RESEARCH PROGRAM ON Livestock

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Progress report on phenotyping indigenous Tunisian sheep breeds for resistance to ticks

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ICARDA Science for resilient livelihoods in dry areas

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CGIAR is a global partnership that unites organizations engaged in research for a food-secure future. The CGIAR Research Program on Livestock provides research-based solutions to help smallholder farmers, pastoralists and agro-pastoralists transition to sustainable, resilient livelihoods and to productive enterprises that will help feed future generations. It aims to increase the productivity and profitability of livestock agri-food systems in sustainable ways, making meat, milk and eggs more available and affordable across the developing world. The Program brings together five core partners: the International Livestock Research Institute (ILRI) with a mandate on livestock; the International Center for Tropical Agriculture (CIAT), which works on forages; the International Center for Research in the Dry Areas (ICARDA), which works on small ruminants and dryland systems; the Swedish University of Agricultural Sciences (SLU) with expertise particularly in animal health and genetics and the Deutsche Gesellschaft für Internationale Zusammenarbeit (GIZ) which connects research into development and innovation and scaling processes.

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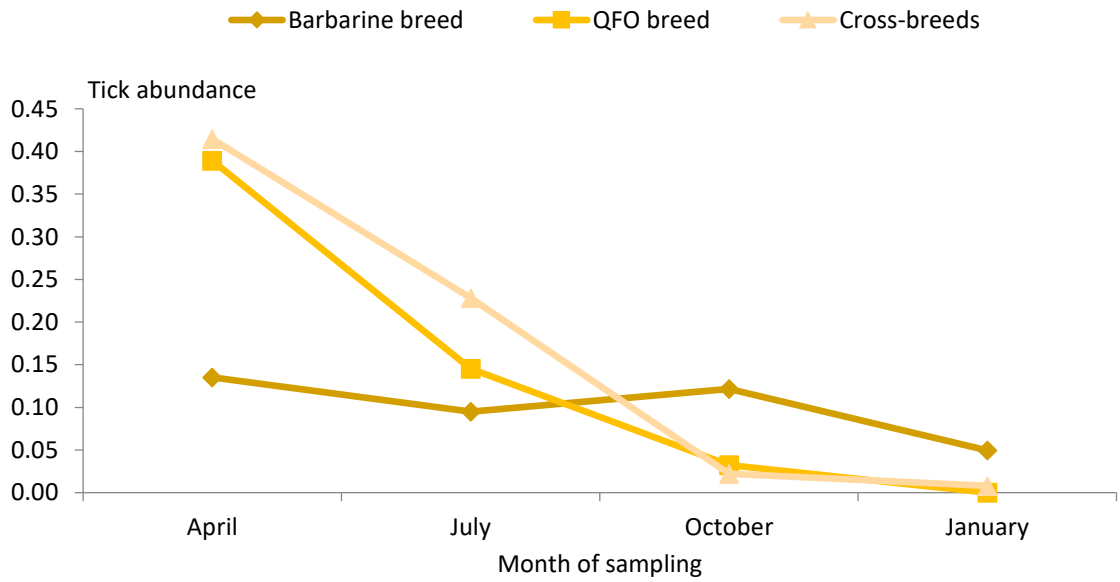
1 NSVM: The National School of Veterinary Medicine of Sidi Thabet, Tunisia
2 ICARDA – The International Center of Agricultural Research in the Dry Areas

Contents



I Background

Sheep are playing important role in national economy and it's a vital activity for thousands of households in Tunisia. Two main breeds exist and are unequally distributed along the country: Barbarine (Fat-tailed breed) and Queue Fine de l'Ouest (thin-tailed breed). Barbarine is the dominant sheep breed in Tunisia (64%), it is well adapted to extreme climatic conditions and low quality fodder, beside good mothering ability and resistance to pathogens (Ben Salem et al., 2011). The second important breed QFO (30%), is from Algeria and is also well adapted, especially to cold temperatures and to mountainous landscape. Molecular genetic diversity was recently investigated for all Tunisian sheep breeds using microsatellites markers (Ben Sassi-Zaidy et al., 2014) and Random Amplified Polymorphic DNA (RAPD) markers and showed a high genetic variability. Such findings will pave the way to manage genetic programs (Ben Sassi-Zaidy et al., 2014; El Hentati et al., 2012; Kdidi et al., 2014) and select resistant breeds to pathogens.

Indeed, in Tunisia, sheep are facing several bacterial, viral and parasitic diseases that impede their development, the selection of resistance breeds could be an alternative to the overuse of several drugs (antibiotics, antiparasitic drugs). Among parasitic diseases, tick-borne pathogens (TBP): *Theileria* spp., *Babesia* spp., *Anaplasmosa* spp., *Borrelia* spp., *Mycoplasma* spp. (Belkahia et al., 2017; Rjeibi et al., 2016, 2015; Said et al., 2016) affect productivity of sheep and induce huge economic losses. Beside the pathogens they transmit, ticks cause also skin irritations and blood spoliation that could in some cases lead to anaemia. Although the main measure to control ticks is using acaricides, resistance to acaricides is spreading in tick populations, it constitutes in several regions of the world the main limit anti-tick and anti-TBD control programs (Abbas et al., 2014; Kunz and Kemp, 1994). The predominant tick species in sheep in Tunisia is belonging to *Rhipicephalus sanguineus* group, with a peak of activity in August (Elati et al., 2018). Most of studies on tick-borne pathogens carried out in Tunisia were focusing on comparing tick infestation prevalence between domestic animals species, or age, or geographic area and few focused on breed sheep. A study of Elati et al. (2018), found that 103 out of 284 (7.3%) and 303 out of 362 (16.7%), from the Barbarine and QFO, respectively were infested by ticks. These studies are insufficient to argue that Barbarine breed is most resistant to tick infestation and further investigations are needed to confirm this trend.

Host resistance to ticks is influenced by multiple factors such as: age, gender, physiological status, coat characteristics, climate and environment, (Marufu et al., 2011) grooming behaviour and genetic background (Raberg et al., 2007). Although, resistance to tick infestation was widely investigated in cattle (de Castro et al., 1991; Mansfield et al., 2009) and experimental animals (Rechav et al., 1994; Rechav and Fielden, 1997) few studies in sheep were performed.

It was established that tick infestation resistance phenotype is heritable (Robbertse et al., 2017). Heritability for tick resistance assessed by tick counts have been estimated at 0.32 to 0.59 in Norwegian sheep (Grova, L.l Sae-Lim, P.; Olesen, 2014), while it ranged from 0 to 0.89 in cattle in South Africa (Mapholi et al., 2017). Since heritability for tick resistance is moderate (Budeli et al., 2009), it will be interesting to identify and select resistant females to transmit resistance to their offspring.

The resistance to ticks is expressed by the following indicators: (i) reduced numbers of engorged ticks; (ii) decreased weight of engorged ticks (smaller blood meals); (iii) reduced number and viability of ova (decreased weight), (iv) shortening in duration of tick feeding (Rechav, 1992).

In sheep, the genetic resistance was investigated mainly for fly (Scholtz et al., 2011) and nematodes (Bishop, 2012). As the genetic resistance to tick infestation is immunologically mediated, the study of the immunological reactions is required to understand this phenomenon. Both innate and acquired immunity are involved in tick resistance (Maharana et al., 2011) and it was reported that sheep develop resistance after one tick infestation (Abdul-Amir and Gray, 1987). Other authors reported that resistance to tick appear after repeated tick infestations (Wada et al., 2010), the fourth one in cattle, according to Barriga et al. (1991). The histological examination of tick site attachment, the dosage of circulating T and B lymphocytes (Boppana et al., 2004), cytokine synthesis (Piper et al., 2010) and examination of gene expression in skin and hypersensitivity (Marufu et al., 2013) were investigated by several authors mainly for tick resistance in cattle but few in sheep.

The measurement of phenotypic and genotypic indicators regarding the tick infestation resistance in both Barbarine and QFO sheep breeds, will provide useful information and will pave the way to select resistant breeds and implement efficient control measures against ticks, as it was the case for cattle in Australia (Allen, 1994).

Despite the resistance to ticks have been widely studied elsewhere; it has never been investigated in Tunisia, where tick infestation is considered as a huge problem in livestock.

The aim of this work will be to assess which sheep breed among the Barbarine and QFO is most resistant to ticks and identify the genetic drivers of this resistance. Phenotypic indicators will be associated to genome analysis using the 600K SNP Chip, which will provide a better resolution of the sheep genomic profiles.

II Study hypothesis

According to the study of Elati et al., 2018, the breed Barbarine might be more resistant to the tick infestation than QFO breed.

1. The most resistant sheep breed to tick infestation will express lower tick counts;
2. The most resistant sheep breed to tick infestation will display less or not anaemia, less parasitemia, good body score condition, high inflammation signs at the tick attachment site;
3. The most resistant sheep breed will show specific genetic markers using SNP (single nucleotide polymorphism) genotyping.

III Methodology

3.1. Study area

In Tunisia, there are 5 bioclimatic stages. To represent each stage, we selected the following areas (Figure 1):

- Humid: Ain Draham (Jendouba district)
- Sub-Humid: Mornaguia (Manouba district)
- Semi-arid: Saouaf (Zaghouan district)
- Arid sup (high steppes): Sbitla (Kasserine district)
- Arid sup (low steppes): Bir Ali (Sfax district)
- Saharian: Tatouine (Tataouine district)

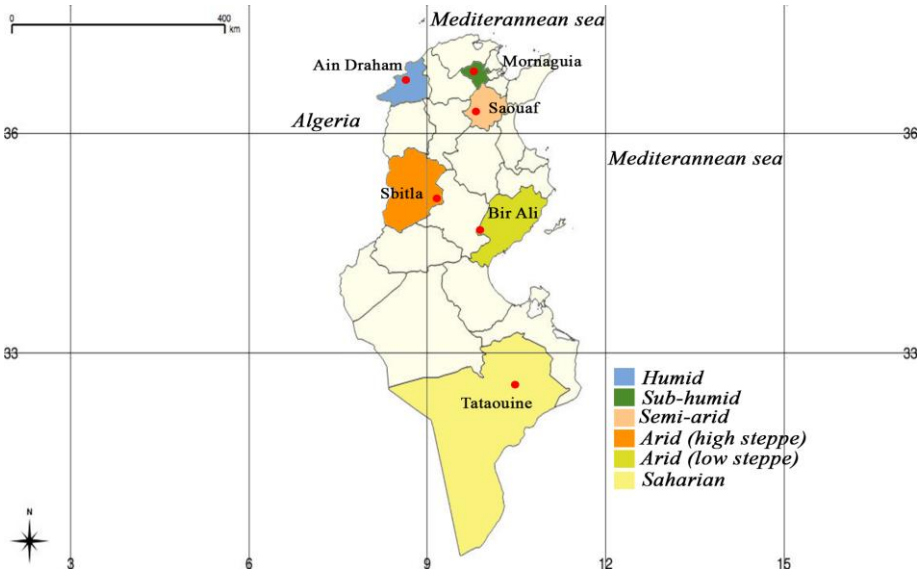


Figure 1: Location of the studied area on the map of Tunisia

3.2. Animals

Only adult females (at least 2 year old) were selected for resistance assessment, because they got previous contact with ticks and are immunized. The animals from breeds Barbarine, QFO and crossbreeds were selected, ear tagged at the first visit and then sampled once per season.

3.3. Sampling protocol

Each visit, data about general status (temperature measure, mucous inspection and body score measurement) was monitored and blood, sera, ticks and feces were also collected (Table 1). The data about herd management (feeding, watering, grazing…) were recorded at the first visit.

Table 1: Type of samples to collect for the study

Field samples collection	*Volume/Quantity*	*Laboratory processing/ analysis*
Total blood	5 ml	Hematological analysis Staining Giemsa DNA extraction (PCR)
Sera	5 ml	Antibodies against TBP
Feces	10g	Worms and eggs counting
Ticks	All	Identification Half preserved Half ➔ DNA extraction

3.4. Laboratory analyses

Table 2: Brief description of the main methods used in laboratory

Measurement	Method brief description	Progress
Tick count	Animal ears were checked for tick presence. Ticks were removed and placed in alcohol (70°) until macroscopic identification	Done
Tick identification	Ticks are identified under stereo-microscope at the genus and species levels according to (Walker et al., 2003)	Undergoing
Blood count	An hematology report was obtained for blood samples collected in EDTA Vacutainers using Auto Haematology analyser BC-2800Vet® (ShenzenMindray Bio-Medical Electronics Co., Ltd, Hamburg, Germany). The report comprises: White blood cells (109 l-1), Hematocrit (PCV) (%), Red Blood Cell count (×1012 m-1), Hemoglobin (g d-1), Mean Corpuscular Volume (MCV) (fl), Mean Corpuscular Hemoglobin (MCH) (pg), Mean Corpuscular Hemoglobin Concentration (MCHC) (g dl-1), Red Blood Cell Distribution Width (RDW), Index of Red Blood Cells Distribution (IDR) (%), Platelets (109 l-1), Average Platelet Volume (VPM) (fl), Index of Platelets Distribution (IDP) and Plateletocrite (Pct) (%).	Done
Giemsa staining	Giemsa-stained blood smears were examined under 1000 magnification. For each slide, 50 microscopic fields were examined.	Done for April, July and October Visits
Coprology: Fecal Egg Count (FEC)	The coproscopic survey is realized for each feces sample. Qualitative coprology allows identification of gastro-intestinal eggs and others such as are Trichures, Coccidies, *Nematodirus*, *Moneizia* and pulmonary larvae. Quantitative corology examination allowed the counting of these eggs.	Ongoing
DNA extraction	DNA will be extracted from 300 µL of anti-coagulated blood of each sheep using the Rapid Genomic DNA purification kit (Blood)®(BioBasic, Canada) according to the manufacturer's instructions and stored at -20°C until use.	Ongoing
Molecular identification of tick-borne pathogens	Catchall primers (RLB-F and RLB-R) which detect Theileria spp. Babesia spp. and Anaplasma/Ehrlichia spp. pathogens will be used. Reactions will be performed in 25 µl volume containing 1 x PCR buffer, 1.5 mM MgCl2, 200 µM of each deoxyribonucleotide triphosphate, 0.125 µg of Taq hot start Ab, 0.1 U of Uracil DNA glycosylase, 25 pmol of each primer and 1.25 U of Super Taq DNA polymerase (Vivantis, Chino, CA, USA). Forty PCR cycles will be performed with a thermocycler (ESCO Swift MaxPro). Each cycle consist of a denaturing step of 1 min at 94°C, an annealing step of 1 min at 50°C, and an extension step of 1.5 min at 72°C. A final extension step of 10 min at 72°C will complete the program	Not yet
SNP		

3.5. Data analyses

Individual data (ID, breed, age, region, temperature, body score, blood report, Giemsa staining and tick numbers) were entered manually into Excel sheet.
The following epidemiological indicators were calculated for overall animals and according to sheep breeds:

- Tick infestation prevalence (%) = 100 x (Number of infested sheep/Number of examined sheep)
- Tick infestation intensity = Number of ticks/Number of infested sheep
- Tick infestation abundance = Number of ticks/Number of examined animals

Chi square test was used to compare prevalences according to regions, season and breeds while Fisher exact test was used for small samples. To compare tick infestation intensity and tick abundance among regions and season, an analysis of variance test was performed with SPSS (version 21, IBM, USA). All tests were considered significant at threshold 0.05.

IV Preliminary results

A total of 1555 samples (blood, sera) and 235 tiks were collected during four seasons: April, July, October 2018, and January 2019 (Table 10). The main breeds involved in our study, are the Barbarine breed, the QFO breeds and crossbreeds. The sample started with 461 animals in April, with almost the same mean proportion (33%) of each sheep breed during the four visits (p=0.8).

4.1. Overall tick infestation indicators

The overall tick infestation prevalence decreased significantly from April to January, also the number of collected ticks (p<0.001)(Table 3).

Table 3: Total animals infested with ticks and total ticks collected according to seasons

	Apr-18	Jul-18	Oct-18	Jan-19
Total animals	461	389	366	339
Total infested	59	46	18	4
Total ticks	146	63	20	6

The tick infestation intensity was higher in April than in July and October. In January, was recorded the lowest tick infestation intensity and abundance (Table 4).

Table 4: Tick infestation prevalence and intensity and tick abundance according to seasons

	Apr-18	Jul-18	Oct-18	Jan-19	p
Tick infestation prevalence (%)	12.8	11.8	4.9	1.1	0.001
Tick infestation intensity	2.475	1.370	1.111	1.500	0.001
Tick abundance	0.317	0.129	0.043	0.018	0.003

4.2. Tick infestation according to region

In April and July, the most infested regions were Saouef and Fernana, respectively (p<0.001). In October and January, Tataouine in Southern Tunisia, at the Saharian bioclimatic stage, was the most infested area (p<0.001)(Table 5).

Table 5: Tick infestation prevalences according to regions and seasons

	Infested/Examined (%)	Infested/Examined (%)	Infested/Examined (%)	Infested/Examined (%)
	April 2018	*July 2018*	*October 2018*	*January 2019*
Mornaguia	7/90 (7.7)	6/60 (10)	2/61 (3.2)	0/61
Fernana	12/75 (16)	20/65 (30.7)	0/50	0/56
Tataouine	0/59	0/44	12/41 (29.2)	4/34 (11.7)
Sbitla	3/89 (3.3)	1/88 (1)	1/76 (1.3)	0/74
Bir Ali	0/66	6/57 (10.5)	3/58 (5.1)	0/54
Saouaf	37/82 (45.1)	13/74 (17.5)	0/63	0/60
Totals	59/461 (12.8)	46/389 (11.8)	18/366 (4.9)	4/339 (1.1)
p	<0.001	<0.001	<0.001*	<0.001*

*Performed with Fisher exact test

4.3. Tick infestation according to breeds

The preliminary results showed a difference on tick infestation among sheep breeds (Table 6).

4.3.1. Tick infestation prevalences according to breeds

The Barbarine breed was significantly less infested with ticks than QFO breed and crossbreed in April and July (p=0.009 and p=0.05), respectively, but in October, they were more infested than the other breeds (p=0.008) (Table 6, Figure 2).

Table 6: Tick infestation prevalences according to sheep breeds

	April 2018	**July 2018**	**October 2018**	**January 2019**
Barbarine	10/148 (6.76)	9/116 (7.76)	11/107 (10.48)	3/101 (2.97)
QFO	19/149 (12.75)	12/124 (9.68)	4/124 (3.23)	0/112 (0)
CB	30/164 (18.29)	25/149 (16.78)	3/135 (2.5)	1/125 (0.81)
p	*0.009*	*0.05*	*0.008*	*Not applicable*

4.3.2. Tick infestation intensity according to breeds

The tick infestation intensities varied in April and January among the three sheep breeds (Table 7, Figure 3).

Table 7: Tick infestation intensities according to sheep breeds

	April 2018	July 2018	October 2018	January 2019
Barbarine	2.00	1.22	1.18	1.67
QFO	3.05	1.5	1.00	0.00
CB	2.27	1.36	1.00	1.00
p	*0.06*	*0.4*	*0.3*	*Not applicable*

4.3.3. Tick abundance according to breeds

The tick abundance was significantly different between sheep breeds in October. The tick abundance in Barbarine breed was higher than in QFO and crossbreeds (Table 8, Figure 4).

Table 8: Tick abundance according to sheep breeds and seasons

	April 2018	July 2018	October 2018	January 2019
Barbarine	0.14	0.09	0.12	0.05
QFO	0.39	0.15	0.03	0.00
CB	0.41	0.23	0.02	0.01
p	*0.06*	*0.07*	*0.005*	*0.07*

V Conclusion

The aim of this work is to study the genetic resistance of the Barbarine sheep to tick infestation. The preliminary results are favorable for a difference in tick infestation among sheep breeds. The QFO breed is probably most infested than Barbarine breed. However, this is different among seasons and among regions. To highlight that there is tick resistance in one breed, we need supplementary observation and analyses. We first need to identify the ticks at species level. Then, we need to fix some factors, to minimize the bias such as choose one region (same abiotic and biotic factors), with one herd management type. The observation of tick infestation for one year is not enough to state about phenotypic tick resistance and we should go forward to follow the sample, one more year. Phenotypic measurements (age, breed, region, anemia, herd management type, hematological parameters, FEC and abundance of infestation) will be performed and will be compiled in a single phenotype database. Genome analysis will be carried out using the 600K SNP-Chip which will provide a better resolution of the sheep genomic profiles. All the data still to be deeply analyzed for phenotypic tick resistance evidencing between sheep breed. In another hand the SNP study, will provide information about the genetic mechanism of tick resistance, if exist. For this, a proper statistical analysis should be done to select the most suitable individuals for the SBP study. DNA extraction is undergoing now in the laboratory and its quality is checked simultaneously through Universal PCR testing.

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Annexes

Table 9: Tick infestation among the sheep breeds

	April 2018	July 2018	October 2018	January 2019
Mornaguia • Barbarine • QFO • CB	7/90 2 4 0	6/60 6 0 0	2/61 1 1 0	0/61 0 0 0
Fernana • Barbarine • QFO • CB	12/75 0 0 12	20/65 0 1 19	0/50 0 0 0	0/56 0 0 0
Tataouine • Barbarine • QFO • CB	0/59 0 0 0	0/44 0 0 0	12/41 10 0 2	4/34 3 0 1
Sbitla • Barbarine • QFO • CB	3/89 0 2 1	1/88 0 0 1	1/76 0 0 1	0/74 0 0 0
Bir Ali • Barbarine • QFO • CB	0/66 0 0 0	6/57 0 6 0	3/58 0 3 0	0/54 0 0 0
Saouaf • Barbarine • QFO • CB	37/82 8 13 16	13/74 3 5 5	0/63 0 0 0	0/60 0 0 0
Totals	59/461	46/389	18/366	4/339

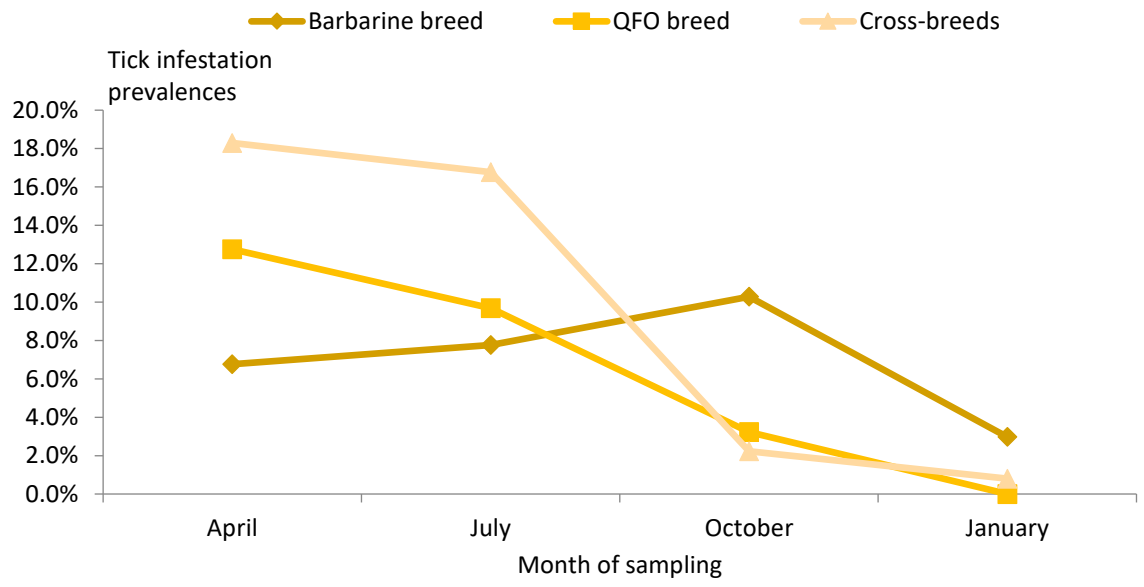

Figure 1: Tick infestation prevalences according to sheep breeds and season

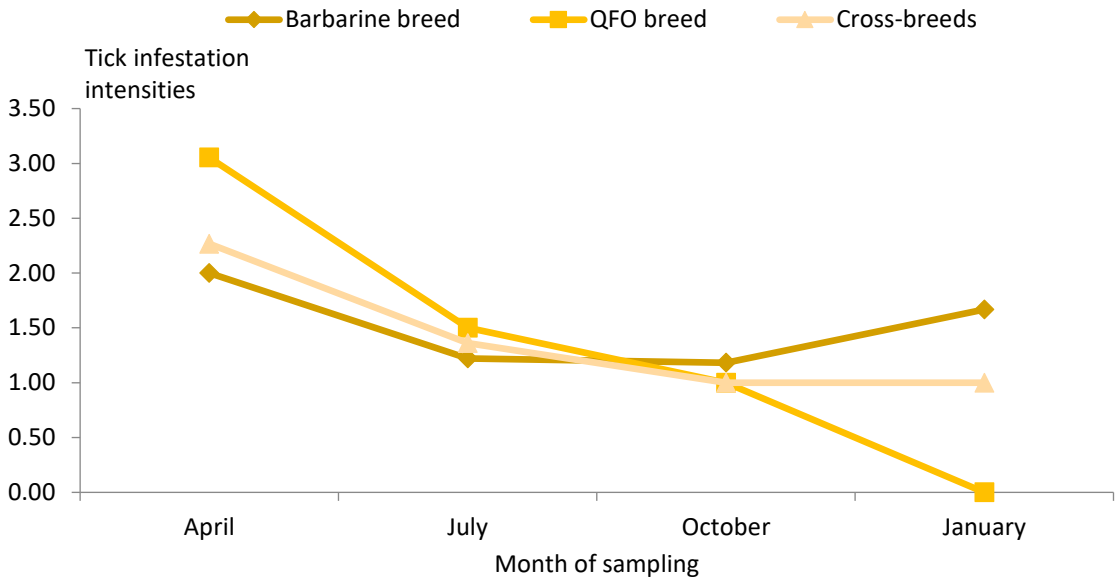

Figure 2: Tick infestation intensities according to sheep breeds and season

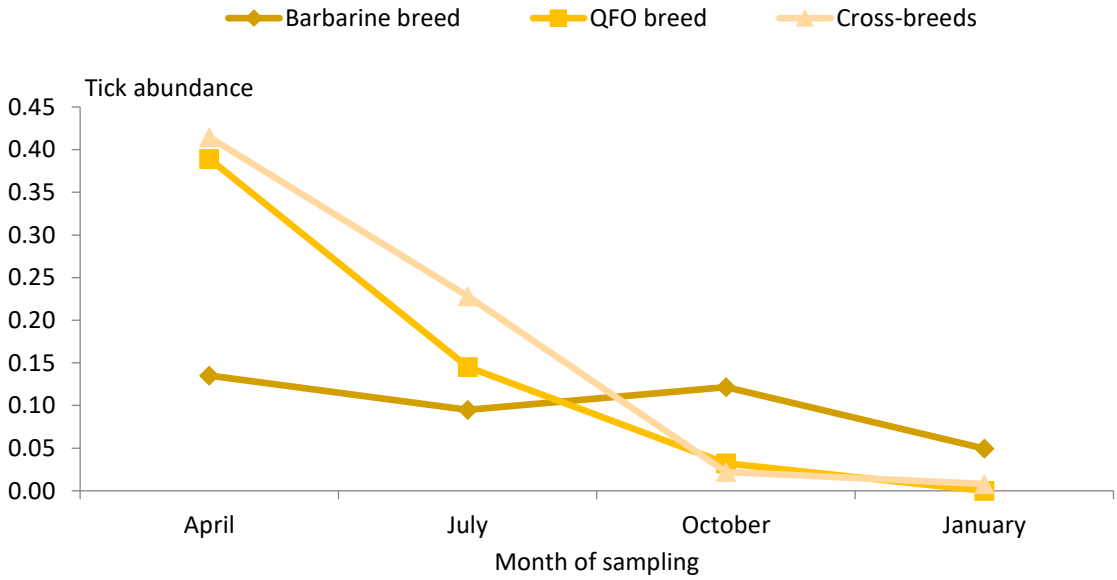

Figure 3: Tick abundance according to sheep breeds and season