% (40.2-72.5)	69.8% (59.8-78.2)	76.1% (69.6-81.6)	<0.001
Montafone breed	4.5% (1.5-13.4)	62.9% (45.7-77.3)	a	12.7% (8.7-18.2)	<0.001
Red Angus breed	a	2.9% (0.4-18.3)	1.0% (0.1-7.2)	1.0% (0.3-4.0)	0.395

a – no observation

Table 3. Distribution of 197 study farms by the presence of other species of animals in three cantons in Bosnia and Herzegovina, given as proportion (95% CI).

Other domestic animals	Region			Overall	p-value
	Una-Sana Canton (n=66)	Canton 10 (n=35)	Central-Bosnia Canton (n=96)		
Horses	9.1% (4.1-19.0)	2.9% (0.4-18.3)	6.3% (2.8-13.3)	6.6% (3.9-11.1)	0.477
Sheep and Goats	36.4% (25.6-48.7)	22.9% (11.7-39.9)	26.0% (18.2-35.8)	28.9% (23.0-35.7)	0.248
Poultry	45.5% (33.8-57.7)	5.7% (1.4-20.6)	31.3% (22.7-41.3)	31.5% (25.3-38.3)	<0.001
Companion animals					
Cats	66.7% (54.3-77.1)	71.4% (54.2-84.1)	40.6% (31.2-50.8)	54.8% (47.8-61.7)	<0.001
Dogs	81.8% (70.5-89.5)	48.6% (32.4-65.0)	43.2% (33.5-53.4)	57.1% (50.1-63.9)	<0.001
Wild animals observed					
Wild birds	90.9% (81.0-95.9)	91.4% (76.1-97.3)	56.3% (46.1-65.9)	74.1% (67.5-79.8)	<0.001
Rodents	57.6% (45.3-69.0)	51.4% (35.0-67.6)	37.5% (28.3-47.7)	46.7% (39.8-53.7)	0.035
Game	51.5% (39.4-63.4)	31.4% (18.1-48.7)	47.9% (38.0-58.0)	46.2% (39.2-53.2)	0.14

Table 4. Distribution of 197 study farms by characteristics for feeding and water supply in three cantons in Bosnia and Herzegovina, given as proportion (95% CI).

	Region			Overall	p-value
	Una-Sana Canton (n=66)	Canton 10 (n=35)	Central-Bosnia Canton (n=96)		
Feeding					
Crops - cereals	95.5% (86.6-98.5)	100%	92.7% (85.4-96.5)	94.9% (90.8-97.3)	0.236
Industrial by-products	80.3% (68.8-88.3)	68.6% (51.3-81.9)	65.6% (55.5-74.5)	71.1% (64.3-77.0)	0.121
Pasture	81.8% (70.5-89.5)	88.6% (72.8-95.7)	91.7% (84.1-95.8)	87.8% (82.4-91.7)	0.168
Hay	100%	100%	97.9% (91.9-99.5)	99.0% (96.0-99.7)	0.345
Silage	74.2% (62.2-83.5)	31.4% (18.1-48.7)	14.6% (8.8-23.2)	37.6% (31.0-44.6)	<0.001
Water source					
Public	80.3% (68.8-88.3)	68.6% (51.3-81.9)	71.9% (62.0-80.0)	74.1% (67.5-79.8)	0.372
Private	10.6% (5.1-20.8)	8.6% (2.7-23.9)	13.5% (8.0-22.1)	11.7% (7.9-17.0)	
Both	9.1% (4.1-19)	22.9% (11.7-39.9)	14.6% (8.8-23.2)	14.2% (10.0-19.9)	

Table 5. Distribution of 197 study farms by the occurrence of reproductive problems in three cantons in Bosnia and Herzegovina, given as proportion (95% CI).

Variable	Region			p-value
	Una-Sana Canton (n=66)	Canton 10 (n=35)	Central-Bosnia Canton (n=96)	
Abortion	69.7% (57.5-79.7)	45.7% (29.9-62.4)	30.2% (21.8-40.2)	<0.001
Stillbirth	34.8% (24.2-47.2)	37.1% (22.7-54.3)	14.6% (8.8-23.2)	0.003
Metritis	45.5% (33.8-57.7)	57.1% (40.2-72.5)	27.1% (19.1-39.9)	0.003
Retention of placenta	72.7% (60.6-82.2)	62.9% (45.7-77.3)	38.5% (29.3-48.7)	<0.001

Table 6. Candidate variables associated with the severe reproductive and infectious outcomes with P<0.2 from the univariable logistic regression, used as candidate variables for the multivariable logistic modelling.

	AB	MT	RT	SB	N. caninum*	C. burnetii*	C. abortus*
Region	x		x			x	x
Herd size (quartiles)	x			x	x	x	
Crossbreed						x	
Simmental breed				x			x
Montafone breed	x					x	x
Red Angus breed		x					
Technical equipment					x	x	x
Workers			x		x	x	
Area of the barn quartiles					x	x	
Cleanliness degree	x						
Wooden floor			x	x	x	x	x
Concrete floor			x	x	x	x	x
Laying area		x		x			x
Calving pen			x			x	
Calf pen			x			x	x
Keeping calves in calf pen		x	x	x	x	x	
Crops							
By-product	x		x				x
Silage			x		x	x	x
Herd-to-herd contact		x	x	x	x	x	x
Horses					x		
Small ruminants					x		x
Cats		x	x		x	x	x

Poultry	x						
Birds		x		x	x	x	x
Hedgehog	x						
Rodents	x					x	x
Straydogs	x	x		x	x	x	x
Dogs	x		x		x		x
R		x					
N. caninum*	x		x	x			

AB - Abortions; MT- metritis; RT- retained placenta; SB- stillbirth; * Seropositive herds

Table 7. Results from the final multivariable logistic models for the four reproduction outcomes and the infection outcomes

	Abortions	Stillbirth	Retention	Metritis	N. caninum*	C. burnetii*	C. abortus*
Rodents' presence	2.1 (1.0-4.2); p=0.047						4.0 (1.0-15.5); p=0.044
Dogs' presence	2.2 (1.1-4.6); p=0.033				3.00 (1.57-5.73); p=0.001		4.7 (1.5-14.6); p=0.008
N. caninum positive herd	3.9 (1.9-7.9); p<0.001	6.5(2.1-14.6); p<0.001					
Hedgehogs' presence	0.4 (0.2-0.8); p=0.007						
Calving pen presence		7.3 (1.4-38.4); p=0.019				17.8 (2.4-134.0); p=0.005	
Keeping calves in calf pens		Baseline		Baseline			
In groups		2.7 (1.1-6.5); p=0.031		0.36 (0.16-0.83); p=0.016			
Workers						Baseline	
Permanently employed						0.2 (0.1-0.7); p=0.011	
Silage - feeding			3.1 (1.4-6.8); p=0.004				

Herd-to-herd contact			2.3 (1.1-4.6); p=0.019			2.8 (1.0-7.8); p=0.046	
Crossbreed						0.4 (0.1-0.7); p=0.049	
Montafon breed						3.8 (1.2-11.8); p=0.024	
Wooden floor presence		2.3 (1.0-5.2); p=0.048				0.2 (0.1-0.5); p=0.001	
Stray dogs					4.05 (2.12-7.72); p<0.001	3.8 (1.4-10.2); p=0.01	
Cats’ presence			0.5 (0.3-1.0); p=0.038				
Birds							10.9 (3.6-33.5); p<0.001
Laying area				0.5 (0.3-0.8); p=0.002			
Retention of placenta				9.4 (4.0-22.2); p<0.001			

* Seropositive herds

Table 8. Results from the confirmatory Structural Equation Model, based upon the multivariable logistic models found in Table 7.

Outcome	Variable	OR	95% CI	p-value
Abortion (AB)	*N. caninum* seropositive herd (NC)	4.3	2.2-8.4	<0.001
Stillbirth (SB)	*N. caninum* seropositive herd (NC)	6.7	2.8-15.6	<0.001
	Keeping calves in the calf pen (calfpen_c)-baseline			
	In groups	2.6	1.1-6.4	0.032
	Wooden floor in the barn (f_wood)	2.5	1.1-5.9	0.030
Retention of the placenta (RT)				
	Feeding by silage (silagef)	3.1	1.4-6.8	0.004
	Herd-to-herd contact (contact)	2.3	1.2-4.6	0.019
	Cats' presence in the farm (cats)	0.49	0.25-0.96	0.038
Metritis (MT)	Retention of the placenta (RT)	10.1	3.9-26.3	<0.001
	Keeping calves in the calf pen (calfpen_c)-baseline			
	In groups	0.36	0.15-0.82	0.016
	Laying area in the barn (layarea)	0.46	0.28-0.78	0.004
N. caninum seropositive herd (NC)	Dogs' presence in the farm (dogs)	3.0	1.6-5.7	0.001
	Presence of the straydogs (straydogs)	4.1	2.1-7.7	<0.001
C. burnetii seropositive herd (CB)	Presence of the calving pen in the barn (calvingpen)	17.8	2.4-134.0	0.005
	Seasonally employed workers (workers)-baseline			
	Permanently employed workers	0.18	0.05-0.68	0.011
	Herd-to-herd contact (contact)	2.8	1.0-7.8	0.046
	Presence of the straydogs (straydogs)	3.8	1.4-10.2	0.01
	Presence of the crossbred cows (cross)	0.39	0.15-0.99	0.049
	Presence of the breed Montafone (mont)	3.8	1.2-11.8	0.024
	Wooden floor in the barn (f_wood)	0.17	0.06-0.49	0.001
C. abortus seropositive herd (CA)	Dogs' presence in the farm (dogs)	4.7	1.5-14.6	0.008
	Occurrence of the wild birds in the farm (birds)	10.9	3.6-33.5	<0.001
	Occurrence of the rodents in the farm (rodents)	4.0	1.0-15.5	0.044

Fig 1. The distribution of 197 study farms located in three cantons of Bosnia and Herzegovina

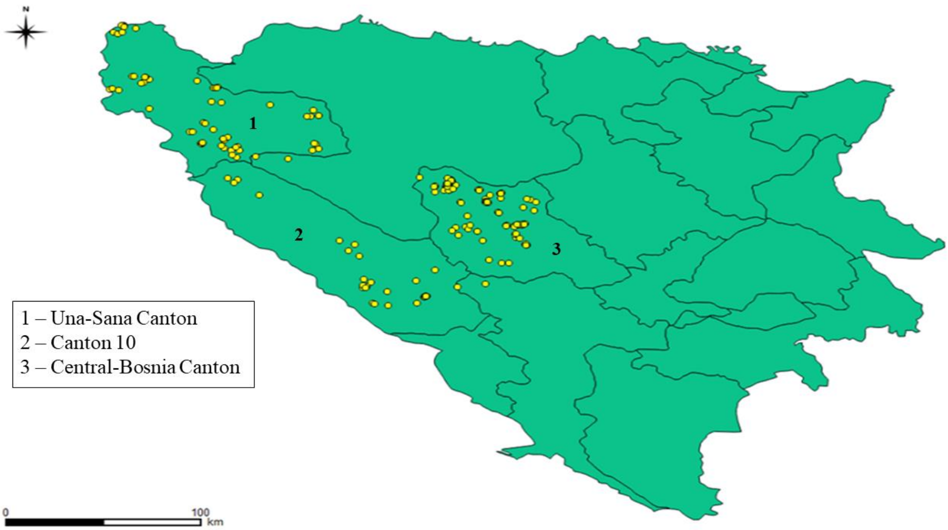
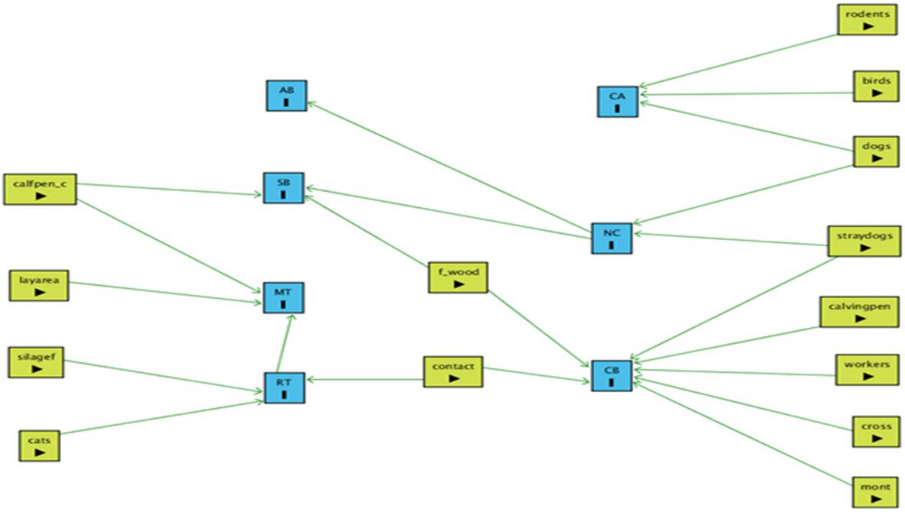


Fig 2. The suggested causal model based upon results from the confirmatory Structural Equation model. The graph was produced using the DAGitty software [20].



Outcomes: AB – Abortion; SB – Stillbirth; MT – Metritis; RT – Retained placenta; CA – C. abortus seropositive herd; NC – N. caninum seropositive herd; CB – C. burnetii seropositive herd.

Predictors: calfpen_c – Keeping calves in the calf pen individually (baseline) or in groups; layarea – Laying area in the barn; silagef – Feeding animals by silage; cats – Cats' presence in the farm; f_wood – Wooden floor in the barn; contact – Herd-to-herd contact; rodents – The occurrence of the rodents in the farm; birds – The occurrence of wild birds in the farm; dogs – Dogs' presence in the farm; straydogs – The occurrence of straydogs in and around farm's facilities; calvingpen – The presence of the calving pen in the barn; workers – Seasonally (baseline) or permanently employed workers at the farm; cross – The presence of the cross breed at the farm; mont – The presence of the Montafone breed at the farm.

Fig 3. Main production constraints of 197 study farms in three cantons in BH shown on herd size quartiles

Paper III

Reproductive performance in a selected sample of dairy farms in Una-Sana Canton, Bosnia and Herzegovina

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Abstract

The production of the milk and dairy products and their placement on the market represent a constant profit for the farmers/producers in BH. The profitable operation of the dairy farms is influenced by the reproductive performance of the lactating animals. This study assessed individual animal reproductive characteristics in selected dairy farms and described their reproductive performance indicators.

The median age at first insemination was 493 days (5^{th} -95^{th} percentile range 429-840), while the age at first calving was 802 days (5^{th} -95^{th} percentile range 708-1168). The median pregnancy proportion at first insemination was 40% (5^{th} -95^{th} percentile range 17-62), while the cumulative pregnancy rate calculated at day-60, day-80, day-100 and day-120 shown that approximately 64% of all pregnancies happened before day-120. The calculated inter-service intervals showed that approximately 69% of the repeat breeding animals came back to the oestrus in the period 18 to 24 days. This is an indication of very good oestrus detection in selected dairy farms. The mean number of services per pregnancy was 2.61 (range 1-12). The median calving-to-first-insemination interval was 62.5 days (5^{th} -95^{th} percentile range 16-408). The calving-to-conception interval was 101 day (5^{th} -95^{th} percentile range 36-506). Finally, the calving interval was 385 days (5^{th} -95^{th} percentile range 329-773).

There is a need for an organised, regular and more comprehensive recording system for the reproduction of dairy cattle among dairy farms in Una-Sana Canton. The calculated reproductive measures indicated an undulant trend in reproductive performance among selected dairy farms in Una-Sana Canton. Knowing the apparent reproductive indicators described in this study, the farmers and veterinary authorities may identify and correct areas in their management that contribute to the reproductive underperformance.

Keywords: reproduction, indicators, management, underperformance, monitoring

Implications

Reproduction in dairy cattle is one of the most important factors for the intensification of the dairy industry in Bosnia and Herzegovina (BH). The monitoring of reproductive performance in dairy cattle, however, is not adequately done. Knowing the apparent reproductive indicators described in this study, the farmers and veterinary authorities in BH may identify and correct areas in their management that contribute to the reproductive underperformance in dairy cattle.

Introduction

The reproductive performance of modern dairy cows worldwide has decreased over the past 50 years. The observed reproductive decline has been partially explained by the intensification of the production, with continuously higher milk yield and larger herds (Lucy, 2001). One recent study suggested the declining trend in reproductive performance has slowed down (Philipsson, 2011), whilst others have suggested that the correlation between increased production and decreased reproductive performance is still significant although the effect is small and modulated at the herd level (Rearte *et al.*, 2018). Milk quotas within the European Union (EU) were lifted in 2015 (European Commission, 2015). This has led to an expansion in milk production in Central and Western Europe. Increased milk production represents another stress which can eventually lead to the decline in reproductive performance in dairy cattle in regions of the EU with high milk production. This potential loss can be overcome by the improvement of key areas in dairy cows' fertility management such as genetic selection, nutritional management, control of infectious diseases and control of cow- and bull fertility (Crowe *et al.*, 2018). The expanding milk production into South-eastern Europe and Baltic countries may reduce some pressure on already high-producing regions of the EU, but then one may experience new challenges with potential reproductive losses.

Although not a member of the EU, BH plays a role in the EU milk supply, having received the official permit to the export of milk and dairy products to the EU (Anonymous, 2016). Consequently, the intensification of the production, with continuously higher milk yield, and potential consolidation of dairy herds are primary aspirations and future plans of the dairy industry in BH. Dairy production, in general, has been gradually increased in BH over the last 20 years, although there were considerable annual variations in the amount of produced milk and the number of dairy cows (BHAS, 2015). There was a continual decrease in the number of registered dairy cows in BH throughout the period 2006-2016, while the total milk production increased, reaching 701 million litres in 2016 (Anonymous, 2017).

However, a number of limiting factors still prevent the country from maximising its potential milk production. The agricultural land and farm properties are fragmented with extensive and unwieldy farming practices, and stocking densities are typically low (Anonymous, 2015). Also, BH is still a country in political and economic transition, and agricultural conditions for more intensive production growth are yet not directly comparable with EU countries (EUROSTAT, 2016). These limiting factors are partially mitigated by the national programmes that are results of the alignment of national legislation with the EU regulations. The optimal bovine reproduction remains one of the essential factors required to achieve the goals of the dairy industry in BH. Hence, there is a need for the monitoring of reproductive performance indicators in order to maximise the production efficiency and milk yield, but also to comprehend the effect size of changes caused by the intensification in the production. However, the information on the current status of reproductive performance is mostly unknown in dairy herds in BH.

Traditionally, dairy farmers aim to produce one calf per cow per year to ensure dairy herd replacement and to optimise milk production. However, herd health programmes (including breeding programmes, nutritional strategies and biosecurity), data management strategies, precision farming systems, and databases are scarce or absent in most farms in BH. In addition, different legislations levels (state, entity or cantonal) and poorly developed herd health services, breeding organisations and automatic recording systems seem to additionally hamper the dairy industry development in BH. Establishing fertility- and merit indices are important for monitoring individual, herd and population reproduction efficacy. Moreover, these indices partially contribute to a better understanding of the causality of reproductive problems, and also assist decision-making process and economic evaluation in the dairy production (Eaglen *et al.*, 2013, Miglior *et al.*, 2017). Internationally, low pregnancy rates have been accompanied by a reduction in bulk milk production and calves born per year, which negatively influences the economic profitability of the dairy farm (Ferguson and Skidmore, 2013). Since the reproductive performance of dairy cattle may be influenced by a number of individual and environmental factors, substantial variations are seen across the country.

Therefore, this study aimed at identifying and describing the individual animal reproductive performance in a subset (Softic *et al.*, 2018) of investigated dairy herds in Una-Sana Canton of BH.

Materials and Methods

Study design and study population

The follow-up study was carried out in the North-western part of BH (Una-Sana Canton) (Figure 1). Farm visits and data collection were completed in the period November-December 2016. To describe individual animal reproductive performance, individual timeline data were recorded. The timeline data referred to the list of all reproductive events in chronological order from birth to either the end of the cow's reproductive life or the last reproductive event. Reproductive events were recorded as calendar dates, and the selection strategy is schematically presented in Figure 2.

The target population comprised of commercially farmed dairy cows in the Una-Sana Canton. Since there was no complete list of all dairy farms in the Una-Sana Canton, the sampling frame consisted of dairy farms from the available municipal lists of cooperatives with dairies. In addition, the selection of farms was based on a subset of dairy farms assembled retrospectively from a previous study on farm management and reproductive infections in dairy cattle, as described in (Softic *et al.*, 2018). The general inclusion criterion was the existence of an on-farm written recording system for reproductive data at the individual animal level. The study sample consisted of animals that had entered their reproductive life and were enrolled in the farm records. Visited farms with only memorised data or insufficient written data were excluded from the study. New-born animals, non-inseminated heifers and animals with incomplete or missing timeline data were not included in the study. The aim was to retrospectively collect all individual data for five years. Given all farms that met the inclusion criteria had appropriate written data in the period 2009-2016, this period was defined as the study period of interest. Animals whose timeline did not start with the birth date, but whose available data had no interruption of the chronological continuity were kept in the study. In such cases, the chosen starting point on the timeline was either i) the date of the first service that resulted in a pregnancy and calving or ii) the calving date, after which they started a new reproductive cycle or finished their reproductive life. Newly introduced animals were included in the study if their records started with the date of introduction to the farm and continued with the reproductive events.

Data collection and calculation

A data collection form was developed before the study was initiated for recording reproductive events. Farms' handwritten papers, diaries and daily tables were used in the preparation of the database. Each animal timeline data was reviewed and put into the database only if it met the inclusion criteria. The database was established in a Microsoft Excel® spreadsheet. After calculating and reviewing data in Excel using filter functions and pivot analyses, data were transferred to Stata SE/15 for Windows (StataCorp, College Station, TX, USA) for further analyses. Reproductive measures calculated in this study are; age at first service, age at first calving, the pregnancy proportion at first service, number of services per pregnancy, calving to first service interval, calving to conception interval, and calving interval.

A heifer was defined as a sexually mature female with no previous calvings. The age at first service is relevant only for heifers and was calculated from the following formula:

$$Age(1st\ service) = First\ service\ date - birth\ date$$

Subsequently, age at first calving was calculated as:

$$Age\ (1st\ Calv) = First\ calving\ date - birth\ date$$

These measures were calculated and presented at the farm level. The pregnancy proportion (PP) at the first service was calculated from the available data. This proportion was calculated for all included farms, according to the formula:

$$PP = \frac{Number\ of\ pregnant\ heifers}{Number\ of\ inseminated\ heifers} x\ 100$$

A cow was considered pregnant if she did not return to oestrus after breeding and before calving, or if the pregnancy was confirmed by rectal palpation. Cows that were inseminated more than three times at regular intervals were classified as repeat breeder cows (Gustafsson and Emanuelson, 2002). Since natural service was uncommon but was appropriately recorded, natural breeding and artificial insemination were compiled as one measure. In repeat breeding cows, all artificial insemination or natural service events were coded as 0 or 1 (cow bred no/yes). The sum of all insemination events, followed by the calving date was the number of inseminations per pregnancy at the individual animal level. The average annual number of inseminations per pregnancy was calculated at the farm level, as follows, where n represents a number of cows at the farm at a given time (year).

$$Average\ no.of\ services\ per\ pregnancy = \frac{1}{n}\sum(services\ per\ pregnancy)$$

Further, the insemination index was calculated for all farms, as an overall average number of services per pregnancy (artificial insemination or natural breeding) of all cows present at the farm (data not shown). Multiple serviced animals without subsequent calving and animals not confirmed to be pregnant were excluded from the calculation. Additionally, the inter-insemination interval was defined as the number of days between two consecutive inseminations/services. It was calculated in repeat breeding animals to identify the characteristics of oestrus and oestrus detection.

The calving to first service interval (CFI) was defined as the number of days from the calving until the cow's first artificial insemination or natural service:

$$CFI = First\ service\ after\ calving - calving\ date$$

This reproductive measure was calculated at the individual and farm level. Also, the CFI is presented as an annual average at the farm level. Similarly, the calving to conception interval (CCI) was the number of days from calving until the effective insemination service.

$$CCI = Effective\ insemination\ service - calving\ date$$

This reproductive measure was calculated and presented at the individual and farm level. For the multiparous animals, the calving interval (CI) was calculated as follows:

$$CI = Calving\ date(n + 1) - Calving\ date(n)$$

Given the previously calculated measures, we were able to check the gestation period of all individuals that had two consecutive calvings. The gestation period was defined as the difference between CI and CCI. Cows with the gestation period below 270 days were considered as cows that experienced foetal loss. Such cows were kept in the calculation, only if they had subsequent calving after a foetal loss. Consequently, their CI was considered as the difference between two normal calvings. The CI was then averaged and presented as the average annual CI, according to the formula:

$$CI(average) = \frac{1}{n} \sum (CIn)$$

where *n* represents the number of cows in the herd at a given time (year). Additionally, the calving index was calculated as an overall average CI of all cows on the farm. The moderate continental climate in BH is characterised by four annual seasons: Spring (March 21st- June 21st), summer (June 21st – September 23rd), autumn (September 23rd – December 21st), and winter (December 21st – March 21st). Accordingly, we determined the calving season for each calving and recorded it in the dataset afterwards.

Descriptive statistics

The median and 5-95 percentile range were calculated for continuous variables. Frequencies were calculated for dichotomous and categorical variables. The Kruskal-Wallis test was used for assessing the statistical difference between continuous dependent (reproductive measures) and categorical independent (farms) variables. Also, data were presented using box plots, bar graphs and histograms.

Results

Twenty-four managers of dairy farms were approached, and twenty-two of them (92%) agreed to participate in this study. Five of twenty-two dairy farms (23%) were excluded due to unreliable data recordings, leaving 17 farms in the final dataset. For individual cows, 57% (310/544) of individual data recordings were excluded from the study due to inappropriate written data or only providing memorised data. Finally, recordings for 234 animals from the 17 dairy farms were confirmed eligible and included in the study. The median number of animals per farm was 20 (range: 9 - 40). Further, the median number of heifers per farm was five (range: 0 - 12), while the median number of cows per farm was 14.5 (range: 4 – 26). The most frequent breeding method was artificial insemination, and it was represented in all of the included farms. The use of natural service sires was the additional option in case of long-lasting failures in conception. All farmers (17/17) reported the use of natural service sires at least for one cow in the period 2009-2016. The most common breed was Simmental (126/234) and Simmental crossbreeds (77/234), while the rest of the animals were Holstein-Friesian and Holstein-Friesian crossbreeds (26/234).

The median age at first artificial insemination or natural service (AFI) was 493 days (5^{th} -95^{th} percentile range 429-840), and the median age at first calving (AFC) was 802 days (708-1168). Figure 3 shows the variations in the AFI and the AFC among selected dairy farms. The median pregnancy proportion at the first insemination service was 40% (17 - 62) (Figure 4a). The cumulative pregnancy rates after Day-60, Day-80, Day-100 and Day-120 are shown in Figure 4b. The cumulative pregnancy rate after day-120 was approximately 64% at the population level. A total of 33% of the first inseminations (artificial and natural) resulted in a pregnancy (Figure 5a), while the mean number of services per pregnancy (NSP) varied substantially over selected dairy farms (Figure 5b). A total of 68.7 % of calculated inter-service intervals were distributed in the range of 18 to 24 days, while 10.8% of them were in the range of 36 to 48 days. (Figure 6). The calving-to-first-insemination interval (CFI) and the calving-to-conception interval (CCI) were unevenly distributed within- and between selected farms (Table 1). The median calving interval (CI) and its distribution over selected farms are shown in Table 1. The median calving to first insemination service interval (CFI) at the population level was 62.5 days (31-408), while the median calving to conception interval (CCI) was 101 days (36-506). Finally, the median CI for all selected farms was 385 days (range 329-773 days) (Table 2.) Since we were able to track reproductive events in the period 2009-2016, the annual and overall distributions of CFI, CCI, and CI are shown in Table 2. All visited farmers followed all-year-round calving, with an approximately equal proportion of calving per each season. We have compiled all dates of recorded calvings, and found out that 19% of them (108/573) have occurred in spring (March 21st- June 21st), 29% (163/573) in summer (June 21st – September 23rd), 26% (149/573) in autumn (September 23rd – December 21st), and 27% (153/573) in winter (December 21st – March 21st).

Discussion

Dairy farming in BH faces several challenges in reproductive management of dairy cattle to maintain milk production and farm profitability. The importance of reproductive performance benchmarking has not been previously adequately addressed in BH. Thus the scope of the study was to apply and to calculate known indicators of reproductive performance and provide initial data as a reference for further application. Thus, the present study describes key reproductive performance indicators at the individual animal level in selected dairy farms of Una-Sana Canton.

The average number of heifers and dairy cows per visited farm is small in comparison with the average herd sizes in EU countries (European Commission, 2016). Taking into account limiting factors in the bovine industry in BH, especially small stocking density, maintaining a constant number of cattle per farm and its gradual increase is a difficult task for dairy farmers. Thus, the optimal replacement of the herds is a primary managerial effort for dairy farmers in BH. The rearing of young stock is the most common source of replacement to avoid the costs of purchasing new animals. Farmers sought to optimise the age at first insemination (AFI) to introduce their heifers in the (re)production as early as possible. However, the mean AFI was substantially different among selected dairy farms as shown in this study. This indicated the lack of planning the heifer management, but also the lack of rearing standards and professional decision-making. Also, this finding highlights the need for continuous education of farmers in order to avoid the observed reproduction delays. In addition, there is a need for the introduction of heifer rearing goals and professional follow up. Farms with a smaller number of heifers had a lower median AFI and AFC, but also fewer heifers whose AFI and AFC deviates from the farm average. The observed trend loosely suggests that this association may be partially explained by the farmer’s adjusting the breeding programme to each heifer. On the other hand, farmers on farms with a higher number of dairy cows were directed toward more intensive milk production. It could mean that heifers were occasionally bred at the predefined time, e.g. when reaching the certain body weight. However, individual variations of the AFI in such farms was not followed up, and more research is needed.

The age at first calving (AFC) in the selected dairy farms was partially influenced by the AFI (Figure 3). Achieving a lower AFC has been found to be associated with improved reproductive performance and lifetime production, as well as timely successive calvings (Eastham *et al.*, 2018). Moreover, earlier studies have shown that the AFC may substantially contribute to the total rearing costs (Mohd Nor *et al.*, 2012, Boulton *et al.*, 2017). We observed substantial differences in the AFC between visited farms and given the median AFC of 802 days, our findings suggest that some of the visited dairy farmers might be affected by some economic burden due to the elevated median AFC in relation with the general goal at 22 to 24 months (730 days) (Le Cozler *et al.*, 2008, Heinrichs *et al.*, 2017).

This study reported an overall pregnancy proportion at first service of 40%, although there was a wide inter-farm variation. The pregnancy proportion at first service measured per farm (data not shown) coincided with the AFC, i.e. farms that experienced higher AFC also had lower pregnancy proportion. This trend is assumed to be related to farm management on smaller farms, as managers reported to follow the heifers more closely. Others have found that reproductive performance and health of dairy cattle is not universally better in smaller farms (Simensen *et al.*, 2010). Furthermore, the cumulative pregnancy rate at the population level showed that one-third of all pregnancies happened after Day-120 post-partum (Figure 4b). Although the median CCI of 101 days at the population level did not indicate the existence of reproductive problems in the selected dairy farms in Una-Sana Canton, the one-third of pregnancies happened after Day-120, indicating animals with high CCI in each visited dairy farm. The median values of the CCI in such skewed distribution likely failed to demonstrate individual variations within the herd. On the other hand, the combination of these reproductive measures is of particular interest. It gives the possibility that animals which got pregnant after Day-120 and cows with the high CCI can be evaluated by veterinary services separately from the rest of the herd. Accordingly, the improvement of the veterinary advisory service and its structured approach to animals whose indicators suggest a reproduction problem arises as one of the primary goals in the dairy sector in BH. Although not commonly used in BH, timed AI can be introduced as an alternative cost-effective and viable solution for this group of animals, as previously reported (Ribeiro *et al.*, 2012, Macmillan *et al.*, 2017). Animals with a chronic reproductive problems could also be removed from the dairy herd, although farmers reported that reproductive culling is not a common management practice in their farms in Una-Sana Canton.

The observed inter-service interval of 18-24 days for more than two-thirds of repeat breeding heifers and multiparous animals at the population level indicated a very good oestrus detection. Remnant et al. (2018) reported that inter-service interval of 19-26 days indicated that this period is the true latent distribution for the inter-service interval with the optimal reproductive outcome, suggesting day-22 with the increased probability of conception (Remnant *et al.*, 2018). However, we found that a total of 9.6% of inter-service intervals were longer than 48 days (Figure 6). Apart from being farmer-dependent, the extension of the inter-service interval over the 48 days may be the result of some other reproductive problems, such as anoestrus (Plozza *et al.*, 2016). In addition, such an extension may be a result of the occurrence of embryonal or foetal death (Dobson *et al.*, 2008). Our finding calls for targeted control of this group of repeat breeding cows. Importantly, caution is needed in the interpretation of our results. In the current study, however, inter-service intervals were only calculated for farms that had satisfactorily written records, thus excluding some of the largest herds.

Artificial insemination (AI) was the dominant type of breeding for dairy heifers in the selected dairy farms. AI is exclusively performed by veterinarians or veterinary technical staff who visit the farms after the farmers' call. The timing of AI might be influenced by the farm demographics which farmers in this study reported to be of concern in relation to repeat breeding. In addition, most of the farmers reported that they have side income which results in inappropriate or less time for oestrus detection. Irrespective of satisfactory oestrus detection found in this study, this may in part have contributed to the persistence of repeat breeding animals. On the other side, larger farms rely on non-family and seasonally employed labour. Considerable difference in farm workers' experience and competences could potentially influence the oestrus detection. Similarly, managing farm labour has been found to be of the greatest challenges in dairy farming (Bewley *et al.*, 2001). Based on data from the farms visited in this study, managers of larger farms, i.e. farms with the greater number of animals did not keep appropriate farm records in comparison with managers of small farms, and their ability to identify a reproduction problem at the individual animal level might be reduced. All visited farmers combined natural breeding and artificial insemination to reduce periods of repeat breeding. However, the results indicate that such managerial decision had either a slight or no effect on the CCI, i.e. the number of repeat breeders remained the same. Herds using natural services, rarely register the number of services per pregnancy. However, in the current study, the mean NSP was 2.61, and this number reflects both natural services and artificial inseminations. This points to reproductive shortfalls compared with other studies (Washburn *et al.*, 2002, Toledo-Alvarado *et al.*, 2017). Farms with low pregnancy rates (first service and cumulative) at individual animal level had the highest NSP. Likely, the NSP was dependent on a large number of factors such as the oestrus display, oestrus detection, timing of service, sire fertility and sperm quality, subclinical diseases and management features. Other studies are needed to investigate all aspects of increased NSP.

Farmers had different views on how long they should wait in re-starting the cows' reproduction after calving, and consequently, we were not able to calculate the voluntary waiting period directly from the farm records. However, farmers reported that they usually wait for two consecutive oestruses, after which they re-start with inseminations. This information is regarded imprecise since the period of voluntary waiting remains undefined in most cases. A recent study on management practices associated with reproductive performance in dairy cattle reported that the lack of a well-established VWP (<50 days) was associated with shorter CFI and consequently shorter CCI (Fodor *et al.*, 2018). Other studies have advocated a voluntary waiting period of at least 60 days, and the timed AI has been advised to be 73 days (Miller *et al.*, 2007). Studies that are aimed at investigating and establishing the optimal VWP for dairy farms in BH are needed.

However, we were able to calculate the calving-to-first-service interval (CFI). The overall median CFI was 62.5 days and varied substantially among the selected dairy herds (Table 1). This is low compared to the report for Norwegian red cattle which also shows a substantial variation in the population (85.3 days, SD±41.9) (Refsdal, 2007). Further, within-farm variations substantially affected the estimation of the farm's median CFI in this study. The individual CFI can be extended by several factors such as nutrition (Thatcher *et al.*, 2010, Rodney *et al.*, 2018), endometritis (Gilbert *et al.*, 2005), and poor oestrus detection. Elkjær et al. (2013) reported that even mild uterine infection could have an adverse effect on CFI (Elkjaer *et al.*, 2013). Similarly, the uterine infection was also one of the observations, associated with poor reproductive performance as presented in an earlier paper by our group (Softic *et al.*, 2018).

The calving interval (CI) was calculated as the traditionally used fertility indicator, and the median CI of 385 days indicates a relatively good reproductive performance in selected dairy farms in Una-Sana Canton. Similar to our findings, another study reported an average CI of 12.6 months in the Norwegian Red cattle (Refsdal, 2007). However, caution is needed in the interpretation of the CI. Since the CI is calculated retrospectively and represents the sum of all previous reproductive measures, it could be influenced by wide individual variations within the dairy herd. Given that our study revealed that one-third of pregnancies happened after the 120 days in milking (Figure 4b) and the average gestation period, there is the legitimate expectation that CIs for such pregnancies were greater than 400 days. The identification of those animals is the primary aim of recording animal performance.

Given that the system for animal identification and traceability has already been established in BH, our study indicate the need for the upgrading such system with the animal performance recording. A comprehensive recording system could help the veterinarians/advisors and farmers to determine if their reproductive management reflects the number of pregnant animals promptly. In addition to pregnancy recorded, the unavailable data of culled or sold individuals and 57% of individuals not included in the calculation due to the inappropriate written data, might influence the actual CI of the investigated population. The calculated reproductive measures indicated an undulant trend in reproductive performance among selected dairy farms in Una-Sana Canton, which is similar to the recent breeding programme set by national authorities.

This may be the optimal level of reproductive performance for this subset of farms, taking into account the study limitations.

External and internal validity of the study

This study described key reproductive performance indicators in dairy farms in the Una-Sana Canton. Given that dairy farmers have more or less the same managerial approach to dairy cattle rearing across the entire territory of the country, our findings may refer to other areas in BH. Also, the Simmental is the most common breed of cattle in BH, and thus, governmental and agricultural authorities have prescribed a breeding programme regarding maintaining and improving productive, reproductive and exterior characteristics of this breed (Anonymous, 2018). Reproductive goals in the breeding programme are set for AFI (14.5 – 16 months); AFC (24 – 26 months), average productive life (7 -8 years), CCI (100 days), NSP (1.8), and CI (<376 days). Although results of this study show the undulant trend in reproductive performance in commercial dairy farms (Tables 1 and 2), there is a substantial similarity of reproductive performance in dairy cattle in Una-Sana Canton with reproductive goals set in the recent breeding programme. This is of great importance for future dairy operations in Una-Sana Canton; however, there are several aspects of the reproduction that should be improved. Also, the obvious lack of records regarding reproduction and reproductive culling could influence the reproductive measure, and the actual reproductive performance could be weaker than shown in this study. In addition, the expected future intensification of the production in BH could, however, substantially contribute to the more serious decline in reproductive performance, as this trend is evident throughout the world (Lucy, 2001). The farms used in this study were farms with appropriate written records, and the external validity of this study is limited.

In summary, the recording of reproductive performance in dairy farms in Una-Sana Canton of BH is based on written or even memorising data, with no proper recording system in bigger farms. There is a need for an organised, regular and more comprehensive recording system in the reproduction of dairy cattle among dairy farms in Una-Sana Canton and BH as a whole. Knowing the apparent reproductive indicators described in this study, the farmers and veterinary authorities in BH may identify and correct areas in their management that contribute to the reproductive underperformance in dairy cattle.

Acknowledgement

This study was supported by the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Norway and Veterinary Faculty in Sarajevo, University of Sarajevo, Bosnia and Herzegovina. AS was supported by a grant of the Quota scheme (Norwegian State Educational Loan Fund – Lånekassen).

Declaration of interest

The authors declare no conflict of interest in this article.

Ethics statement

The study was submitted to and approved by the Ethics Committee of the Veterinary Faculty in Sarajevo of the University of Sarajevo, Bosnia and Herzegovina.

Software and data repository resources

None of the data were deposited in an official repository.

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Table 1 *The calculated reproductive measures and their median distribution (5th – 95th percentile range) in selected dairy farms in the Una-Sana Canton of Bosnia and Herzegovina.*

Farm ID	CFI[1]	CCI[2]	CI[3]
1	59.5 (35-100)	110.5 (39-223)	398 (344-477)
2	62.5 (44-176)	96 (62-242)	350.5 (332-442)
3	50 (30-102)	94 (34-320)	388 (321-618)
4	62 (20-98)	131(50-342)	388 (335-473)
5	69.5 (39-389)	126 (44-389)	425.5 (328-606)
6	55 (38-130)	73 (38-215)	359 (324-491)
7	63 (43-209)	113 (52-235)	393.5 (339-445)
8	60 (41-93)	83 (42-134)	353.5 (322-435)
9	61 (42-121)	116 (59-329)	385 (337-537)
10	63 (44-145)	121 (67-235)	392 (346-497)
11	55.5 (21-84)	100.5 (21-185)	386 (341-444)
12	76.5 (38-277)	111.5 (38-470)	380 (344-584)
13	75 (52-106)	88 (42-138)	372 (335-765)
14	79 (60-134)	126 (60-239)	409 (350-560)
15	80 (25-142)	103.5 (56-188)	403 (334-444)
16	62.5 (60-276)	104 (61-387)	390.5 (340-769)
17	63 (53-124)	63 (19-207)	413 (343-483)

1-Calving-to-first-insemination interval; **2**-Calving-to-conception interval; **3**-Calving interval

Table 2 *The median annual distribution of reproductive parameters in the follow-up period 2009-2016 in selected dairy farms in the Una-Sana Canton of Bosnia and Herzegovina*.

	2009	2010	2011	2012	2013	2014	2015	2016	Overall
n	6	17	25	39	72	97	125	75	234
CFI[1]	62.5	63	62	61	63	60	62	66	62.5
R[2]	42-86	36-408	40-104	31-121	40-121	38-106	38-143	40-169	31-408
n	7	17	24	39	72	95	127	a	234
CCI[3]	63	101	98.5	99	102.5	103	118	a	101
R	42-158	36-506	46-218	43-255	54-221	39-244	52-326	A	36-506
n		7	15	24	38	67	87	109	234
CI[4]	a	347	402	396	383	381	385	394	385
R	a	320-378	343-773	329-545	328-497	330-513	327-506	331-584	320-773

1-Calving-to-first-insemination interval; **2**-5th – 95th percentile range; **a**-no data; **3**-Calving-to-conception interval; **4**-Calving interval;

Figure 1 The locations of the selected dairy farms in the Una-Sana Canton, Bosnia and Herzegovina.

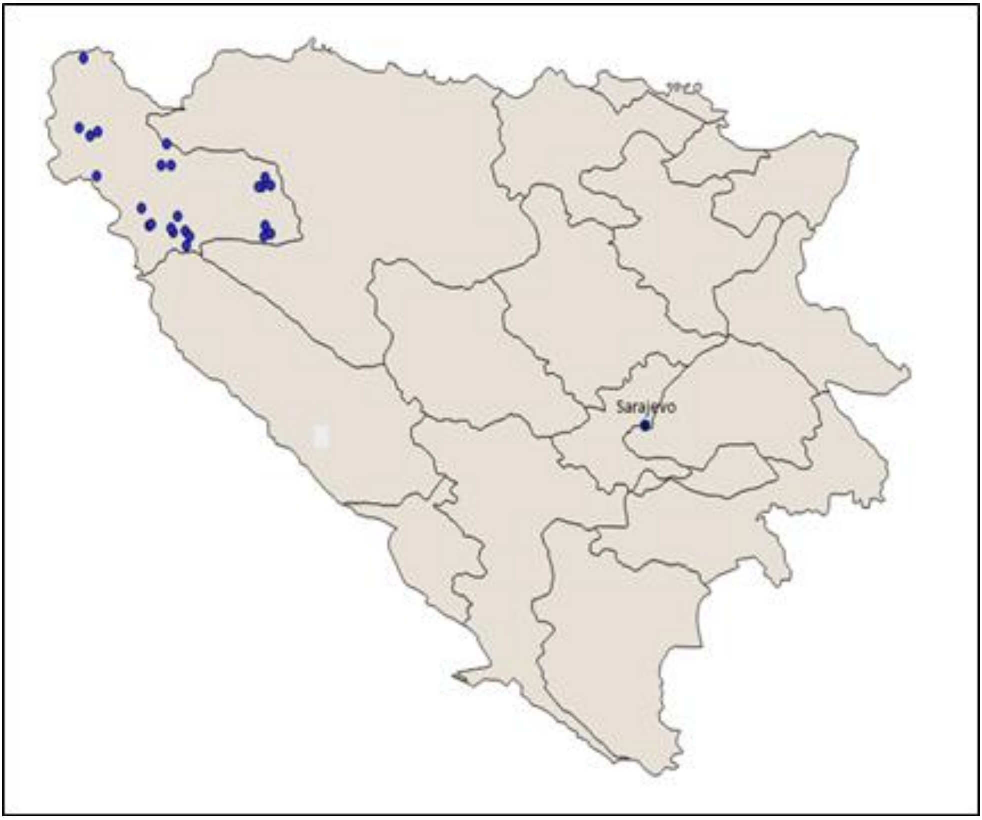


Figure 2 a) The scheme of the recording of reproductive events in selected dairy farms in Una-Sana Canton; b) The calculation of reproductive indicators from available dairy farms' records

AI – artificial insemination; AFI – the age at first insemination; AFC – the age at first calving; NSP – the number of services per pregnancy, ISI – inter-service intervals; CFI – calving to first insemination interval; CCI – calving to conception interval; CI – calving interval

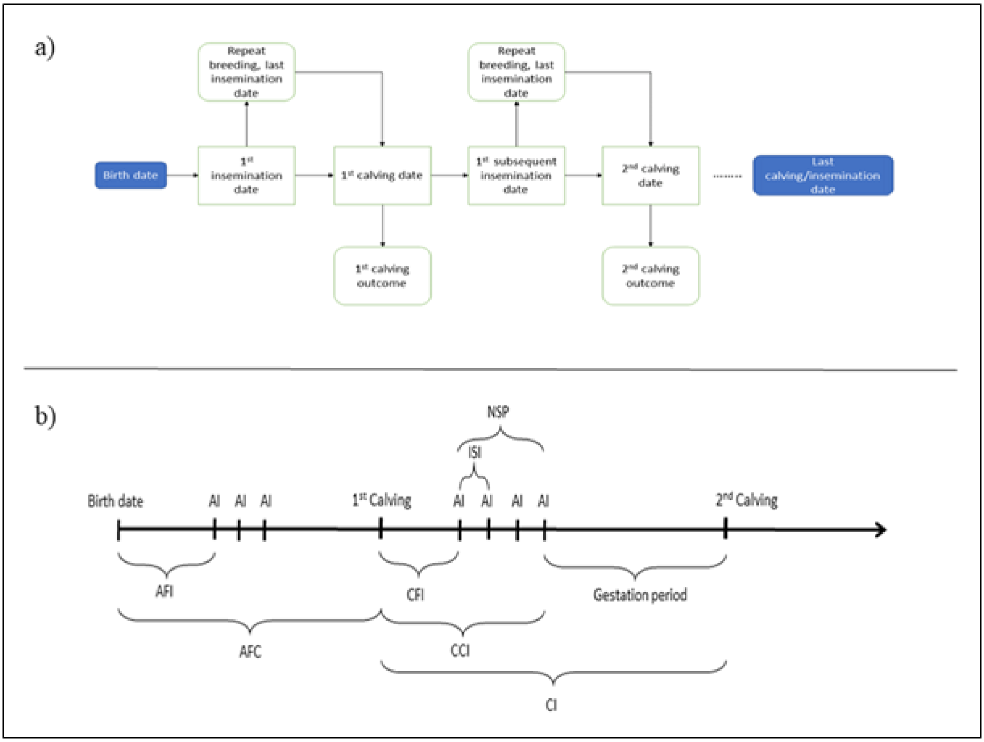


Figure 3 Distribution of the age-at-first-insemination (artificial or natural breeding) and the age-at-first-calving measures in heifers among selected dairy farms in Una-Sana Canton, Bosnia and Herzegovina

AFI – the age at first insemination; AFC – the age at first calving

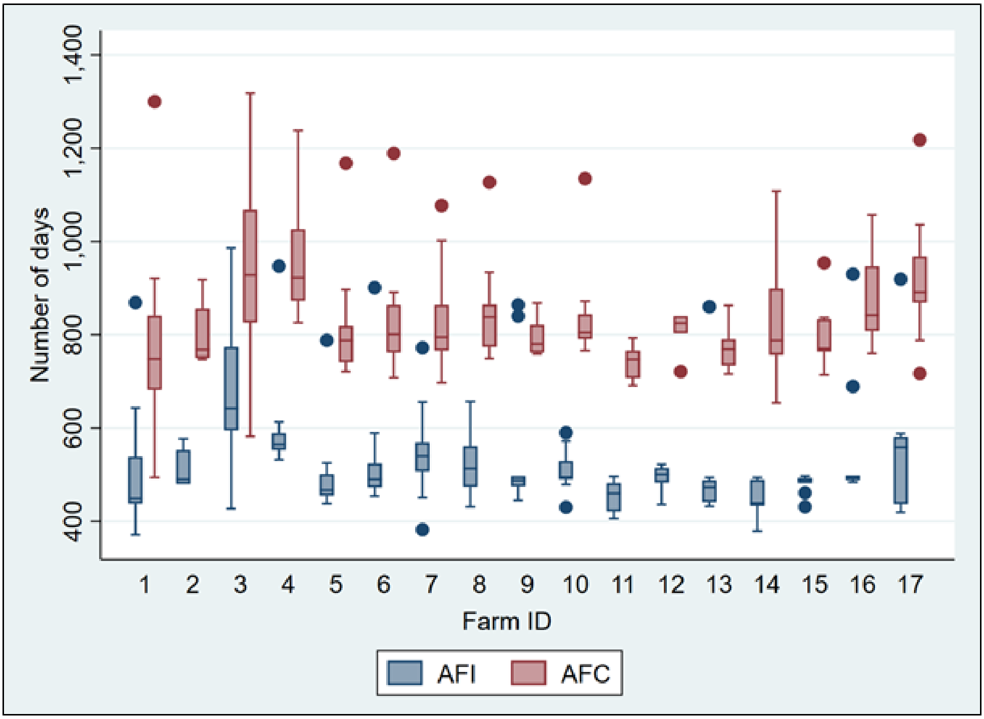


Figure 4 The pregnancy proportion expressed as a) The pregnancy proportion for heifers at the first insemination service (median 40%) and b) The population level cumulative pregnancy rate at Day-60, Day-80, Day-100 and Day-120 days after the previous calving in the selected dairy farms in Una-Sana Canton, Bosnia and Herzegovina.

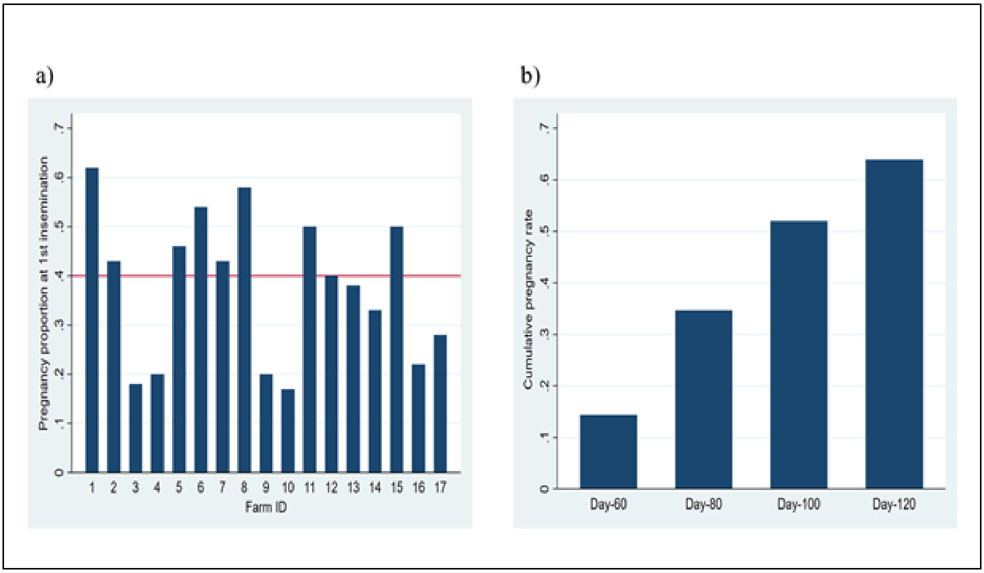


Figure 5 The number of services (artificial insemination and natural breeding) per pregnancy expressed as a) the percentage of successful services distributed by the number of attempts b) the mean number of services per pregnancy (2.61) distributed by the dairy farm in the selected area of Una-Sana Canton, Bosnia and Herzegovina.

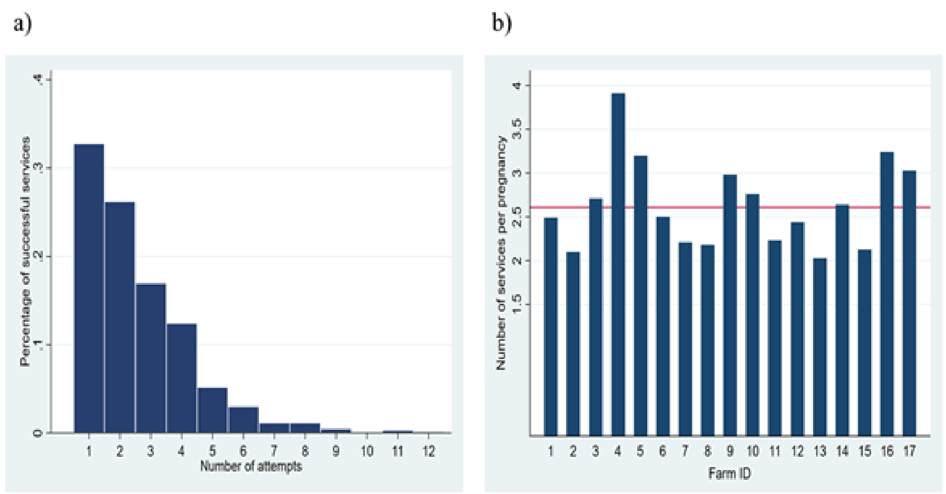
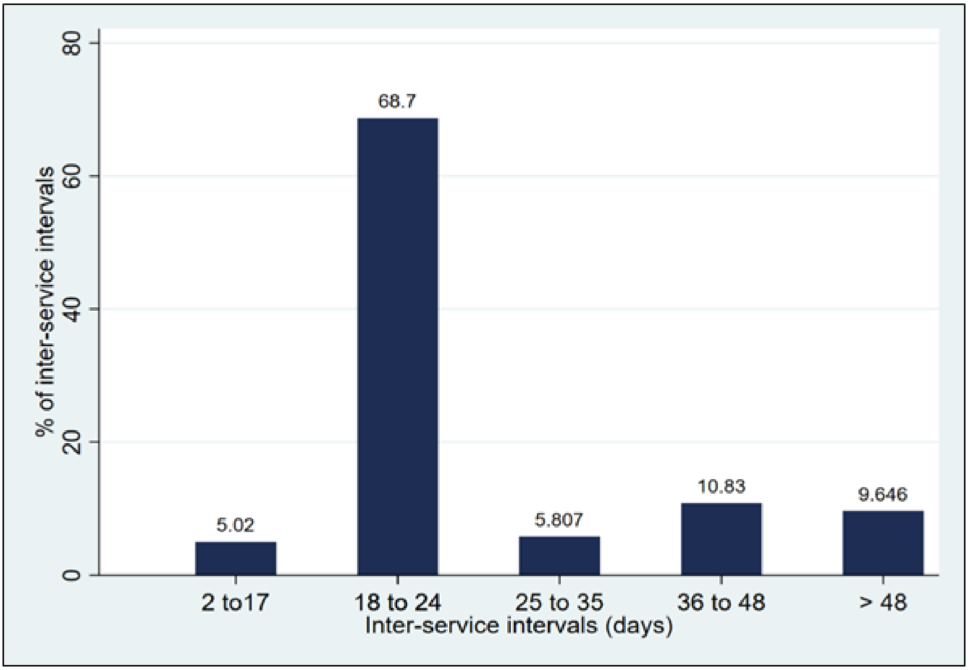


Figure 6 Inter-service intervals histogram. Repeat breeding services recorded and compiled at the population level showed that 68.7% of returns to the oestrus occurred in the 18 to 24 days period. Considering good oestrus detection, the target is set on 55% as described in (Smith *et al.*, 2014)



ISBN: 978-82-575-1774-8
ISSN: 1894-6402

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