Aus dem Institut für Parasitologie und Tropenveterinärmedizin
des Fachbereichs Veterinärmedizin
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Improvement and development of methods to propagate *Theileria annulata in vitro* and to study the *T. annulata* life cycle based on cell culture techniques and an artificial tick feeding system

Inaugural-Dissertation
zur Erlangung des Grades eines
Doctor of Philosophy (PhD)
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vorgelegt von
Khawla Elati
Biologin aus Ksour, Tunesien
MSc. Molecular Biology, B.Ed. Science Biotechnology

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Gedruckt mit Genehmigung des Fachbereichs Veterinärmedizin der Freien Universität Berlin

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Dekan: Univ.-Prof. Dr. Uwe Rösler

Erster Gutachter: Univ.-Prof. Dr. Ard Nijhof

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Dritter Gutachter: Prof. Dr. Philipp Olias

Deskriptoren (nach CAB-Thesaurus): cattle, *Theileria annulata*, theileriosis, cell culture, leukocytes, biological markers, gene mapping, merozoites, erythrocytes

Tag der Promotion: 30.08.2024

Printed and/or published with the support of the German Academic Exchange Service

Dedication

This thesis is dedicated to my parents, brothers and husband who have given me love and support without compromise over the years.
My heartfelt appreciation to my friend Barbara Mehlitz.
Thanks all for being there for me.

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List of abbreviations

ANOVA	Analysis of variance
APC	Allophycocyanin
ATFS	Artificial tick feeding system
BP	Biological processes
CC	Cellular components
CO2	Carbon dioxide
CTFR	Cell trace far-red
DDAO-SE	dichloro dimethyl acridin one succinimidyl ester
DEG	Differentially expressed genes
DIC	Differential interference contrast microscopy
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Flow cytometry activated sorting
FBS	Foetal bovine serum
GDT	Generation doubling time
GO	Gene ontology
GUTS	Ground-up tick supernate
HEPES	N[2-hydroxyethyl] piperazine-N -[2-ethanesulfonic acid
IFAT	Immunofluorescence antibody test
KEGG	Kyoto Encyclopedia of Genes and Genomes
LaGeSo	Landesamt für Gesundheit und Soziales
MF	Molecular function
mVYM	modified Vega Y Martinez solution
NaHCO3	Sodium hydrogencarbonate

N_2	Nitrogen
O_2	Dioxygen
PCA	Principal component analysis
PCR	Polymerase chain reaction
PPE	Percentage of parasitized erythrocytes
RBCs	Red blood cells
RNA seq	RNA sequencing
RPMI-FBS	RPMI medium containing Foetal bovine serum
RPMI-SF	RPMI serum-free
RT-qPCR	Real time quantitative PCR
SFM	Serum-free medium
SFM	Serum-free medium
SP	Signal peptide
Tams-1	*Theileria annulata* merozoite surface 1
TaSP	*Theileria annulata* surface protein
TBD	Tick-borne Diseases
TMD	Transmembrane domain
TT	Tropical theileriosis

General introduction and thesis objectives

Ticks are blood-sucking arthropods with a worldwide distribution that can transmit numerous pathogens to humans and animals. This includes pathogens causing diseases such as Lyme borreliosis, tick-borne encephalitis and Crimean Congo Hemorrhagic fever in humans (Brites-Neto et al. 2015; Estrada-Pena 2009). In domestic animals, losses due to tick-borne diseases such as theileriosis, anaplasmosis and babesiosis hamper livestock production and amount to millions of euros. In addition to the pathogens they transmit, tick bites can also cause allergic reactions and heavy tick infestations can cause skin irritation at the attachment sites, anaemia, and losses in body weight and milk production (Boulanger et al. 2019; Sonenshine et al. 2014a, 2014b).

Tropical theileriosis (TT or also called Mediterranean theileriosis) is a tick-borne disease caused by the protozoan *Theileria annulata*, and affects cattle in several countries around the Mediterranean basin, the Middle East and Asia (Gharbi et al. 2020). Recently, *T. annulata* infections were also recorded outside of the known distribution range in Nigeria (Mamman et al. 2021), Benin and Burkina Faso (Ouedraogo et al. 2021) and Côte d'Ivoire (Grace et al. 2023) (Figure 1).

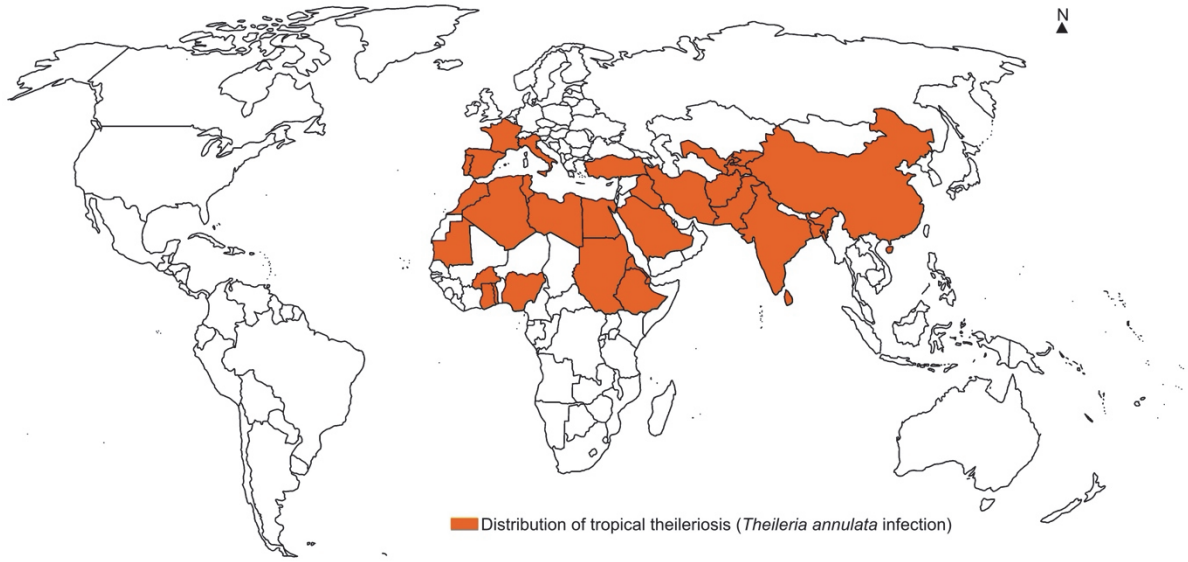

Figure 1: Current geographical distribution of TT (image credit: Khawla Elati)

The clinical symptoms of TT include a febrile syndrome, enlarged lymph nodes, extensive haemorrhages and extravascular haemolytic anaemia, occasionally leading to jaundice, particularly in subacute or chronic forms of the disease (Sergent et al. 1945). The pathogen is transmitted by several tick species of the *Hyalomma* genus, such as *Hyalomma scupense* (Bouattour et al., 1996), *Hyalomma dromedarii* (d'Oliveira et al., 1997), *Hyalomma anatolicum*

(Hussein et al. 2012), *Hyalomma lusitanicum* (Elfegoun et al. 2013) and *Hyalomma excavatum* (Aktas et al. 2004).

Sporozoites of *T. annulata* mature in the salivary glands of infected ticks and are transmitted with tick saliva to the host during the uptake of a blood meal. In cattle, the sporozoites invade mononuclear cells where they develop into macro- and microschizonts, which cause a lymphoproliferative disease by inducing transformation of the infected host cell, leading to its uncontrolled proliferation, a phenotype similar to cancer (Tretina et al. 2015; von Schubert et al. 2010). The microschizonts produce merozoites, which are subsequently released from the mononuclear cells and invade erythrocytes to become erythrocytic merozoites or piroplasms. Ticks become infected with *Theileria* when they imbibe piroplasms with a blood meal. Sexual reproduction subsequently takes place in the ticks' midgut; kinetes penetrate the midgut and migrate to the salivary glands (Schein 1975) (Figure 2).

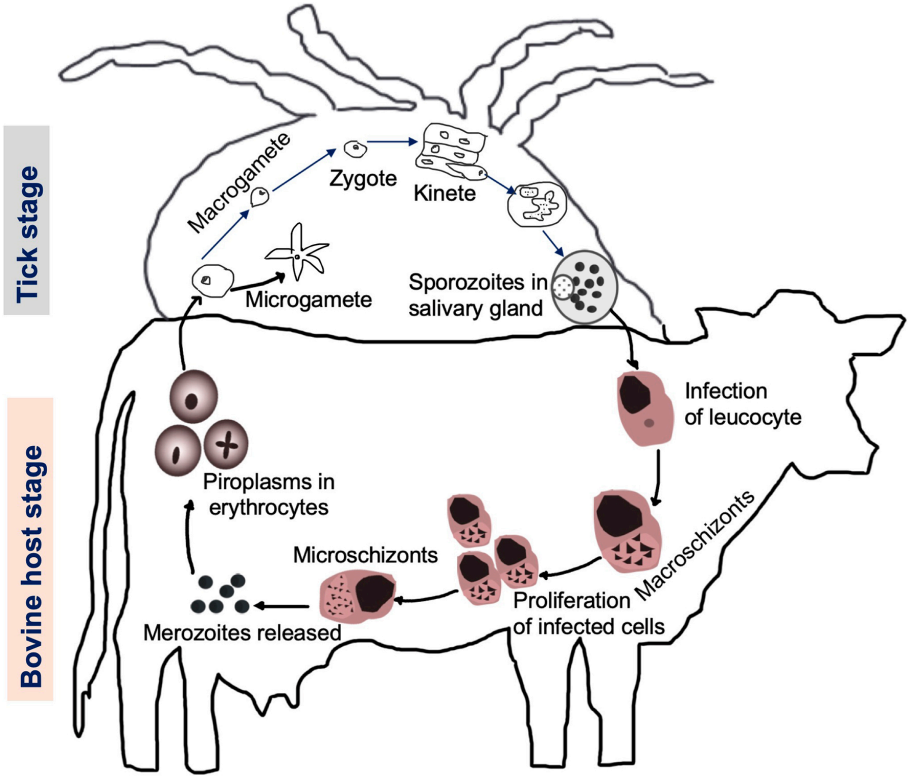

Figure 2: Life cycle of *Theileria annulata* (credit image: Khawla Elati)

Age, breed, physiological status and breeding system are the main risk factors of TT while gender is not considered as a promoting factor of this tick-borne disease (Bouattour et al., 1996; Flach and Ouhelli, 1992).

This protozoan disease is responsible for severe losses in term of mortality, milk production, weight and costs related to treatment (Ayadi et al. 2016; Gharbi et al. 2006a; Robinson 1982).

Control of the disease is mainly based on the treatment of clinical cases with buparvaquone, a drug that has to be applied at the early stage of the disease, the use of chemical acaricides for tick control or upgrading stables by smoothing their outer and inner surfaces to remove tick shelter sites to control endophilic ticks such as *H. scupense* (Gharbi et al. 2011). Despite the availability of control measures the disease is still a serious constraint to livestock productivity. In Tunisia, losses in live weight for subclinical cases were estimated to be 14.7% per case, with a total cost of 4049.570 TD (2515.32 euros) per case and the decrease in mean milk production in lactating carrier cows was estimated to be 0.7 litres per day (Gharbi et al. 2006a, 2015). Previous reports of resistance of Tunisian *T. annulata* strains namely ST2/12, ST2/13 and ST2/19 against buparvaquone (Mhadhbi et al. 2010, 2015), and the occurrence of acaricide resistance in *Hyalomma* ticks (Gaur et al. 2016; Shyma et al. 2012), are cause for concern that the impact of TT may increase in endemic regions and particularly on cattle smallholder's livelihood.

Due to the veterinary and economic importance of the disease, the use of a live attenuated vaccine in endemic countries is justified. In this context, attenuated vaccines were successfully developed in countries such as Iran, India, China and Turkey (Onar 1989; Shkap et al. 2007; Yin et al. 2008), as well as African countries such as Morocco (Ouhelli et al. 1989) and Sudan (Saaid et al. 2020; Sharieff et al. 2017). In Tunisia, the attenuated schizont infected cell line 'Beja' was developed and used at passage 280 to immunize cattle under field conditions (Darghouth 2008). This vaccine proved to be particularly effective when applied to control TT in regions of endemic stability and thus has great economical potential in small dairy herds in Tunisia (Gharbi et al. 2006b). However, in contrast to the high level of protection recorded in endemic situations with a low to moderate infection pressure, live attenuated vaccines are not sufficiently effective against heavy experimental heterologous challenge (Darghouth, 2008; Darghouth et al., 1996). This situation suggests the potential for enhancing the vaccine efficacy to protect animals in situations of substantial tick infestations, a scenario frequently observed in certain regions of Africa.

The improvement of vaccines against TT relies on the use of experimental animals and the production of live attenuated vaccines requires the use of animal sera, particularly foetal bovine serum (FBS). In addition, the maintenance of *Hyalomma* tick colonies for studies on TT and tick-pathogen interactions also requires experimental animals. The dependency on the use of animals and animal products are of both ethical and biosafety concerns. Further 3Rs (Reduce, Refine, Replace) studies are therefore needed to develop new experimental models with reduced animal use to study vector-pathogen interactions, and to develop, characterise and produce attenuated vaccines. The 3Rs principle for humane animal experimentation aims to Replace, Reduce and Refine the use of experimental animals in scientific research. The term was introduced by Russel and Burch, (1959) and aims to improve

animal welfare in studies using animal models. This principle provides an ethical framework within which all experiments involving laboratory animals must be conducted and encourages research into alternatives.

The 3Rs concept is explored in the four main chapters of this thesis, each of which represents a different approach to the application of the 3Rs principle in *Theileria* research. These approaches are introduced in **Chapter 1** by a review article on the current status of tropical theileriosis in Africa, its economical and veterinary impact, available control options and their limitations that justify the development of novel tools to study and develop new TT control options. **Chapter 2** focuses on the evaluation of a serum-free medium for the development and production of different *T. annulata*-infected cell lines to replace the use of FBS in *T. annulata* cultivation. The results were compared with cell lines cultured in RPMI 10% FBS, the classic complete medium.

Chapter 3 presents the results of a comparative transcriptome analysis of virulent and attenuated passages of *T. annulata*-transformed bovine leukocytes (Beja strain, Tunisia). This study highlighted several important genes that showed changes in their expression profiles upon attenuation and could be further investigated as potential attenuation markers. This may improve our knowledge of the attenuation process in *T. annulata* and contribute to the 3Rs principle by replacing/reducing the use of animals required for cell line attenuation validation.

In **Chapter 4**, the generation of infected erythrocytes *in vitro* is described. Combined with the artificial feeding of *Hyalomma* ticks, *T. annulata*-infected erythrocytes may serve as source material for the *in vitro* infection of ticks, which could lead to a completion of the life cycle of *T. annulata in vitro* in the future. This would also contribute to the 3Rs principle of humane animal research by reducing the use of animals required for this purpose. We describe a method to induce merogony in the Ankara *T. annulata* cell line, followed by the infection of red blood cells (RBCs) with the generated merozoites or microschizonts to obtain piroplasm-containing erythrocytes. The invasion of *T. annulata* merozoites into RBCs was confirmed by three techniques. This model could help researchers to understand the invasion process as well as the molecular mechanisms that control the interaction between the parasite and host cells.

In **Chapter 5**, we adapted an *in vitro* feeding method originally developed for three-host ticks such as *Ixodes ricinus* for the successful feeding of all life stages of two- and three-host *Hyalomma* ticks (*H. excavatum, H. dromedarii* and *H. scupense*) that are vectors for *T. annulata*. This system may be useful for the maintenance of tick colonies and the *in vitro*

infection of *Hyalomma* ticks with cultured *T. annulata* piroplasms, thus closing the *T. annulata* life cycle *in vitro*.

The final chapter of the thesis, **Chapter 6,** presents a general discussion and synthesis of the presented findings. It discusses the advantages and limitations of each of the studies presented in this thesis and ways to further improve the developed methods.

Chapter 1:

Literature review

Current status of tropical theileriosis in Northern Africa:

A review of recent epidemiological investigations and implications for control

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The content of this chapter was published in *Transboundary and Emerging Diseases*.

Transbound Emerg Dis. 2020;67(Suppl. 1):8–25. doi: 10.1111/tbed.13312

Abstract

Tropical theileriosis caused by the apicomplexan hemoparasite *Theileria annulata* is a tick-borne disease that constraints livestock production in parts of Europe, Asia and Africa. Four *Hyalomma* tick species transmit *T. annulata* in at least eight African countries (Mauritania, Morocco, Algeria, Tunisia, Egypt, Sudan, South Sudan and Ethiopia). The two dominant *T. annulata* vector ticks present in Africa, *H. scupense* and *H. anatolicum*, underlie two different patterns of transmission, which in turn greatly influence the epidemiology of tropical theileriosis. *H. dromedarii* and *H. lusitanicum* are also capable of transmitting *T. annulata* in North Africa, but their roles are associated with specific production systems and agroecological contexts. The emergence of resistance to the most widely used theilericidal compound, buparvaquone, continues to limit the effectiveness of chemotherapy. In addition, acaricide use is increasingly becoming unsustainable. Deployable *T. annulata* attenuated live vaccines established from local strains in Tunisia, Sudan and Egypt are available, and recent work has indicated that these vaccines can be protective under conditions of natural transmission. However, vaccination programmes may vary over space and time due to differences in the prevalence of disease amongst cattle populations, as well seasonal variation in vector activity. We review recent descriptive and analytical surveys on the epidemiology of *T. annulata* infection with reference to (a) demographic aspects such as breeds and ages of cattle herds previously exposed to distinct *T. annulata* infection pressures and (b) seasonal dynamics of tick activity and disease transmission. We then discuss how the wider endemic patterns that we delineate can underpin the development and execution of future vaccination programmes. We also outline options for integrated control measures targeting tick vectors and husbandry practices.

1. Introduction

The African continent covers a surface of 30.7 million km^2 representing 20.4% of the earth's total land area. The continent presents a huge variety of climates according to Köppen–Geiger climate classification (Peel, Finlayson, & McMahon, 2007). Livestock is one of the fastest-growing sectors in agriculture and represents one of the most important resources for the livelihood of rural people in Africa (Thiec, 1996). Africa breeds about 14% of the world's cattle population but contributes only 3% of world milk and 16% beef productions (Herrero, Havlik, McIntire, Palazzo, & Valin, 2014). The low productivity of the African livestock sector is globally due to poor livestock management and also to the occurrence of several health constraints and in particular arthropod-borne endemic infections (Minjauw & McLeod, 2003). This situation is exacerbated by climate change and anthropogenic factors. Such changes also affect vectors that transmit animal and human pathogens and create suitable conditions for the

emergence and re-emergence of different diseases including those transmitted by ticks. Tropical theileriosis is one of the most important tick-borne diseases affecting different cattle breeds (taurine and zebu cattle) as well as buffaloes (Darghouth, Preston, Kilani, & Bouattour, 2011). The disease is a serious impediment to livestock development in several African and Asian countries causing huge economic losses (Cicek, 2009; El Hussein, Majid, & Shawgi, 2004; Gharbi, 2006; Gharbi et al., 2011; Inci et al., 2007; Minjauw & Mcleod, 2003). The epidemiology of tropical theileriosis is complex as it involves mammalian host, pathogen and vector interactions. In turn, these interactions are influenced by livestock production systems, their agroecology and the political and socio-economic environments. It is worth mentioning that the epidemiology of tropical theileriosis has received much attention as exemplified by the range of studies including both published work and grey literature in the form of scientific and technical reports.

Accordingly, the present review analyses issues related to the vector ticks, the prevalence and distribution of the disease, the transmission dynamics, endemic states and socio-economic impact of theileriosis. The outcome of this analysis is expected to provide scientific basis for future vaccination programmes and other control measures.

2. Tick vectors

In Africa, the vectors of *T. annulata* belong to four *Hyalomma* tick species: *H. scupense, H. anatolicum, H. dromedarii* and *H. lusitanicum*. The role of these ticks in transmission of *T. annulata* under experimental conditions is well documented (Mustafa, Jongejan, & Morzaria, 1983; Ouhelli, 1985; Ouhelli & Pandey, 1984; Schein, Büscher, & Friedhoff, 1975). Both feeding tropism and plasticity in response to changes in the ecosystems are known to influence the vector capacities of *Hyalomma* ticks under conditions of natural transmission for *T. annulata* as observed in Mauritania (Jacquiet et al., 1990, Table 1). A detailed description regarding the occurrence of these vector tick species in Africa and their biological characteristics are summarized in Tables 1 and 2, respectively.

Table 1: *Theileria annulata* vector tick species present in the eight Africans countries endemic for tropical theileriosis

Country	Vector tick	Host	References
Mauritania	*H. dromedarii*	Dromedaries Cattle	Jacquiet et al. (1990, 1994)
Morocco	*H. scupense* *H. lusitanicum*[a]	Cattle	Ouhelli and Pandey (1982), Ouhelli and Flach (1990), Flach, Ouhelli, Waddington, Oudich, and Spooner (1995), Laamari, Mrifag, Boukbal, and Belghyti (2012)

Algeria	*H. scupense* *H. lusitanicum*[a]	Cattle	Sergent et al. (1924), Benchikh-Elfegoun, Benakhla, Bentounsi, Bouattour, and Piarroux (2007), Benchikh-Elfegoun, Gharbi, Djebir, and Kohil (2013)
Tunisia	*H. scupense*	Cattle	Bouattour et al. (1996), Bouattour, Darghouth, and Daoud (1999), Gharbi and Darghouth (2014)
Egypt	*H. anatolicum* *H. dromedarii*[a]	Cattle and water buffaloes Dromedaries	El Kammah et al. (2001), Al-Hosary, Ahmed, & Seitzer (2015), Al-Hosary, Ahmed, Nordengrahn, & Merza (2015), Youssef et al. (2015)
Sudan	*H. anatolicum* *H. dromedarii*[a]	Cattle Cattle	FAO (1983), Ali et al. (2006), El Hussein et al. (2012), Ahmed *in* Guma, Hussien, Salih, Salim, & Hassan, 2015, Mohamed et al. (2018)
South Sudan	Not identified	Cattle	Salih, El Hussein, Kyule et al. (2007), Salih, El Hussein, Seitzer, & Ahmed (2007)
Ethiopia	*Hyalomma* spp. (species not identified)	Cattle	Gebrekidan et al. (2014)

3. Agroecological zones and transmission patterns

Reports of tropical theileriosis are available for Morocco and Algeria (Sergent et al., 1924), Tunisia (Ducloux, 1905), Egypt (Mason, 1922; Nagaty, 1947) and Sudan (El Hussein, Hassan, & Salih, 2012; El Hussein, Mohamed, Osman, & Osman, 1991). More recently, disease cases have been reported in Mauritania (Jacquiet et al., 1990), South Sudan (Salih, El Hussein, Kyule et al., 2007; Salih, El Hussein, Seitzer, & Ahmed, 2007) and Ethiopia (Gebrekidan et al., 2014). Although *T. annulata* infections have also been reported from Eritrea and Libya (Carpano, 1930), these nonetheless need to be confirmed.

On the basis of the Köppen–Geiger climate classification (Peel et al., 2007), a variety of climates can be distinguished from these *T. annulata* endemic regions. These include the following: (a) warm Mediterranean climate occurring in the Northern parts of Morocco, Algeria, Tunisia and parts of Libya, (b) semi-arid climate mainly found in the central parts of Morocco, Algeria, Tunisia and in restricted zones of Libya, as well as in Eastern Ethiopia and in a region extending over southern parts of Sudan and norther parts of South Sudan, (c) desert climate, either of cold or warm type, which extends over large parts of Morocco, Algeria, Tunisia and Libya, to Mauritania, the Nile Basin zone including Egypt and the larger part of Sudan, the African red sea shores and nearby Ethiopian eastern regions, and (d) a variety of different climates such as tropical, subtropical, equatorial, monsoon and oceanic types, prevailing in the Central to Southern parts of the continent (Figure 2).

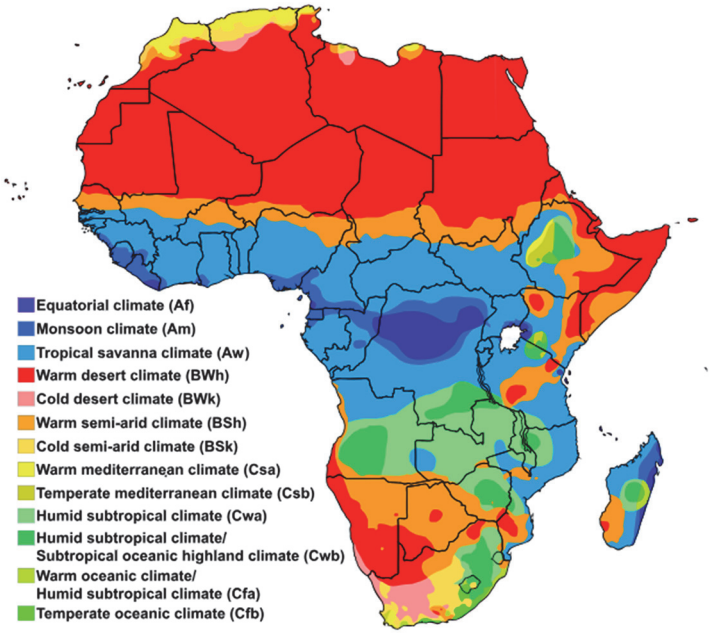

Figure 1: Köppen-Geiger climate type map of Africa (https://commons.wikimedia.org/wiki/File:Africa_Köppen_Map.png)

3.1. Mediterranean pattern of transmission of *T. annulata* in Africa

Relatively cold winters and hot summers with rainfalls occurring mainly in autumn and winter seasons define the Mediterranean climate of North Africa. Under these conditions, tropical theileriosis is typically a summer disease. Its transmission dynamics depend on moderate tick infection rates and intensities, and the moderate to low tick burdens. Taking these together, the infection pressure seems to be low for cattle (Bouattour, Darghouth, & Ben, 1996; Darghouth, Bouattour, Ben Miled, Kilani, & Brown, 1996; Ziam et al., 2015). In this region, disease cases are observed between May–June and August–September (Bouattour et al., 1996; Boussaadoun, Gharbi, Sayeh, Soudani, & Darghouth, 2015; Elfegoun, Gharbi, Merzekani, & Kohil, 2018; Ziam et al., 2016), where the number of cases is highest approximately two weeks after the peak activity of *H. scupense* adults (Bouattour et al., 1996). A similar pattern of distribution of

confirmed clinical cases of tropical theileriosis has been observed in the region of Sidi Thabet (Tunisia) over a period of 16 years (Fatnassi, 2010). Nevertheless, the disease season seems to be more spread out from May to October in the littoral Gabes Oases in Southern Tunisia, (Gosrani, 1999). In this context, several aspects of the biology of *H. scupense* are relevant to this pattern of transmission. These include (a) absence of overlap between the activity periods for adults (June–August) and immature ticks (September–November, Bouattour et al., 1996; Gharbi et al., 2013), (b) long hibernation periods, (c) one tick generation per year as evidenced by a single and short season of adult tick activity in summer (Bouattour et al., 1996; Gharbi et al., 2013), (d) dominant domestic behaviour with the non-parasitic stages sheltering in farming

structures (barns, sheds and pens), (e) a marginal, but monotropic, population infesting cattle outside farming structures including grazing areas and rangelands, emphasizing hence the risk of re-emergence in barns (Bouattour, unpublished data) and (f) very selective trophic preference to cattle suggesting that acaricide use can be restricted to cattle.

Hyalomma lusitanicum has been shown to be a natural vector of *T. annulata* in Spain (Viseras, Hueli, Adroher, & García-Fernández, 1999). However, its role as a *T. annulata* vector in North Africa remains unclear since it is not widely distributed in Africa, with the exception that it has been found in specific locations in Algeria and Morocco (Table 2). If it turns out to be a vector for *T. annulata* in North Africa, it's potentially transmission season would be longer than that noted with *H. scupense*, since adult *H. lusitanicum* are active from May to September. *H. lusitanicum* infestation of cattle occurs outside the housing directly on pastures and rangelands, where there are reservoir hosts for juvenile and adult ticks (wild mammals and other ungulates, Walker et al., 2003). Due to this behaviour, this tick species is more difficult to control than *H. scupense*. Furthermore, the finding of unfed adults of *H. lusitanicum* on cattle from November to May might indicate that this tick is able to hibernate on cattle (behavioural diapause) under the Mediterranean climate (Yousfi-Monod & Aeschlimann, 1986).

3.2. A transmission pattern adapted to arid and desert climates

Hyalomma anatolicum is the major vector in the vast endemic regions of Africa experiencing arid or desert climates (Walker et al., 2003). *H. anatolicum* adapts easily to animals housing, where it behaves as a domestic monotropic three hosts tick closely associated with farming structures and feeding at all stages on cattle (Walker et al., 2003). However, this tick retains an important wild reservoir population with telotropic behaviour. In arid regions with relatively cold weather, *H. anatolicum* could exhibit morphogenetic and behavioural diapauses according to the temperature (Walker et al., 2003).

In African regions where the tick is active across the year, high infestation burdens are recorded on cattle particularly in crossbred and pure-bred exotic cattle (Walker et al., 2003).

This vector is abundant in Sudan (Ali et al., 2006; El Hussein et al., 2012; FAO, 1983) and Egypt (Al-Hosary, Ahmed, Nordengrahn, & Merza, 2015; El Kammah, Oyoun, El Kady, & Shafy, 2001) where it is responsible for the transmission of tropical theileriosis. Elsewhere, the distribution of this tick species remains limited to semi-arid and arid regions of Algeria and Morocco where it does not seem to act as a natural vector of tropical theileriosis. This is probably because of the occurrence of a wild cycle where immature ticks are essentially feeding on small mammals. There is still a need to better understand the adaptation of *H. anatolicum* to a domestic behaviour and its role as a natural vector of *T. annulata* in other

North African countries. As opposed to *Hyalomma scupense* in the Mediterranean transmission pattern, *Hyalomma anatolicum* is active along the year in the main endemic arid and desert regions of North Africa where seasonal and daily environmental changes are limited as in Sudan and to some extent in Egypt (Mohammed et al., 2018; Walker et al., 2003). Furthermore, adults and juveniles of this tick species may be present simultaneously on cattle over long periods as observed in Northern Sudan from September to March (Ahmed, El-Hussein, & El-Khider, 2005). This results in increased risks of exposure of crossbred and pure-bred exotic cattle to high infection pressure (Table 2). In different regions of Upper and Lower Egypt, the infection is more common during the hot season (from April to September; Abdel-Rady, Kot, Mahmoud, & Ellah, 2008; Abou-ELHassan, 1997; Al-Hosary, 2009, 2013; El Masry, Dessouky, & Abo-El Kheir, 2006; Fadly, 2012). However, the transmission of *T. annulata* may be maintained over the year as reported by Al-Hosary et al. (2018) in the Egyptian Oases. Similarly, a retrospective study conducted in Sudan from 1983 to 2001 showed that tropical theileriosis clinical cases are reported throughout the year with a bimodal distribution pattern (Zakia, Rahman, & Osman, 2003). Interestingly, the first peak was observed during the hot–dry summer season (May– July) and is thought to be related to the stress caused by extremely high temperature. However, the second peak occurred during the dry cold winter season (November–January) and is associated with higher tick vector activity (El Ghali & El Hussein, 1995; Zakia et al., 2003).

The ability of *H. anatolicum* to adapt to different types of arid to desert climates results in the occurrence of tropical theileriosis along the year according to a north–south gradient of decreasing seasonal and daily changes. This adaptation offers the tick a significant comparative advantage over other tick species under the predicted scenarios for climate change (United Nations Economic and Social Commission for Western Asia, 2017), particularly in the context of North African countries where this tick species might gain importance in the future.

Hyalomma dromedarii is also common in arid and desert regions where camels are found. This tick species is one of the several *Hyalomma* species capable of experimentally transmitting *T. annulata* (Mustafa et al., 1983; Ouhelli, 1985; Robinson, 1982). As detailed in Table 1, *H. dromedarii* was described for the first time in 1990s as a ‘new’ natural vector of *T. annulata* in Mauritania (Jacquiet, Colas, Cheikh, Thiam, & Ly, 1994; Jacquiet et al., 1990). Subsequently, a report from Egypt implicated this tick in the transmission of *T. annulata* on the basis of molecular detection in camels and microscopic observation of the parasite in the tick salivary glands (Youssef et al., 2015). In addition, in Sudan *H. dromedarii* has also been implicated as possible vector of *T. annulata* on the basis of epidemiological observations (Ahmed in Guma et al., 2015; Mohammed et al., 2018) and experimental findings (Mustafa et al., 1983). However, it remains to be clarified whether these ticks can transmit the parasite to

cattle. This can be accomplished by isolation of *T. annulata* sporozoites from infected ticks and subsequent in vitro infection of bovine mononuclear cells or in vivo infection of naive cattle. An important issue common to both transmission dynamics relates to the evolution of transmission patters under the influence of climate change and other anthropogenic factors affecting the agroecosystems (Box 1). Indeed, changes occurring at the level of the agroecosystems could affect the population dynamics of the domestic or/and wild preferred hosts for immature and adult tick stages.

Competent vector tick species could adapt to these changes, by evolving to monotropism with a preference for large ungulates and specifically cattle, resulting in suitable biological conditions for the expression of the vector capacity for *T. annulata*. Furthermore, introduction of new husbandry practices associated with exotic dairy cows and in particular intensive housing may lead tick populations to acquire a domestic or peri-domestic behaviour as reported with *H. anatolicum* (Walker et al., 2003) and *H. dromedarii* in Mauritania (Jacquiet et al., 1994). Some of these changes are discussed and illustrated by examples in Box 1. In this context, it is important to carry out further studies on the drivers for emergence and invasion/ regression processes involving the four *Hyalomma* vectors of *T. annulata* and to model the putative distribution of these vectors under different plausible change scenarios expected in the endemic African regions.

Box 1: Main drivers of change for vector ticks distribution under the context of the endemic African countries for *Theileria annulata*.

The risks of emergence of new *Hyalomma* tick species capable of transmitting *T. annulata* in Africa have not been systematically investigated. Although the majority of *Hyalomma* species present in Northern Africa may be competent for transmitting *T. annulata*, their vector capacity under natural conditions depends on feeding preferences. Accordingly, the risks of emergence of new vector *Hyalomma* species in Africa depend on the evolution of the tick feeding behaviour. This evolution could occur under three different scenarios:
(1) When endemic competent tick species which were not previously transmitting *T. annulata* under field conditions evolve to monotropism with a preference for cattle in response to modifications in the population dynamics of their preferred hosts resulting from changes in local agro-ecological systems. The establishment of a transmission cycle with such new vectors could happen if tropical theileriosis is already endemic in the region or when carrier animals are introduced in the cattle population due to animal movement but in the absence of previously established transmission cycles. This scenario has been observed for *H. dromedarii* in southern Mauritania (Jacquiet et al., 1994) and *H. lusitanicum* in Spain (Viseras et al., 1999) and could potentially occur with *H. anatolicum* and *H. lusitanicum* in Algeria and Morocco particularly in cattle farms where these two potential vector species cohabit with the *T. annulata* vector tick *H. scupense*. Similar risks are possible with *H. dromedarii*, since its engorged nymphs can be found on cattle maintained nearby dromedaries as has been the case in the continental Oasis of Hazoua (Southern Tunisia; Hniche, 2006).
(2) When an invasive and competent tick species establishes successfully in a new zone already endemic for *T. annulata*. The expression of the vector capacity of the invasive tick species requires that this tick adapt to monotropism with a preference for cattle. This situation started to develop in 2000s in Tunisia, when *H. dromedarii* established successfully in a farm of the Sidi Thabet village in an endemic region for *T. annulata* (North of Tunisia).

The tick, which was introduced with camels coming from central Tunisia adapted easily to feed on available domestic ungulates. Extensive acaracide treatments were necessary to eradicate it from the animal buildings and surrounding areas (unpublished data). These observations illustrate clearly the potential of *H. dromedarii* to invade and establish successfully around animal housing not only in the oases but also in the unusual context of the upper semi-arid Mediterranean climate with cold winters in Northern Tunisia. For these reasons, *H. dromedarii* should be regarded as a potentially emerging *T. annulata* vector species in Africa that may have strong competitive advantages over the endemic tick species if the aridity of the region will increase under the effect of climate change.

(3) The spread, under the influence of several driving forces (see below), of known natural vector ticks and the pathogen via animal movements to non-endemic zones. The discovery of tropical theileriosis in Mauritania is one of the best example illustrating this scenario. A disease outbreak occurred in Nouakchott following the introduction of Moor zebus from South Trarza region into herds of dairy cows imported from France (Jacquiet et al., 1990, 1994). The South Trarza region (Senegal river valley) was already endemic for *H. dromedarii* and *T. annulata*. It is possible that *H. dromedarii* has been transmitting tropical theileriosis in the Senegal River Valley for a long time, but this had not become clear due to the state of endemic stability associated with the local zebu cattle production system.

Additional recent field reports have confirmed the extension of *Hyalomma* vector ticks for *T. annulata* to new regions. In Sudan, *H. anatolicum* has been recorded southward below 14°N (El Ghali & Hassan, 2012) and also northward beyond Khartoum region, which is its usual northern limit (El Hussein et al., 2012; A. R. El Hussein, unpublished data). Similarly, in Southern Tunisia few specimens of *H. scupense* were collected from cattle in the littoral Oasis of Gabes (Gosrani, 1999) where a low incidence of tropical theileriosis was recorded. This tick was also found in few farms of the continental Tunisian Oasis of Hazoua, albeit not in association with tropical theileriosis cases (Hniche, 2006). In both oasis, cattle are typically introduced form the Northern regions of Tunisia where *H. scupense* is endemic.

It should be emphasized that emergence of new *Hyalomma* vectors for *T. annulata* in Africa will depend on the existence of enabling conditions, particularly the presence of basic reproduction ratios (Ro) levels for both of the vector tick and the transmitted pathogen that are compatible with their successful establishment under new transmission patterns or within a new region. This Ro has been expressed in an equation incorporating the following key variables adapted to the transmission contexts of *T. annulata* in Africa: (a) Density of ticks; (b) Probability of a tick to feed on *T. annulata* carrier cattle; (c) Transmission efficiency of *T. annulata* by the new vector; (d) Interstadial vector tick survival rates; (e) Ticks birth rates; (f) Numbers and density of *T. annulata* carrier cattle; (g) Cattle mortality rates; and (h) Rate at which carrier cattle lose their infectivity (Randolph In Ogden & Lindsay, 2016). Taking into account the frequency of the cattle carrier state for *T. annulata* and its long duration in endemic regions, variables 1–6 appear to be the most critical ones in the case of *T. annulata*.

It should also be emphasized that records in the same sites of non-endemic tick species, which are, isolated and not reproducible over time and with the different tick instars, should not be overlooked as they most likely correspond to unsuccessful establishment of the tick in the new habitat. This seems to be the case with the single *Hyalomma anatolicum* specimen recorded in 1970 by Van Den Ende in Southern Tunisia.

The occurrence of new transmission patterns for *T. annulata* is also conditioned by anthropogenic factors such as livestock policies, new economic opportunities and trade, eco-climatic and insecurity crises resulting in forced livestock migration, changes in production.

Table 2: Distribution and main biological characteristics of the four *Hyalomma* tick species transmitting *T. annulata* in Africa (the general information on the ticks biology and distribution are taken from Estrada-Peña, Pfäffle, & Petney, 2017 and Walker et al., 2003).

	H. anatolicum	***H. scupense***	***H. dromedarii***	***H. lusitanicum***
General distribution in Africa	Mainly in desert and semi-arid climates particularly in Sudan, Egypt, and Somalia, with foci in Algeria (Benchikh-Elfegoun et al., 2013; Benchikh-Elfegoun et al., 2018), and Morocco (H Ouhelli and Pandey, 1982; Sahibi et al., 1998). Previously signalled in Libya, however an extensive three years survey on thousands of animals did not confirm (Gabaj et al., 1992).	Mainly in warm Mediterranean climate regions: Morocco, Algeria, Tunisia, possibly in parts of Libya, with an extension to warm desert climate arid and desert regions of North-Central Sudan and in few locations in Egypt.	All regions of Africa with desert climate northern to the equator: Mauritania, Morocco, Algeria, Tunisia, Libya, Egypt, Sudan, Eritrea, Djibouti, Somalia, Ethiopia, Chad, Mali.	North of Morocco and Eastern Mediterranean region of Algeria.
Transmission of *T. annulata*	Major natural vector in Egypt (El Kammah et al., 2001; Al-Hosary et al., 2015) and Sudan (FAO, 1983). No evidence up to date of any role in the transmission of tropical theileriosis in Algeria and Morocco.	Major natural vector in Morocco, Algeria (Sergent et al., 1928) and Tunisia.	Major natural vector in Mauritania (Jacquiet et al., 1994, 1990).	Major natural vector in Spain (Viseras et al., 1999). Potential secondary vector in parts of Morocco and Algeria.

Life cycle and hosts	i/ Tick species with a strong biological adaptation to arid conditions, ii/ Three hosts domestic monotropic cycle on cattle, with however a possible telotropic behaviour, iii/ Feeding on cattle as well as other ungulates and dromedaries iv/ Possibility of a two hosts ditropic cycle on hares. v/ Endophilic tick species with frequent adaptation to a domestic behaviour associated to animal housing with a moulting in the buildings fabrics,	i/ Two hosts tick, ii/ Preference for large ungulates particularly cattle with a dominant monotropic behaviour, also feeding on horses, much less frequent on other herbivores, iii/ Endophilic tick with a domestic behaviour and close association to animal housing, the non-parasitic stages are hiding in cracks and crevices. Long diapause of engorged nymphs during the winter.	(i) Most commonly two hosts life cycle with immatures feeding on camels, small and medium size mammals, birds and reptiles. Three hosts life cycle less frequent with a telotropic behaviour, ii/ Preferences for camels, but easily feeding on others herbivores (cattle, small ruminants and horses), iii/ Exophilic tick highly adapted to extreme dryness, life cycle established around animal barns.	i/ Adults feeding on large and medium size mammals and particularly cattle, immatures mainly feeding on lagomorphs, ii/ Exophilic tick species with a three hosts life cycle.
Activity periods in Africa	Along the year when seasonal and daily environmental changes are limited as for instance in Sudan and to some extent in Egypt; adult ticks activity in the summer under cold arid climate (Walker et al., 2003; Mohammad et al., 2018).	Under cold Mediterranean climates the adults are active during the summer with a peak in July and the larvae and nymphs are active during the autumn. This dynamic is leading to the completion of one cycle per year (Gharbi and Darghouth, 2014).	Along the year when seasonal and daily environmental changes are limited, decreased tick activity during the winter under cold arid climate (Gharbi et al., 2013).	The presence of active juveniles for May to September is consistent with adults activity period from April to September-October with a peak in spring (Laamari et al., 2012; Rahali, 2014).
Infection rates and intensities with *T. annulata*	Variable data recorded in Sudan with infection prevalence ranging from 8.6 to 86%, and infection intensities (average number of infected salivary gland acini/infected tick) of 1.3 and 37% (Walker et al., 1982) (Salih et al., 2007).	Low to moderate vector infection rates of 12.8-21.8% (Bouattour et al, 1996) (Flach and Ouhelli, 1992, Flach et al, 1992); and low infection intensities of 5.32 with 62% of infected ticks having 1-2 infected acini in Tunisia (Bouattour et al, 1996).	A *T. annulata* specific PCR revealed 73 and 57% positive H. dromedarii ticks form two Mauritanian sites (d'Oliveira et al. 1997).	Transmission of *T. annulata* by H. lusitanicum not documented in Africa.

Tick abundance and prevalence in Africa	One of the most abundant species in Egypt (Al Hosary et al., 2018) and Sudan (Ahmed et al., 2005; El Ghali et al,. 2012). Reported as a minor tick species on cattle in semi-arid and arid regions of Morocco (Ouhelli and Pandy, 1983, Sahibi et al., 1998) and Algeria (Benchikh Elfegoun et al., 2013) with up to 7.3 % of total tick counts. Never recorded in Tunisian at the exception of a single specimen collected in the South (Van Den Ende, 1970). Likely occurrence of a wildlife cycle in North Africa.	One of the most abundant cattle tick species in semi-arid and sub-humid to humid regions in Morocco, Algeria and Tunisia when barns conditions are suitable for the tick (Rahali et al., 2014, Bouattour et al., 1996), seems less frequent in humid regions of Algeria (Benchikh Elfegoun et al., 2013). Recorded on few occasions in Sudan ((Hoogstral, 1956 in El Ghali and Hassan 2012) (Mohammed et al., 2018).	Adult ticks recorded on cattle in Libya at an infestation prevalence of 40% (Gabaj et al. 1992). Engorged nymphs collected from cattle maintained close to camels in the Tunisian continental oasis of Hazoua (Hniche, 2006).	H. lusitanicum is more frequent in humid and sub-humid regions having a meso-mediterranean vegetal cover with up to 10,7% of ticks collected from cattle (Laamari et al., 2012), much less frequent under arid climate where it only accounted for 1.09% of the collected ticks (Benchikh Elfegoun et al., 2013). Not reported up to date in potentially suitable bioclimatic regions of Northern Tunisia although of having been described in an Algerian border region to Tunisia (Benchikh Elfegoun et al., 2006 and 2013).

4. Recent epidemiological investigations into tropical theileriosis in North Africa

4.1. Prevalence of *T. annulata* infections

As shown in Table 3, the lowest prevalence values noted in all the endemic countries were below 10%, irrespective of the detection assays used. On the other hand, the highest prevalence for each country was above 50% and, in some cases, was as high as 90% (Bouattour et al., 1996, Darghouth et al., 2004; Gharbi et al., 2006; Salih et al., 2007).

The broad similarities in the prevalence values in the studied countries could be explained by: (i) widespread introduction of Holsteins either as purebred or through cross breeding policies to support the increasing demand for milk products, (ii) increased cattle movements due to insecurity and improved transportation, (iii) changes in climate towards increased and extended aridity over the region affecting vector distribution and activity. In addition, a significant positive association of infection prevalence and cattle age has been recorded in several endemic countries such as Mauritania (Jacquiet et al., 1994), Morocco (Aithamou et al., 2012), Tunisia (Darghouth et al., 1996), Sudan (Abaker et al., 2017, Salih et al., 2007), and to a certain extent in Algeria (Ayadi et al., 2017). However, no such associations were identified in Egypt (El-Masry et al., 2006; Al-Hosary et al., 2013; Al-Hosary et al., 2018)

The observation made in Egypt and Mauritania suggests a possible genetic background for susceptibility of certain breeds to *Theileria* infection. The finding that Egyptian buffaloes show lower *Theileria* infection prevalence compared to cattle (Table 3) and the record of moderate seroprevalence in Mauritanian Moor zebu cattle associated to the absence of disease cases (Jacquiet et al., 1994), are together supporting this assumption. Innate immune mechanisms against *T. annulata* (Ahmed et al., 2008; Echebli et al., 2014) may have a role to play in view of the emerging concept of trained or memory innate immunity (Netea and van Crevel, 2014). Further investigations are nonetheless required to ascertain its relevance, as well as to analyse the role of the above cattle breeds susceptibility to *Hyalomma* ticks.

The economic impact of tropical theileriosis is highly noticeable in endemic regions of Africa and in particular in the more intensive production systems based on exotic breeds and their crosses. The mean daily live weight gain in naive crossbred calves at primary infection was found to be reduced by an average of 14.7% (Gharbi et al., 2006). Furthermore, the highest proportion of the economic losses is due to subclinical forms of infection (Gharbi et al., 2006). In carrier purebred dairy cows, a mean milk yield decrease of 0.7 litres/day was recorded (Gharbi et al., 2015). In Algeria, milk yield losses in crossbred cows treated for tropical theileriosis were estimated to an average of 2.76 litres/day/cow over a period of 63 days (Ayadi et al., 2016). In Egypt, tropical theileriosis causes productivity losses in local cattle breed and water buffaloes and case fatalities especially in exotic cattle breeds (Michael et al., 1989). In

Sudan, *T. annulata* infection is associated with huge production losses ranging between 4 to 6 million US dollars in the Khartoum province (Latif, 1994), in the same district, the fatality rate in calves and heifers was 22% and 30% respectively (Ali et al., 2006).

Table 3: *Theileria annulata* infection prevalence in cattle in African countries

Country	Region (by alphabetic order)	Technique	Animal species	Positive/examined (%)	Sampling methodology & type of survey	References
Mauritania	Assaba	IFAT	Cattle	51/510 (10)	Stratified random sampling according to cattle population demography Cross sectional survey	(Jacquiet et al., 1994)
	Brakna			11/98 (11)		
	Gorgol			37/187 (20)		
	Guidimaka			11/74 (16)		
	Hodh Gharbi			27/245 (11)		
	Tagant			10/29 (34)		
	Trarza			172/661 (26)		
	Nouakchott	IFAT	Cattle	4/49 (8.16)	Non-probability sampling in an outbreak herd and region Cross sectional survey	(Jacquiet et al., 1990)
Morocco	Al Hajeb	IFAT	Cattle	32/134 (23.9)	Simple random sampling Cross sectional survey	(Aithamou et al., 2012).
	Kenitra			3/123(2.4)		
	Meknès			25/140 (17.9)		
	Sidi Kacem			30/121 (24.8)		
	Sidi Slimane			4/150 (2.7)		
	Doukkala	ELISA	Cattle	745/1819 (41)	Two stages random sampling Cross sectional survey	(El Haj et al., 2002)
	Gharb			491/1819 (27)		
	Haouz			1200/1819 (66)		
	Loukkoss			509/1819 (28)		
	Tadla			855/1819 (47)		
	Doukkala	Blood smears	Cattle	47/97 (48.5)	Non-probability sampling Longitudinal survey	(Flach and Ouhelli, 1992)

Algeria	El Eulma (Sétif)	Blood smears	Cattle	22/134 (16.4)	Simple random sampling	(Ayadi et al., 2017)
		PCR		67/134 (50)	longitudinal survey	
	Algiers	RT-PCR	Cattle	17/41 (41.4)	Double random sampling in farms with history of piroplasmosis Cross sectional survey	(Ziam et al., 2015)
	Ain Defla			6/40 (15)		
	Bejaia			8/39 (20.5)		
	Blida			34/40 (85)		
	Bouira			3/38 (7.9)		
	Boumerdes			20/40 (50)		
	Medea			2/37 (5.4)		
	Tipaza			11/44 (25)		
	TiziOuzou			7/39 (17.9)		
	Eastern Algeria (Annaba and El Tarf)	Blood smears	Cattle	29/54 (53.7)	Non-probability sampling Cross-sectional survey	(Ziam and Benaouf, 2004)
Tunisia	Amdoun (Béja)	IFAT	Cattle	80/292 (27.4)	Stratified random sampling according to cattle population demography Longitudinal survey	(Darghouth, 2000)
	Amdoun (Béja)	IFAT	Cattle	13/192 (6.8)	Non-probability sampling Cross-sectional survey on the same initial sample surveyed by (Darghouth, 2000)	(Jouini, 2006)
	Bizerte	Blood smears	Cattle	114/735 (15.5)	Non-probability sampling in outbreak farms. Cross sectional survey	(Boussaadoun et al., 2015)
	Ariana (Sidi Thabet)	IFAT	Cattle	93/306 (30.4)	Stratified random sampling according to cattle population demography Longitudinal survey	(Darghouth et al., 1996)
	Ariana (Sidi Thabet)	IFAT	Cattle	8/239 (3.3%)	Non-probability sampling	(Ben Nasser, 2009)

					Cross-sectional survey on the same initial sample surveyed by (Darghouth et al. 1996)	
	Ariana (Hessiene)	IFAT	Cattle	50/54 (92.1)	Non-probability sampling targeting herds in endemic stability for tropical theileriosis Longitudinal survey	(Bouattour et al., 1996)
	Ariana (Hessiene)	Blood smears	Cattle	46/72 (63.9)	Non-probability sampling targeting herds in endemic stability for tropical theileriosis Longitudinal survey	(Darghouth et al., 2004)
		IFAT		64/72 (88.9)		
	Ariana (Hessiene)	IFAT	Cattle	(92.8)	Non-probability sampling targeting herds in endemic stability for tropical theileriosis Longitudinal survey	(Gharbi et al., 2006b)
	Gabes Oasis (South East)	Giemsa blood smears	Cattle	27/102 (26.5)	Non-probability sampling Cross sectional survey	(Gosrani, 1999)
		PCR		5/16 (31.2)		
		IFAT		18/18		
Egypt	Menoufia	Tams-1 PCR	Cattle	24/354(6.77)	Non-probability sampling Cross sectional survey	(Elsify et al., 2015)
			Buffaloes	0/33 (0)		
	Behera		Cattle	14/47 (29.78)		
	Giza		Cattle	4/30 (13.33)		
	Assiut	Blood smears	Cattle	32/139 (23)	Non-probability sampling Cross sectional surveys	(Al-Hosary, 2013; Al-Hosary et al., 2016)
			Buffaloes	1/30 (3.33)		
		TaSP ELISA	Cattle	100/139 (71.9)		
			Buffaloes	11/30 (36.6)		

		Tams-1 PCR	Cattle	30/70 (42.8)		
			Buffaloes	1/20 (5)		
		LAMP	Cattle	36/70 (51.4)		
			Buffaloes	8/20 (40)		
		Reverse Line Blot	Cattle	36/70 (51.4)		
			Buffaloes	9/20 (45)		
	EL-Fayoum	Blood smears	Cattle	25/129(19.3)		
			Buffaloes	2/20 (10)		
		TaSP ELISA	Cattle	78/129 (60.4)		
			Buffaloes	10/20 (50)		
		Tams-1 PCR	Cattle	29/70 (41.4)		
			Buffaloes	2/17 (11.7)		
		LAMP	Cattle	33/70 (47.1)		
			Buffaloes	8/17 (47)		
		Reverse Line Blot	Cattle	33/70 (47.1)		
			Buffaloes	10/17 (58.8)		
	Al –Wady Al-Jadid (Egyptian Oasis)	Blood smears TaSP ELISA Tams-1 PCR	Cattle	145/468 (30.9) 340/468 (72.6) 343/468 (73.2)	Non-probability sampling Cross-sectional survey	(Al-Hosary et al., 2015)
		Blood smears PCR	Cattle	393/1068 (37.8) 679/1086 (63.6)	Non-probability sampling Cross-sectional survey	(Al-Hosary et al., 2018)
Sudan	Atbara	PCR	Cattle	36/52 (69)	Non-probability sampling Cross-sectional survey	(Taha et al., 2013)
	White Nile	ELISA	Cattle	50/82 (61)	Non-probability sampling Cross-sectional survey	(Guma et al., 2014)
	Khartoum (Southern Blue Nile)	Blood smears	Cattle	27/162 (16.7)	Non-probability sampling Cross-sectional survey	(Ali et al., 2006)
		PCR		78/162 (48.1)		
		Reverse Line Blot		106/162 (65.4)		
	Nyala (South Darfur)	Blood smears IFAT	Cattle	43/1200 (3.6) 31/100 (31)	Simple random sampling Longitudinal survey	(Gaafar, 2016)
		Blood smears	Cattle	11/150 (7.3)	Non-probability sampling	(Abaker et al., 2017)

		IFAT PCR		70/150 (46.7) 39/100 (39)	Cross-sectional survey	
	Atbara	ELISA	Cattle	32/40 (80)	Non-probability sampling Cross-sectional survey	(Salih et al., 2009)
	Damazin			12/40 (30)		
	El Damer			37/40 (92.5)		
	El Dieum			8/40 (20)		
	El Obied			11/40 (27.5)		
	Fashir			6/40 (15)		
	Gadarif			4/40 (10)		
	Kassala			4/40 (10)		
	Kosti			15/40 (37.5)		
	Madani			5/40 (12.5)		
	Nyala			2/40 (5)		
	Port Sudan			4/40 (10)		
	Rabak			15/40 (37.5)		
	Sennar			16/40 (40)		
	Um Benin			14/40 (35)		
	Sennar	PCR	Cattle	113/180 (63%)	Non-probability sampling Cross sectional survey	(Mohamed et al., 2018)
South Sudan	Central Equatoria State, Southern Sudan	Reverse Line Blot	Cattle	1/600 (0.2)	Simple random sampling Longitudinal survey	(Salih et al., 2007)
Ethiopia	Northern Ethiopia (Humera)	PCR	Cattle	10/525 (2)	Non-probability sampling Cross sectional survey	(Gebrekidan et al., 2014)

4.2. Distribution of *T. annulata* infections

Tropical theileriosis tends to be widespread within the endemic African countries. This is particularly true for Egypt and Sudan, where infection and clinical cases due to *T. annulata* have been recorded over extended areas in these countries. Such widespread distribution has been seen in Lower, Middle and Upper Egypt and also in the Egyptian oases (Al-Hosary, 2009, El-Ballal et al., 1999, Harfoush, 2001). This distribution is to some extent influenced by a number of risk factors such as the geographic areas, management systems and the state of the cattle housing (Salih et al., 2007). In Algeria, Morocco and Tunisia, where a high diversity of bio-climatic zones over relatively limited geographical areas is observed, several surveys have shown a distribution extending to humid, sub-humid, and semi-arid regions (Aithamou et al., 2012; Ayadi et al., 2016, 2017; Bouattour et al., 1996; Darghouth, 2000; El Haj et al., 2002, Rahali, 2014; Ziam and Benaouf, 2004). The relationship between the distribution and the

bioclimatic conditions seems to be complex, high prevalence has been recorded in both semi-arid and sub-humid regions (Ayadi et al., 2016, 2017; Darghouth et al., 1996; Darghouth, 2000; Rahali, 2014). Other surveys have indicated a positive prevalence gradient from sub-humid to arid regions, such as in Morocco (Aithamou et al., 2012). However, a contrasting pattern has been recorded in Algeria. In this context, and in view of the domestic behaviour of *Hyalomma scupense*, the effect of cattle housing on the epidemiology of tropical theileriosis needs to be better investigated, since it represents a relatively isolated microenvironment compartment directly conditioned by management and husbandry practices while being at the same time subject to the effect of the surrounding biophysical environment. This is illustrated by two surveys carried out in Tunisia more than 10 years later on the same farms previously surveyed using the same diagnostic approach in 1991 (Darghouth, 2000; Jouini, 2006) and 1992 (Darghouth et al., 1996; Ben Nasser, 2009). The results summarised in Table 3 are showing a significant decrease in seroprevalence values following an improvement of the housing conditions associated with the adoption of a Holstein based dairy production system.

4.3. Infection dynamics

The susceptibility of cattle to tick infestation and to *T. annulata* (Bakheit and Latif, 2002, Glass *et al.,* 2005) and the biophysical and socioeconomic conditions result in a transmission dynamics gradient ranging from infection of entire herds to the absence of infection. Accordingly, the transmission dynamics of tropical theileriosis show two characteristics endemic states: endemic stability and endemic instability (Darghouth et al., 1996).

4.3.1. Endemic stability

In the state of endemic stability for tropical theileriosis, the majority of the cattle are local breeds. The numbers of ticks and carrier cattle are sufficient to allow early primary infections of calves at few weeks/months of age, and to ensure continuous superinfection. However, disease is rare due to the low susceptibility of local cattle populations to ticks and *T. annulata* (Bakheit and Latif, 2002, Glass et al., 2005). This is also partly due to exposures to limited *Theileria* quantum at primary infection due to significantly lower tick burdens in calves compared to older cattle as observed in Tunisian and Morocco (Bouattour et al., 1996, Flach and Ouhelli, 1992), and to a lesser extent in Sudan (Mohamed et al., 2018).

The persistence of the carrier state induced by *T. annulata* infection allows endemic stability to be maintained even in regions where tropical theileriosis is strictly seasonal as it is the case in Algeria, Morocco and Tunisia. However, the use of susceptible exotic cattle for artificial insemination programs or as progenitor bulls has resulted in significant increase in both the incidence and the severity of clinical cases in crossbred calves in herds initially known to be at endemic stability state for tropical theileriosis (Hussein et al., 2012). This is illustrated by

the rise of the incidence of clinical cases of tropical theileriosis in calves in Tunisia from 7% of usually mild clinical forms to up to 50% with severe acute disease cases in a group of farms in endemic stability following the introduction of Holstein progenitor bulls (Karoui, 2005).

4.3.2. Endemic instability

The state of endemic instability is not homogeneous as it goes through a variety of situations, where according to the probabilities of *T. annulata* transmission, different cattle age categories continue to be exposed to disease risks due the presence of naïve animals (Darghouth et al., 1996). Two distinct endemic instability states, namely moderate endemic instability and the high endemic instability have been recognised. This distinction has been introduced to rationalise vaccination strategies by focusing more on the cattle categories where significant proportions of naive animals are still expected to be present in endemic herds. The two endemic instability states vary with respect to the level of infection prevalence at which dairy cows, heifers and calf bulls are still exposed to significant disease risks (Darghouth et al., 1996, Darghouth et al., 2011). Endemic instability is associated with vector tick burdens that are lower than for endemic stability infection dynamics. This is illustrated, in particular in high endemic instability, by average summer tick abundance as low as 3 *Hyalomma* ticks/cow/year recorded in a survey carried out in farms with previous tropical theileriosis cases (Lebbi, 1992). In Algeria and Tunisia, disease cases are dominantly observed on purebred and crossbred dairy cows (Darghouth et al., 1996, Boussaadoun et al., 2015, Ziam et al., 2016). This observation is attributable to the dominance of exotic dairy cows in the cattle herds and also to the frequency of high endemic instability in infected farms (Darghouth et al., 1996). In Egypt, animals below one year are considered more susceptible compared to animals aged 5 years and beyond that are better protected by acquired immunity (Abdel-Rady et al., 2008, Al-Hosary, 2009; Al-Hosary et al., 2013). Similarly, in Sudan the disease is mainly affecting heifers and, to some extent, calves of up to one year (Latif, 1994, El Hussein, unpublished data). These data point to the dominance of endemic stability and low endemic instability states in these two countries of the Nile Basin.

5. Towards improvement of the control of tropical theileriosis

The analysis of the epidemiological situations of tropical theileriosis in the African countries clearly indicates that there is an urgent need to optimise the available control tools and to design more innovative science-based strategies. The present control measures against tropical theileriosis relies on chemotherapy, tick control using acaricides and improving cattle barns, as well as vaccination with attenuated cell line vaccines (Darghouth et al., 2011, Gharbi et al., 2011, Mhadhbi et al., 2015). Each of these measures has its own benefits, but also constraints. Thus, there is increasing evidence of buparvaquone resistance (see Box 2);

furthermore, risks of acaricide resistance in *Hyalomma* vector ticks should be better investigated in North Africa. Accordingly, vaccination and barn upgrading should be prioritised as they represent control options that are more sustainable and in better accordance with the One Health approach promoted by the "Office International des Epizooties" (OIE), the Food and Agriculture Organisation (FAO) and the World Health Organisation (WHO). These measures have also been shown to have the best economic potential (Gharbi et al., 2011).

Box 2: Resistance of *Theileria annulata* to the naphtoquinone compound buparvaquone

The first specific theilericide treatment was released in 1984 with the discovery of parvaquone, an hydroxynaphthoquinone (HNQ), from 1988, this compound was replaced by buparvaquone (second generation of HNQ) which is a longer bioavailable and, subsequently, more efficient on *T. annulata* (Kinabo & Bogan, 1988; McHardy & Morgan, 1985). Since then, buparvaquone has been, up to date, widely used in endemic regions for treating tropical theileriosis with a cure rate of up to 90.9% (Hashemi-Fesharki, 1991). From 1990s, several cases of treatment failure with buparvaquone were reported in Tunisia (M. Darghouth, unpublished data), leading then to suspect the emergence of *T. annulata* resistance stocks to buparvaquone. This phenomenon was confirmed later in vivo (Mhadhbi et al., 2010), and the resistance mechanism was associated with single point mutations affecting the cytochrome b gene (Sharifiyazdi, Namazi, Oryan, Shahriari, & Razavi, 2012; Mhadhbi, Chaouch, Ajroud, Darghouth, & BenAbderrazak, 2015; Chatanga et al., 2019). However, another study revealed that the mutation of the gene encoding the parasite enzyme PIN1 represents an additional resistance mechanism to buparvaquone, which acts in the absence of the mutation, by inhibiting the proliferation of the *T. annulata* host cell via blocking the PIN1 enzyme (Marsolier et al., 2015). In absence of other alternatives to naphtoquinones, the emergence of resistant strains of *T. annulata* represents a significant danger for cattle breeding in tropical theileriosis enzootic countries. It is therefore imperative to generate new research for the development of novel theilericide drugs including biological product such as for instance juglone (Marsolier et al., 2015), giving at the same time more importance to vaccination using the already available live attenuated vaccines developed in Africa and to improving their delivery system to the field.

Schizont infected cell lines have already been produced in Tunisia (Darghouth et al., 1996, Darghouth, 2008), Morocco (Kachani et al., 2004, Ouhelli et al., 2004) and Sudan (El Hag, 2010, Sharieff et al., 2006, Sharieff et al., 2003). The protection conferred by these attenuated cell lines under experimental conditions depends on the level of attenuation, the dose of sporozoite challenge, and whether the challenge is homologous or heterologous (Darghouth

et al. 1996, Darghouth, 2008). Vaccine development and its use must take into account the prevailing epidemiological contexts. The main epidemiological features of relevance to this issue are detailed according to the infection transmission patterns in Tables 4 and 5.

Table 4: Guidelines for optimal vaccine profile and application practices based on main epidemiological features of tropical theileriosis under the Mediterranean transmission pattern associated with *H. scupense* as illustrated by the Tunisia context (the information detailed in the table is synthesized from Darghouth et al., 1996, Darghouth, Bouattour, & Kilani, 1999, Darghouth et al., 2011, Darghouth, 2000, 2008)

Relevant main epidemiological features of the Mediterranean transmission pattern for *T. annulata* (example of the Tunisian context)	**Parameters to consider for vaccine development and application**	**Corresponding guidelines for optimal vaccine profile and application practices**
Cattle at risks categories by frequency: Dominantly pure-bred lactating cows, then heifers, bull calves and calves	Intrinsic safety	Pure-bred dairy cows represent the critical cattle category in terms of susceptibility to severe infections and importance of economic losses. Accordingly, the vaccine line should be enough attenuated and well tolerated by pure-bred cows at lactation and pregnancy
Strict summer disease occurring from May–June to August–September	Duration of immunity	At least 6–7 months to cover the following tropical theileriosis season considering that cattle are vaccinated in March-April to allow the vaccine induced immunity to be developed before the theileriosis season (May–June to August–September)
Exposure of naïve cattle to moderate to low infection dynamics (see Table 2)	Vaccine efficacy Vaccine line transmission to ticks	The vaccine should at least provide evidence for protection against disease expression and a full protection against death under moderate to low heterologous tick challenges Low risks of breakthrough cases under field conditions Vaccine not transmissible to ticks to avoid consecutive risks of increasing tick infections and natural transmission dynamics
Smallholder farms of 4–5 cows	Vaccine packaging	Flasks of 5 and 10 doses to reduce waist and vaccine degradation particularly for frozen vaccine to be thawed on farm

Indeed, under field conditions, a Tunisian cell line, was able to protect almost 90% of predominantly Holstein cattle (Darghouth, 2008). Whilst in Sudan, exotic calf breeds immunised with a local attenuated line are only protected against fatality cases (El Hag, 2010). It is worth mentioning that all the available live vaccines cannot eradicate the infection, but rather reduces the risk of occurrence and severity of tropical theileriosis cases, a fact that does not exclude the risks of breakthrough infections. The breakthroughs are likely to be more frequent under high infection pressures as reported in Egypt and in Sudan.

Table 5: Guidelines for optimal vaccine profile and application practices fitted to the main epidemiological features of tropical theileriosis under the arid and desert climates transmission pattern due to *H. anatolicum* as illustrated by the Sudan case

Relevant main epidemiological features of the arid and desert climates transmission pattern for *T. annulata* (example of the Sudan context)	**Parameters to consider for vaccine development and application**	**Corresponding guidelines for vaccine profile and application practices**
Cattle at risks categories by frequency: Dominantly calves up to one year, then heifers and bull claves	**Intrinsic safety**	Well tolerated by cross bred and pure-bred calves and heifers, no need for highly attenuated vaccines as required for North Africa, older animals are already naturally immune and do not require vaccination.
Disease cases occurring along the year	**Duration of immunity**	Long enough to protect the naïve cattle until the first exposure to infected ticks. This is not a critical issue for vaccine development considering the relatively long duration of immunity using attenuated lines and the high risks of infections by *T. annulata*. Vaccinated animals should be protected from natural infection during the month following vaccination to avoid disease risks due to exposure to early infections before installation of vaccine immunity. Long lasting acaricides could be used to protect vaccinated cattle against ticks during the month following immunization.
High transmission dynamics (see Table 2)	**Vaccine efficacy to heterologous challenges**	Since the attenuation process affects the heterologous protection level, it is recommended to reach a reasonable balance between the required attenuation level and vaccine immunogenicity to heterologous tick challenge. Tick challenge doses mimicking the average exposure of calves to infected ticks under field conditions are recommended. Occurrence of significant risks of breakthrough cases in vaccinated calve due to high infection pressures as observed in Sudan (El Hag et al., 2010).
	Vaccine line transmission to ticks	The issue of vaccine transmission to ticks is not as critical as under the Mediterranean transmission pattern, it is however recommended to use a vaccine line coming from a local stock.

It is clear from the present review that a more rational base for improving live vaccine efficacy and safety in Northern Africa is required. Translational research should focus on: (i) the need to better understand the relationship between the levels of natural challenge post vaccination and vaccine efficacy; (ii) the requirement of evidence based approaches to characterise and assess virulence and attenuation mechanisms, (iv) the possibility of combining live attenuated and sub-unit vaccines to enhance efficacy, (v) the need to improve the control measures by combining multiple approaches including tick elimination and upgrading cattle housing, (vi) improvement of vaccine delivery systems to overcome the dependence on liquid nitrogen cold chain.

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Chapter 2:

Cultivation, cryopreservation and resuscitation of *Theileria annulata* transformed cells in serum-free media

The content of this chapter was published as an open access research article at *Frontiers in Veterinary Science*.
Elati K, Zweygarth E, Mhadhbi M, Darghouth MA and Nijhof AM (2022). Cultivation, cryopreservation and resuscitation of *Theileria annulata* transformed cells in serum-free media. *Front. Vet. Sci.* 9:1055022. doi: 10.3389/fvets.2022.1055022

frontiers | Frontiers in Veterinary Science

TYPE Original Research
PUBLISHED 22 December 2022
DOI 10.3389/fvets.2022.1055022

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Parasitology, a section of the journal Frontiers in Veterinary Science

RECEIVED 27 September 2022
ACCEPTED 23 November 2022
PUBLISHED 22 December 2022

CITATION
Elati K, Zweygarth E, Mhadhbi M, Darghouth MA and Nijhof AM (2022) Cultivation, cryopreservation and resuscitation of *Theileria annulata* transformed cells in serum-free media. *Front. Vet. Sci.* 9:1055022. doi: 10.3389/fvets.2022.1055022

Cultivation, cryopreservation and resuscitation of *Theileria annulata* transformed cells in serum-free media

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Introduction: Tropical theileriosis is a protozoan disease caused by *Theileria annulata* that affects cattle in Northern Africa, the Middle East and Asia where vector ticks of the genus *Hyalomma* occur. Various measures are applied to control the disease, including vaccination with attenuated *T. annulata* schizonts. Cultivation of *T. annulata* schizonts is mainly conducted in media containing Fetal Bovine Serum (FBS), which has some disadvantages such as costs, batch- to-batch variation and ethical concerns.

Methods: In this study, we conducted three experiments to evaluate the ability of (1) *T. annulata* strains grown in RPMI with 10% FBS (RPMI-FBS) to adapt and grow in serum-free media (i.e., HL-1, RPMI without FBS supplementation, ISF-1, and M199), (2) a *T. annulata* strain grown in ISF-1 and subsequently frozen in this medium to grow in ISF-1 again after long-term storage in liquid nitrogen, and (3) a *T. annulata* strain freshly isolated from infected bovine lymphocytes to growin ISF-1, also after cryopreservation. Cell numbers, schizont index, the viability and generation doubling time were calculated in all experiments.

Results and discussion: In the first experiment, the Hessiene and Beja cell lines from Tunisia previously cultivated in RPMI-FBS and adapted to serum-free media continued to grow significantly better in RPMI-FBS compared to the serum-freemedia. In the second experiment, a Tunisian cell line (Hessiene) cryopreserved in ISF-1 with 5%[v/v] dimethylsulfoxide (DMSO) grewbetter after thawing in RPMI-FBS compared to ISF-1 with a highly significant difference in cell growth ($p < 0.001$), whereas the third experiment showed that the Ankara cell line had similar growth characteristics in both RPMI-FBS and ISF-1 before and after thawing, with a shorter generation doubling time in ISF-1 than in RPMI-FBS ($p = 0.23$). Our findings suggest that freshly isolated cells can be propagated, frozen and thawed in serum-free media such as ISF-1, but once cells are adapted to cultivation in the presence of FBS or resuscitated from frozen storage, propagation in serum-free media may not perform as well as cultivation in RPMI-FBS.

KEYWORDS

***Theileria annulata*, cell culture, serum-free media, tropical theileriosis, fetal bovine serum**

 10.3389/fvets.2022.1055022

Introduction

Tropical theileriosis (TT) is a disease that affects cattle in several countries in the Mediterranean basin, the Middle East and Asia (1–6). It is caused by the protozoan *Theileria annulata*, which is transmitted by several tick species of the genus *Hyalomma*, e.g., *Hyalomma scupense* in Tunisia (7), *Hyalomma dromedarii* in Mauritania (8) and *Hyalomma anatolicum* in Sudan (4). Despite control measures such as the use of chemical acaricides for tick control, chemotherapy of clinical TT cases with buparvaquone or the use of live attenuated vaccines, the disease is still a serious obstacle to livestock productivity in countries such as Tunisia (9, 10). In Tunisia, the attenuated schizont-infected cell line "Beja" was developed and used at passage 280 to immunize cattle under field conditions. This vaccine proved to be particularly effective when applied to control TT in small dairy herds with endemic instability (11) and was considered to have economical potential in populations with endemic stability in Tunisia (10). However, in contrast to the high level of protection found in Tunisian natural endemic situations where a low to moderate infection pressure prevails, live attenuated vaccines were not sufficiently effective against heavy experimental heterologous challenge (11, 12). This illustrates the necessity to improve the effectiveness of the vaccine in order to better protect animals in the case of heavy tick infestations (13).

Theileria annulata cell lines are typically cultured in media containing animal sera, mostly Fetal Bovine Serum (FBS). The use of FBS raises several issues: (i) risk of contamination of the culture by bacteria, viruses and prions, (ii) ethical concerns as it is harvested from bovine fetuses, (iii) high costs and batch-to-batch variability, making standardization in mass culture procedures difficult. The identification of a standardized serum-free cell culture medium suitable for the cultivation of attenuated *T. annulata* cell lines could be an attractive alternative to classic media supplemented with FBS. Previous studies showed that *T. annulata* cell lines cultivated in RPMI 1640 medium supplemented with 10% FBS could be adapted to serum-free culture conditions using the ISF-1 medium, which also resulted in a shorter generation doubling time (14).

The objectives of this study were to (i) adapt Tunisian *T. annulata* cell lines previously propagated in RPMI medium containing 10% FBS (RPMI-FBS) to serum-free media, (ii) evaluate the growth ability of a cell line after freezing and thawing in serum-free medium, and (iii) check the capability of *T. annulata* strain freshly isolated from infected lymphocytes to grow, be frozen and re-cultured in ISF-1.

Materials and methods

Experiment 1: Adaptation of cell lines previously cultivated in RPMI-FBS to serum-free media

For this experiment, two different *T. annulata*-infected cell lines from Tunisia were used: *T. annulata* Beja (previously also referred to as CL2), isolated in 1989 in the Beja district from a crossbred cow suffering from an acute form of theileriosis and *T. annulata* Hessiene, which was isolated from an infected Holstein cow in the Hessiene region in 2015. Prior to their adaptation to serum-free culture conditions, the *T. annulata* cell lines were cultured in RPMI 1640 (Gibco) medium containing 2 g/L NaHCO3 and 10 % (v/v) heat-inactivated fetal bovine serum (FBS). The medium was buffered with 20 mM HEPES (N[2-hydroxyethyl] piperazine-N′-[2-ethanesulfonic acid]) and supplemented with 2 mM L-alanyl-l-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. This medium is hereafter referred to as RPMI-FBS. The experiment started with *T. annulata* Beja at passage 22 and *T. annulata* Hessiene at passage 23.

For serum-free propagation, four different commercially available media were used: ISF-1 (Biochrom GmbH), HL-1(Lonza, BioWhittaker), M199 (Sigma-Aldrich Chemie GmbH), and RPMI 1640 (Gibco). RPMI-FBS was used as a positive control. ISF-1 was supplemented with L-glutamine (2 mM) and penicillin/streptomycin (2 mM). The other serum-free media were further supplemented with L-glutamine (2 mM), penicillin/streptomycin (2 mM), lipid-rich bovine serum albumin (LR-BSA, Gibco) (3 mg/ml) and insulin, human recombinant, zinc solution (Gibco) (15 mg/ml). The RPMI 1640-based medium without FBS (serum-free) is hereafter referred to as RPMI-SF. All cultures were kept in 25 cm^2 vented cell culture flasks (Corning, NY, USA) and propagated at 37°C in a 5% CO_2-in-air atmosphere incubator.

Experiment 2: Resuscitation of *T. annulata* cells cryopreserved in ISF-1 medium

Cryopreserved *T. annulata* Hessiene strain at passage 42 and 127 were grown in ISF-1 until passage 62 and 147, respectively. Stabilates were prepared in October 2018 using dimethylsulfoxide (DMSO; final concentration 5 %[v/v]) in ISF-1 as a cryoprotectant. Cell suspension aliquots were transferred to 2 ml cryotubes which were frozen at −80°C using a NALGENE® Frosty™ Cryo 1°C freezing container. After being kept overnight at −80°C, the cryotubes were transferred to

liquid nitrogen and stored for 3 years. For resuscitation, the cell suspensions were thawed rapidly in a water bath at 37°C. The cells were then suspended in 10 ml ISF-1 and centrifuged at 950 rpm for 10 min. Pellets were resuspended in ISF-1 medium or RPMI-FBS and transferred into 25 cm^2 vented cell culture flasks and propagated as described above for experiment 1.

Experiment 3: Propagation of early passage *T. annulata* schizont cultures in serum-free medium and their resuscitation after storage at −80°C

A 6-month-old male Friesian calf purchased from a local dairy farm was infected by subcutaneous injection with ground up tick supernate (GUTS) from *Hyalomma lusitanicum* ticks containing sporozoites from the Ankara strain of *T. annulata*. Once the animal started showing symptoms of tropical theileriosis and the infection was confirmed by both Giemsa-stained blood smears and PCR, blood was collected in EDTA tubes as starting material for a fresh schizont culture. All animal experiments were conducted with approval of the commission for animal experiments (LAGeSo, Berlin, registration number G0240/19). Peripheral blood mononuclear cells (PBMC) were isolated with BioColl separation solution 1.077 g/ml (Bio&SELL GmbH, Nuremberg) and the isolated cells were washed with phosphate-buffered saline (PBS) as previously described (15). Cells were placed in 5 ml RPMI-FBS medium and incubated as described above. The medium was renewed every 3 days until a culture was established, which was confirmed by Giemsa-stained cytospin smears. Once a culture was established, passages were made in RPMI-FBS ISF-1 and RPMI-SF (in this experiment, RPMI-SF medium were not supplemented with lipid-rich bovine serum albumin and insulin). After 10 passages, the cells grown in ISF-1 were cryopreserved in the same medium with 2.5% [v/v] DMSO for 3 months at −80°C, then thawed and cultured in (ISF-1), to examine their ability to grow further in serum-free medium, and also in RPMI-FBS for comparison purposes.

Cell growth, generation doubling time, schizont index and statistical analyses

The trypan blue exclusion test was carried out to determine the percentage of living cells. On every third day, 100 µl of the cell suspensions were diluted in 400 µl PBS with 0.5% trypan blue and counted in cell counting chambers (C-Chip Neubauer improved DHC-B02, NanoEntech, Ingbert, Germany). The viability of the cells was determined by calculating the percentage of live cells out of the total number of examined cells. For comparison of the cell growth between different media, the starting cell concentration for all cultures was 2.5×10^5 cells/ml.

Giemsa-stained slides were used to determine the schizont index (SI), which is defined as the percentage of leukocytes infected with schizonts out of the total number of leukocytes examined (15). The generation doubling time (GDT) was based on the number of live cells (time interval in hours/number of generations) and number of generations = [ln(final cell number) – ln(initial cell number)]/ln(2). Ordinary one-way ANOVA test was performed using GraphPad Prism (version 9) to study the variation of cell growth parameters and GDT according to the evaluated media.

Results

Cell growth in serum-free media and generation doubling time

For cultures in M199 medium, the metabolic activity was too low and the medium became alkaline. The number of cells was low ($<5 \times 10^5$/ml) and the culture in M199 was stopped. There was a statistically significant difference in cell growth between the remaining media evaluated (ANOVA test, $p < 0.001$). For both Beja and Hessiene cell lines, the highest cell number was recorded in culture with RPMI-FBS (highest mean cell number was $30.7 \pm 1 \times 10^5$/ml for Beja and $20.3 \pm 2.4 \times 10^5$/ml for Hessiene). RPMI-FBS was the most efficient medium for growing both *T. annulata* cell lines, followed by ISF-1 (highest mean cell number was $20.5 \pm 3.8 \times 10^5$/ml for Beja and $15.4 \pm 1.6 \times 10^5$/ml for Hessiene), HL-1 and RPMI-SF (Figure 1).

The results showed that both cell lines could successfully be propagated in serum-free media (ISF-1, RPMI-SF and HL-1), but RPMI-FBS was superior in terms of growth rate and generation doubling time, which was significantly shorter compared to the serum-free media tested (Table 1).

Ability of cell line cryopreserved in ISF-1 medium to grow after resuscitation

Theileria annulata Hessiene cultures (passage 62 and 147) frozen in ISF-1 with 5% DMSO in liquid nitrogen for a period of 3 years were successfully resuscitated, but could only be propagated for six passages in ISF-1 after thawing, whereas they continued to grow well in RPMI-FBS (ANOVA test, $p < 0.01$) (Figure 2).

The poorer growth was also reflected in the schizont index, which reached 78 and 83.5% for passage 62 and 147 in RPMI-FBS, respectively. The schizont index for cells cultivated in ISF-1 declined rapidly over time (Figure 3).

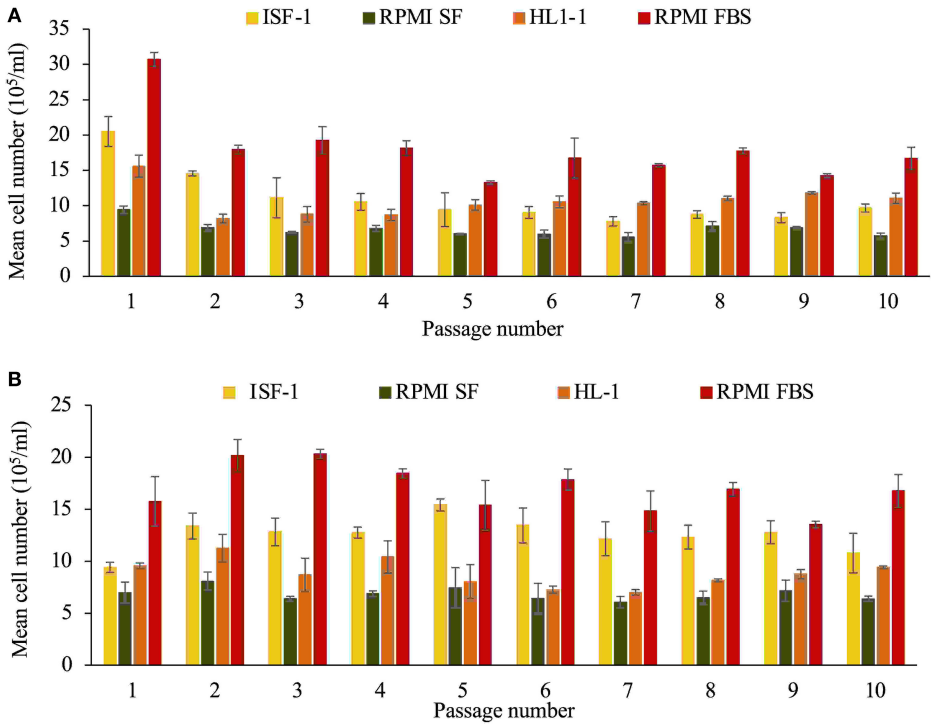

FIGURE 1
Growth of the *T. annulata* Beja **(A)** and Hessiene **(B)** cell lines as measured over ten passages (starting from passage 22 for Beja and passage 23 for Hessiene) in different serum-free media and RMPI supplemented with 10% fetal bovine serum (RPMI-FBS). Bars indicate standard deviation from the mean cell number of each culture replicate.

TABLE 1 Generation doubling time (GDT, in hours) of *Theileria annulata* strains (Beja and Hessiene) grown in different serum-free media and RPMI supplemented with 10% fetal bovine serum (RPMI-FBS).

Cell line	Beja				Hessiene			
Media	RPMI-FBS	ISF-1	RPMI serum-free	HL-1	RPMI-FBS	ISF-1	RPMI serum-free	HL-1
Mean GDT (SD)*	25.89 (2.6)	37.84 (8.6)	52.91 (7.5)	36.1 (4.3)	26.33 (1.8)	31.45 (2.8)	50.96 (4.2)	40.57 (4.9)
p-value **	$p < 0.01$				$p < 0.01$			

*SD, standard deviation.
**As determined by ANOVA one-way test for all media compared amongst each other.

Culture initiated, frozen and resuscitated in serum-free medium

As shown in Figure 4, fresh cultures from the *T. annulata* Ankara strain grew comparable in RPMI-FBS and ISF-1 media, with a mean cell number of 18.3± 2.7 × 10^5/ml and 15.5± 3.2 × 10^5/ml for ISF-1 and RPMI-FBS, respectively (ANOVA test, $p < 0.01$). Cells grew in RPMI-SF for only three passages and the culture was stopped due to the low cell number. The same growth trend as for fresh cells was observed for culture in ISF-1 and RPMI-FBS after a three-month storage at −80°C, with a mean cell number of 16.7 ± 1 × 10^5/ml and 15.2 ± 1.3 × 10^5/ml, respectively. A significant effect of freezing and thawing on cell growth was not observed with ISF-1 (ANOVA test, $p = 0.1$) or RPMI-FBS media (ANOVA test, $p = 0.7$). The Ankara cell line cultivated in ISF-1 had a relatively shorter generation doubling time (27.3 ± 2.7 h) than the cell line cultivated in RPMI-FBS (29.6 ± 3.2 h), but this was not statistically significant (ANOVA test, $p = 0.23$).

For the freshly propagated cells, the schizont index was higher in RPMI-FBS cultivated cells (mean=92.2%±3.5) than in ISF-1 (mean = 86.5%±4) (ANOVA test, $p < 0.01$) (Figure 5).

After having been resuscitated, the mean cell viability of Ankara *T. annulata* cell line was significantly higher in

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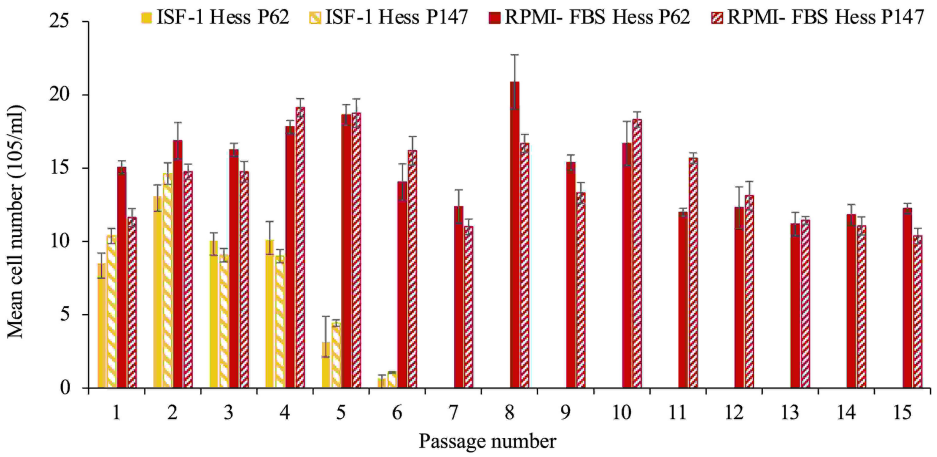

FIGURE 2
Growth of *T. annulata* Hessiene cell culture in RPMI-FBS and ISF-1 for 15 passages starting from passage 62 and passage 147 that were previously frozen in liquid nitrogen and maintained in ISF-1. Bars indicate standard deviation from the mean cell number of each culture replicate.

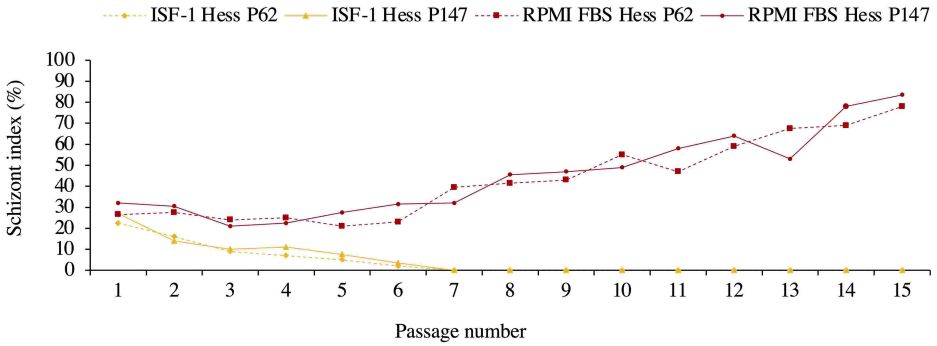

FIGURE 3
Schizont index of Hessiene cell culture passage 62 (Hess P62) and passage 147 (Hess P147) cultivated in ISF-1 and RPMI-FBS.

RPMI-FBS (mean= 96.1%±1.5) than in ISF-1 (80.5%±1.2) (ANOVA test, $p < 0.01$) (Figure 6).

Discussion

Various experiment on cultivating and freezing cell culture in serum-free medium (SFM) for different type of cells and parasites such *Toxoplasma gondii*, *Neospora caninum* (16, 17), *Plasmodium falciparum* (18–20) and *Babesia spp* (20–22) have been reported over the years, with a single publication on the cultivation of *T. annulata* in serum-free media by Zweygarth et al. (14). The use of FBS is a significant cost factor and by its addition to a culture medium, there is always a risk of introducing unwanted biological agents such as viruses and bacteria, which could jeopardize the production of a vaccine and its safety for animals. A previous study on tick-borne haemoprotozoan parasites *Babesia* spp reported that animals vaccinated with a vaccine derived from *in vitro* culture using SFM were 100% protected against babesiosis, while a protection of 83% was recorded in the animals vaccinated with vaccine derived from medium containing 40% FBS (23). These results confirm the ability of a vaccine derived from the *in vitro* cultures with a serum-free medium to protect animals, but it does not show whether this protection is related to the elimination of serum protein and biological agents as was suggested by other studies (24). The use of serum-free media such as ISF-1 could therefore not only be useful for vaccine production but also for studying the interaction between the host immune system and the parasite without the influence of the bovine serum and possible immune effectors and modulators it may contain.

The capacity of serum-free media to substitute RPMI supplemented with 10% FBS in *T. annulata* cultures was the

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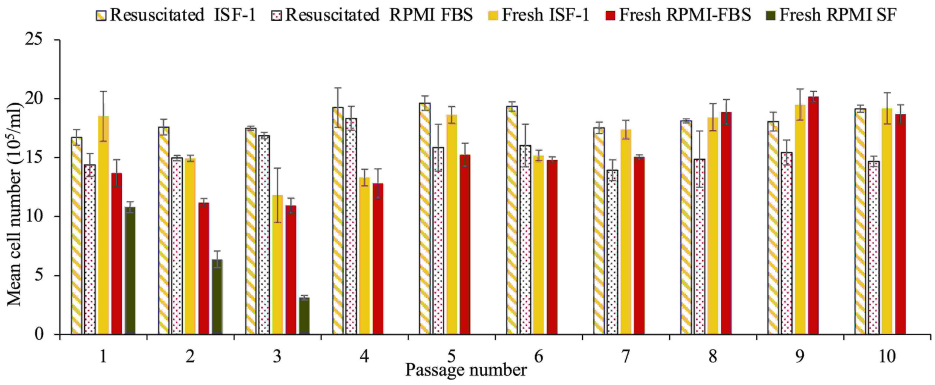

FIGURE 4
Growth of fresh and resuscitated thawed Ankara cell culture maintained in ISF-1, RPMI serum-free and RPMI-FBS. Bars indicate standard deviation from the mean cell number of each culture replicate.

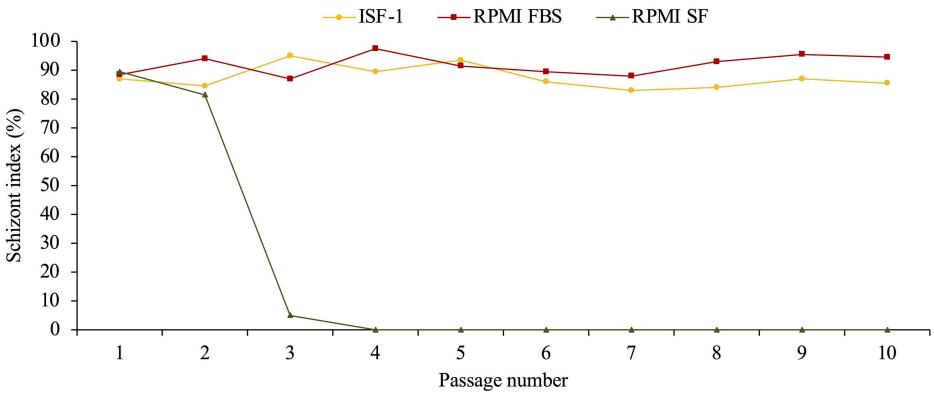

FIGURE 5
Schizont index of Ankara cell line culture (starting from passage 11) maintained in ISF-1 and RPMI supplemented with 10% fetal bovine serum (FBS).

main objective of this study. Attempts were made to grow *T. annulata* cell lines at different passages in different serum-free media (ISF-1, HL-1, RPMI serum-free, M199), as well as early passages of a newly established cell culture in ISF-1 medium. In addition, the performance of a *T. annulata* culture after freezing and thawing in ISF-1 medium and RPMI-SF was examined. RPMI-FBS medium was used as a positive control in all our experiments.

The results of the first experiment to propagate the two Tunisian cell lines previously cultured in RPMI-FBS in serum-free media were generally better for the Hessiene cell line compared to the Beja cell line. Even though RPMI-FBS has a significantly better cell culture performances compared to the serum-free media tested, ISF-1 was the best SFM that supported the cell growth when compared to HL-1, RPMI -SF and M199. Generation doubling time was shorter for cells propagated in RPMI-FBS than other media. Both Tunisian cell lines grew better in RPMI-FBS than in the serum-free media tested. Similar results were previously obtained with a Moroccan cell line that performed less well in SFM compared to RPMI-FBS, in contrast to other cell lines that performed better in ISF-1 compared to RPMI-FBS. The authors attributed that to the batch of FBS used, which may have provided favorable growth conditions for the Moroccan strain, but why this strain did not grow well in ISF-1 was not known (14).

In this first experiment, it was surprising that the cell lines could be propagated in RPMI-SF even though it had the lowest growth rate. This could be due to the addition of bovine serum albumin and insulin to the medium which were absent in the third experiment where cells could not grow in RPMI-SF (experiment 3, Figure 4). Similar studies on *P. falciparum* showed that RPMI 1640 supplemented with bovine

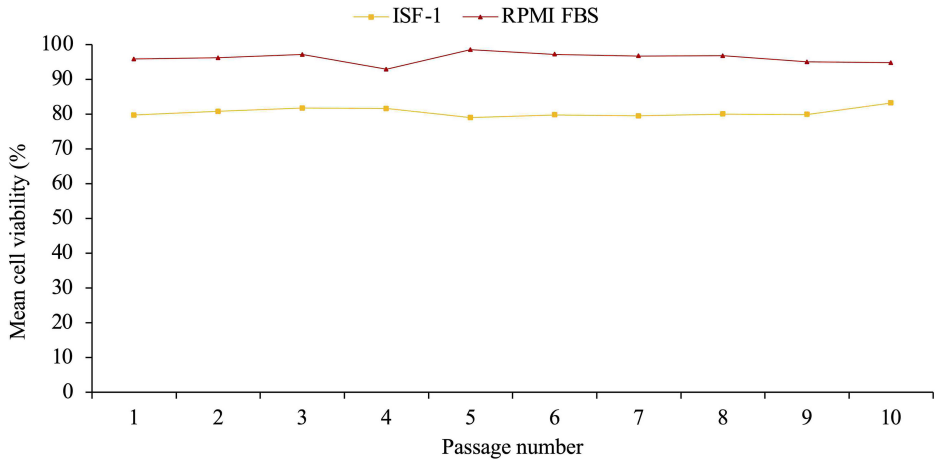

FIGURE 6
Mean cell viability of resuscitated Ankara cell line culture propagated in ISF-1 and RPMI supplemented with 10% fetal bovine serum (FBS).

albumin and lipids-cholesterol-rich mixture could successfully replace the serum in culture media (19). Also, for the culture of *Babesia bigemina*, serum-free medium was supplemented with insulin, transferrin, selenite, and putrescine instead of the use of FBS (25).

In the second experiment, we found that ISF-1 could be used to freeze *T. annulata* cell lines, but it was not possible to successfully resuscitate and re-culture the Hessiene cell line in ISF-1. As the cells were frozen in a low protein medium, the used DMSO concentration of 5 % [v/v] might have been too high and might have had a negative influence on survival, especially when further cultured in a serum-free medium. This was taken into account when we repeated this experiment with the freshly isolated Ankara cell line, which has been propagated after the first passage in ISF-1 and frozen for a short period (3 months) in the same medium containing 2.5% DMSO [v/v]. After resuscitation, the Ankara cell line grew well in both ISF-1 and RPMI-FBS. Our results are supported by previous studies showing that stromal cells from marmoset monkey (*Callithrix jacchus*) could be successfully frozen in serum-free medium containing 2.5% DMSO [v/v] (26). Another study on cryopreservation of regulatory T Cell (Treg) showed that reducing the concentration of DMSO in the freezing medium improves the cell recovery rate, viability and functionality (27). Another point that should be considered in the future is the adaptation type and period. In our study, we used direct adaptation where cells are switched directly from serum supplemented medium to SFM which may have been the reason why cells did not grow in ISF-1 after thawing. A sequential adaptation, whereby cells are transferred to SFM in several steps by reducing the percentage of FBS over a longer adaptation period may be preferred.

In the third experiment using the Ankara cell line, the results revealed that cells could perform well in ISF-1, with a high schizont index and a similar growth trend as RPMI-FBS. This confirmed previous findings describing that the Ankara strain can be propagated in serum-free media such as HL-1, ISF-1 and Hybridomed DIF 1000, whereby ISF-1 gave shorter generation doubling times (14).

The observed variations in cell growth in our cultures are undoubtedly multifactorial and may be partly related to differences in the origin of the cell lines and the batch of medium or FBS used. Similar observations have been made previously, where not all *T. annulata* cell lines used grew in a similar way in serum-free media either (14).

Our results showed that freshly isolated cells proliferate better in serum-free media than cells adapted to RPMI-FBS. If this is the case for all *T. annulata* strains, serum-free media would be an attractive alternative to classical culture media enriched with fetal bovine serum and could play an important role in the research and development of vaccines against *T. annulata*. Furthermore, this will contribute to the 3Rs principle (Replace, Reduce, Refine) in *T. annulata* research by replacing the use of FBS in culture. There is a need for optimization of the culture conditions in serum-free media where special attention should be paid to the effect of cultivating *T. annulata* in serum-free media on strain attenuation and the genetic structure of the cell line since the influence of bovine serum on the attenuation process is not demonstrated. Also, it is not yet known whether the attenuation occur in SF medium at a similar rate as in medium containing FBS.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The animal study was reviewed and approved by LAGeSo, Berlin, Registration Number G0240/19.

Author contributions

Conception and design of the study: KE, EZ, MD, and AN. Acquisition of data: KE and MM. Analysis and/or interpretation of data: KE, AN, and EZ. Drafting the manuscript: KE. Revising and editing of the manuscript: EZ, AN, MD, and MM. Approval of the version of the manuscript to be published: KE, EZ, AN, MM, and MD. All authors contributed to the article and approved the submitted version.

Funding

Part of the work was supported by DFG Project Molecular epidemiology network for promotion and support of delivery of life vaccines against *Theileria parva* and *Theileria annulata* infection in Eastern and Northern Africa (DFG-SE862/2-1 and CL166/4-2). KE was supported by a doctoral scholarship from the German Academic Exchange Service (DAAD).

Acknowledgments

We acknowledge support by the Open Access Publication Initiative of Freie Universität Berlin.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Chapter 3:

Dual RNA-seq catalogue host and parasite gene expression changes associated with virulence of *T. annulata*-transformed bovine leukocytes: towards identification of attenuation biomarkers

The content of this chapter was published as an open access research article in *Scientific Reports.*

Elati, K., Tajeri, S., Obara, I. *et al.* Dual RNA-seq to catalogue host and parasite gene expression changes associated with virulence of *T. annulata*-transformed bovine leukocytes: towards identification of attenuation biomarkers. *Sci Rep* 13, 18202 (2023).

doi: 10.1038/s41598-023-45458-9

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Dual RNA-seq to catalogue host and parasite gene expression changes associated with virulence of *T. annulata*-transformed bovine leukocytes: towards identification of attenuation biomarkers

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The apicomplexan parasite *Theileria annulata* is transmitted by *Hyalomma* ticks and causes an acute lymphoproliferative disease that is invariably lethal in exotic cattle breeds. The unique ability of the schizont stage of *T. annulata* to transform infected leukocytes to a cancer-like phenotype and the simplicity of culturing and passaging *T. annulata*-transformed cells in vitro have been explored for live vaccine development by attenuating the transformed cells using lengthy serial propagation in vitro. The empirical in vivo evaluation of attenuation required for each batch of long-term cultured cells is a major constraint since it is resource intensive and raises ethical issues regarding animal welfare. As yet, the molecular mechanisms underlying attenuation are not well understood. Characteristic changes in gene expression brought about by attenuation are likely to aid in the identification of novel biomarkers for attenuation. We set out to undertake a comparative transcriptome analysis of attenuated (passage 296) and virulent (passage 26) bovine leukocytes infected with a Tunisian strain of *T. annulata* termed Beja. RNA-seq was used to analyse gene expression profiles and the relative expression levels of selected genes were verified by real-time quantitative PCR (RT-qPCR) analysis. Among the 3538 T. *annulata* genes analysed, 214 were significantly differentially expressed, of which 149 genes were up-regulated and 65 down-regulated. Functional annotation of differentially expressed *T. annulata* genes revealed four broad categories of metabolic pathways: carbon metabolism, oxidative phosphorylation, protein processing in the endoplasmic reticulum and biosynthesis of secondary metabolites. It is interesting to note that of the top 40 genes that showed altered expression, 13 were predicted to contain a signal peptide and/or at least one transmembrane domain, suggesting possible involvement in host-parasite interaction. Of the 16,514 bovine transcripts, 284 and 277 showed up-regulated and down-regulated expression, respectively. These were assigned to functional categories relevant to cell surface, tissue morphogenesis and regulation of cell adhesion, regulation of leucocyte, lymphocyte and cell activation. The genetic alterations acquired during attenuation that we have catalogued herein, as well as the accompanying in silico functional characterization, do not only improve understanding of the attenuation process, but can also be exploited by studies aimed at identifying attenuation biomarkers across different cell lines focusing on some host and parasite genes that have been highlighted in this study, such as bovine genes (CD69, ZNF618, LPAR3, and APOL3) and parasite genes such as TA03875.

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Although many tick-borne *Theileria* species infect domestic and wild ruminants, pathogenicity in cattle is mainly confined to *Theileria annulata* and *Theileria parva*. The uniqueness of these *Theileria* species lies in the ability of their schizont stages to multiply and transform infected bovine leukocytes to a cancer-like phenotype[1]. For *T. annulata*, the acute lymphoproliferative diseases that results from infection is termed Tropical Theileriosis (TT). The disease is found throughout north Africa, the Mediterranean basin, the Middle East, India and southern Asia, where an estimated 250 million cattle (*Bos taurus*), as well as buffalos (*Bubalus bubalus*) are at risk[2]. TT is among the most serious constraints to cattle production in the countries where it is found[3–6].

Sporozoites of *T. annulata* mature in the salivary glands of infected *Hyalomma* ticks during feeding and are transmitted to cattle along with tick saliva. In cattle, the sporozoites invade either macrophages or B cells and develop into multinucleated *T. annulata* forms named schizonts that induce hyperproliferation and dissemination of infected cells, causing a leukaemia-like disease[7]. As a result of the rapid expansion of infected bovine cell populations, the local lymph node draining the site of infection often contains large numbers of infected cells that are subsequently disseminated throughout the lymphoid system and to non-lymphoid tissues[8]. The lymphoproliferation typically results in death within three weeks of infection[9]. The mode of oncogenesis by *T. annulata* is little understood, but there is evidence that it involves substantial changes in the infected host cell transcriptome due to parasite-dependent activation of key host cell transcription factors such as Activator Protein 1 (AP-1)[10], nuclear factor kappa B (NF-kB)[11], E2F transcription factor[12] and Hypoxia-inducible factor 1-alpha (HIF1-a)[13]. The transcriptional change in the host supports immortalization, constant proliferation and widespread dissemination. Several host kinases that act upstream of these transcription factors are found to be constitutively activated in presence of live intracellular schizonts, examples include c-Jun N-terminal kinase (JNK)[14], Src kinase[15] and phosphatidylinositol-3 kinase (PI3K)[16]. It is believed that parasite secreted factors contribute to alterations in host cell signalling pathways regulating the above mentioned kinases and transcription factors[17]. However, due to the lack of genetic tools to manipulate the *Theileria* genome, their exact roles in the transformation process remain unclear.

Because of the often acute and fatal nature of *T. annulata* infections, control of TT is challenging. The routine form of control has been the use of acaricides to kill the tick vectors. However, acaricide use is unsustainable, with limitations evident in their high costs and the continuing selection of acaricide-resistant tick populations[18,19]. Similarly, the need to treat animals during the early stages of disease and the high costs of the therapeutic compound (buparvaquone) imposes constraints on the effectiveness of chemotherapy.

Vaccination can only offer a sustainable approach to TT control in cattle if it can be shown to be efficacious and cost-effective. The ability of *T. annulata* to multiply and transform infected leukocytes has been explored to develop an immunization procedure against the disease, which involves attenuating parasitised cells by lengthy serial propagation in vitro[20]. The perception that transboundary use of attenuated *T. annulata* infected cell lines could introduce 'foreign' parasite genotypes and result in enhanced disease problems, led to the need to generate culture-attenuated live vaccines independently for each country using local parasite isolates[3,6,21–23]. In Tunisia, the attenuated schizont infected cell line 'Beja' was developed and used at passage 280 to immunize cattle under field conditions[24]. This vaccine proved to be particularly effective when applied to control TT in regions of endemic infections and thus has great potential for widespread deployment in small dairy herds in Tunisia, where this is the most common production system. However, its use in the field is hampered due to logistic issues of the delivery of the vaccine as this requires a liquid nitrogen chain and biosafety constraints due to the potential vaccine contamination with other pathogens.

A crucial part of the parasitised cell line vaccination strategy is the lengthy in vitro propagation coupled with occasional inoculation into calves to assess attenuation. A cell line is considered to be attenuated if the calves do not develop clinical signs and schizonts and piroplasms cannot be detected in lymph nodes, liver biopsies or Giemsa-stained blood smears, respectively. An attenuated line is considered as a vaccine when it has undergone challenge experiments and immunogenicity tests as well as tolerance tests with the most susceptible cattle categories in the target population (i.e. exotic dairy breeds cows in lactation/pregnancy) this in addition to microbiological and quality control tests[24]. The nature of the mechanism underlying attenuation has remained largely unclarified and could potentially involve, separately or in combination, selection of non-virulent subpopulations, genomic alterations, or changes in gene expression[25]. For example, Darghouth et al. (1996)[24] observed that virulent low passage *T. annulata*-transformed leukocytes often contain multiple parasite genotypes compared to attenuated lines. Interestingly, it has been shown that upon vaccination, *T. annulata* schizonts are transferred from vaccine cells to leukocytes of the recipient animal conferring an attenuated phenotype on them[26]. Thus, virulence of *T. annulata* transformed leukocytes is a parasite encoded trait but it is the infected host cell phenotype that decides on the outcome of an infection; in case of vaccination with attenuated leukocytes leading to establishment of a mild subclinical infection in vaccinated animals that protects against future field challenge. Substantial molecular changes in the long-term passaged *T. annulata* transformed macrophages have been identified and characterised. A relatively well-studied example is transcriptional downregulation of bovine MMP9 that has been observed in several *T. annulata* attenuated cell lines of different geographic origins[27,28]. MMP9 is an infection induced bovine gene that is under control of AP-1[27] and drives tissue dissemination of *T. annulata*-transformed leukocytes[29]. In attenuated macrophages dampened AP-1 activity results in reduced MMP9 expression and activity, partially explaining the diminished dissemination of attenuated cells[27]. Downregulated expression of another host factor, Transforming growth factor-beta 2 (TGF-ß2), has also been linked to reduced dissemination potential of attenuated cells[30]. Recently, downregulated expression of infection-induced microRNAs *mir-126-5p* and *mir34c-3p* was shown to contribute to reduced Matrigel traversal of attenuated macrophages[31,32].

In contrast to these single gene studies, we here followed a more global approach by using comparative Illumina RNA-seq to identify differentially expressed host (*Bos taurus)* and parasite (*T. annulata*) genes between virulent and attenuated *T. annulata*-transformed leukocytes that could explain the attenuated phenotype of long-term passaged cells. In addition, we sought to identify genes that could be used as transcriptionally measurable

candidate biomarkers for attenuation, with could eventually lead to a reduction in the number of required in vivo assessments for attenuation.

Results

Read mapping statistics

We applied a deep sequencing strategy to generate host and parasite transcript abundance profiles by comparing bovine leukocytes infected with virulent and attenuated passages of the Beja *T. annulata* strain from Tunisia. The host and parasite reads retained after sequential rounds of quality filtering were mapped to the ensemble *B. taurus* ARS-UCD1.2 and the *T. annulata* Ankara genome, respectively. The read mapping statistics are summarized in supplementary Fig. 1.

A demonstration that the data set represented differences in gene expression profiles between virulent and attenuated infected cells was provided by a Principal Component Analysis (PCA). As shown in Fig. 1, the PCA explains the clustering according to the passage level (low and high passage), with Principal Component 1 (PC1) represent 50.6% of most variation in the data and PC2 represent 21.3% of the second variation in the data. The correlation coefficient of samples between groups is calculated and drawn as heat maps which shows a Pearson correlation R^2 greater than 0.9, confirming a high similarity between the samples.

Global gene expression changes

Global gene expression changes following in vitro serial propagation T. annulata infected leukocytes

In total, 3538 parasite transcripts were detected across the six replicates sequenced (three from each passage), 149 of which were significantly up-regulated and 65 were down-regulated in the attenuated passage (Fig. 2A). Analysis of the bovine cell transcriptome revealed a total of 16,514 genes, 284 of which were significantly up-regulated, 277 were down-regulated and 15,953 genes were unchanged after attenuation (Fig. 2B).

Broad functional categories represented by the genes that significantly changed expression in the host and the parasite

The gene functions are classified into three groups including biological processes (BP), cellular components (CC) and molecular function (MF). The refined list of 171 differentially expressed bovine genes that exhibited a twofold or greater significant changes between virulent and attenuated cell lines were associated with the cell surface and with functions such as tissue morphogenesis, regulation of leukocyte cell–cell adhesion, regulation of cell adhesion and regulation of leucocyte and lymphocyte cell activation (Fig. 3).

Although there was no significant GO enrichment, the differentially expressed parasite genes were associated with different metabolic pathways such as protein folding, lipid biosynthetic process, cellular lipid metabolic process, chromosome organization, generation of precursor metabolites and energy (Supplementary Fig. 2b). KEGG analysis similarly identified parasite genes enriched in metabolic pathways such as carbon metabolism, oxidative phosphorylation, protein processing in the endoplasmic reticulum and phagosome and biosynthesis of secondary metabolites (Supplementary Fig. 2a).

Transcriptional changes in *T. annulata*-infected bovine leukocytes during long-term in vitro culture

Because the loss of virulence and clinical pathogenicity of high passaged (attenuated) *T. annulata* transformed cells could directly be due to changes in host cell gene expression during the long-term process of in vitro passaging[20,24], the bovine transcriptome of leukocytes of low and high passage were compared resulting in a total

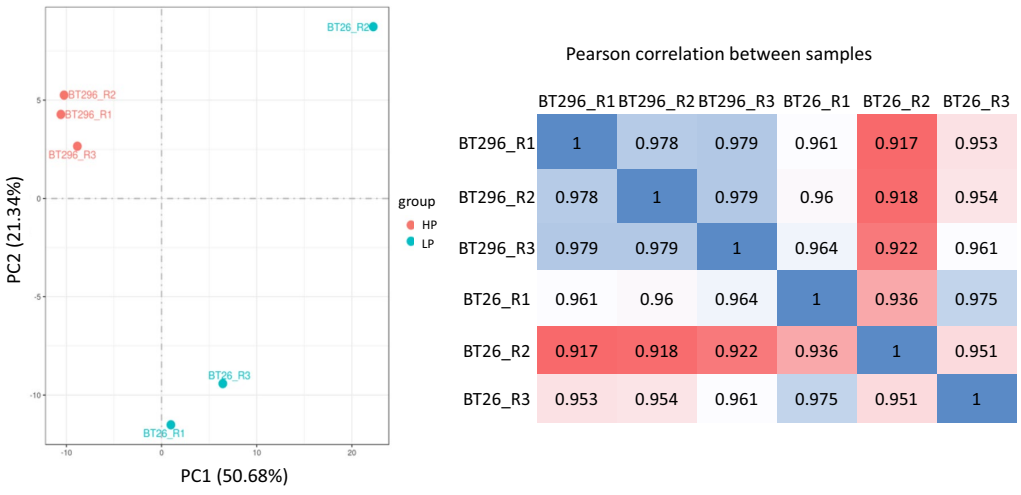

Figure 1. Principal component analysis (PCA) plots and inter-sample correlation heat map (R^2: Square of Pearson correlation coefficient(R)) showing the clustering and the correlation between the samples (BT296 is the attenuated and BT26 is the virulent).

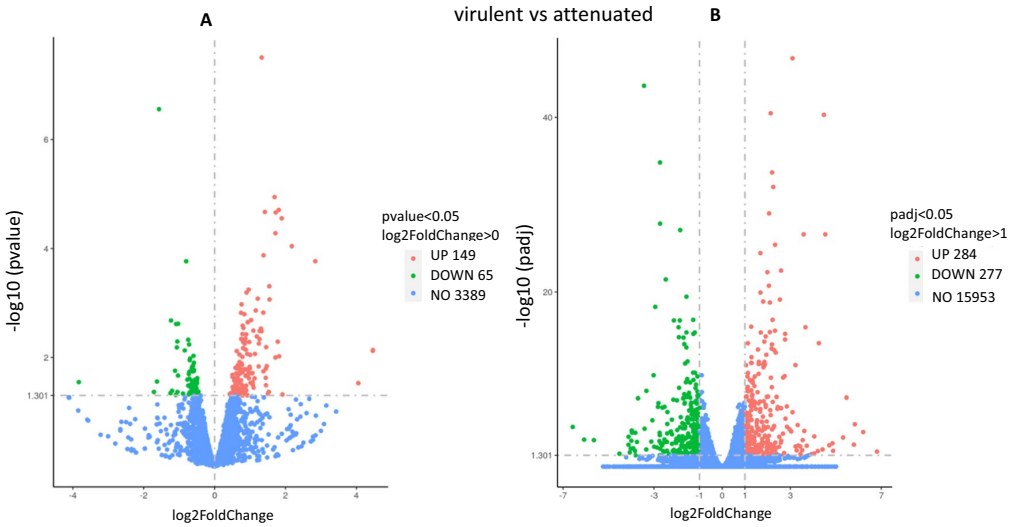

Figure 2. Differential Gene expression Volcano Map showing the up- and down-regulated genes as well as the unchanged genes in the parasite (**A**) and in the host (**B**). The abscissa in the figure is log2 Fold Change, and the ordinate is $-\log10$ *p* adj or $-\log10$ *p* value, the dashed line indicates the threshold line for differential gene screening criteria.

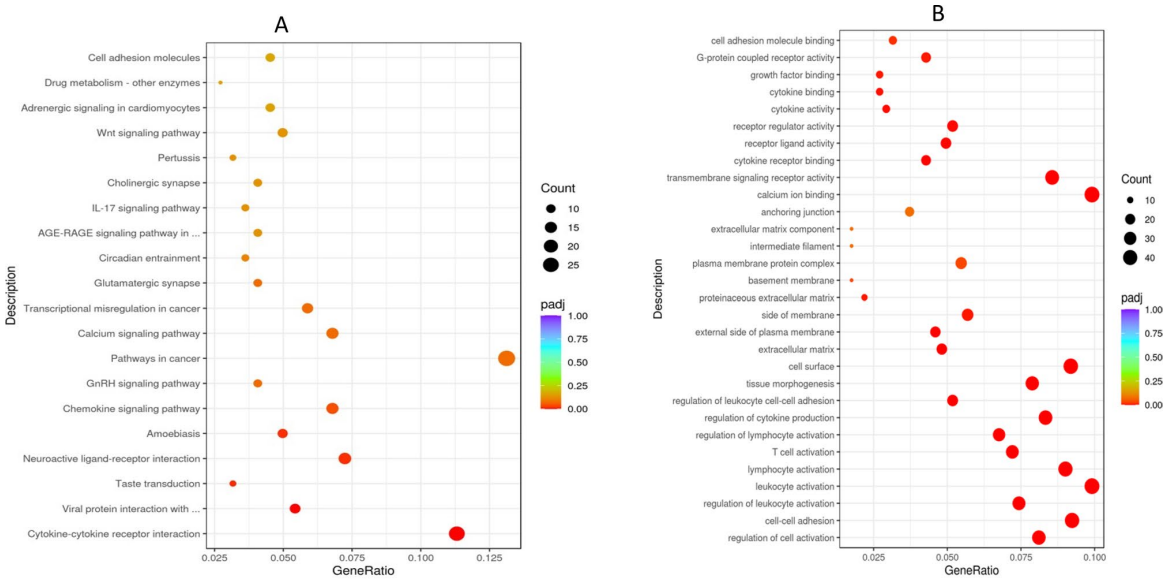

Figure 3. KEGG (**A**) and GO (**B**) enrichment GO enrichment analysis scatter plot of differentially expressed host genes. The abscissa in the graph (A) is the ratio of the number of differential host genes on the KEGG pathway to the total number of differential genes, and the ordinate is KEGG pathway. The abscissa in the graph (B) is the ratio of the differential gene number to the total number of differential genes on the GO Term, and the ordinate is GO Term.

of 561 host genes that were significantly differentially expressed (284 were up- and 277 were downregulated). Based on the padj value, 168 genes were found with a fold change value greater than 2 with significant *p* adj values (< 0.05). The most up-regulated genes in high passage attenuated Beja cells were UPK3BL1 (*Bos taurus* uroplakin 3B-like) and P2RX3 (purinergic receptor), while the most down-regulated genes were ZNF618 (zinc finger protein 618) and the *B. taurus* CD69 molecule. A list of top 20 up and top 20 down-regulated genes is shown in Table 1.

Gene_id	Gene_name	Gene_description	Attenuated (average RPKM*)	Virulent (Average RPKM*)	log2FoldChange	padj
ENSBTAG00000020524	UPK3BL1	Bos taurus uroplakin 3B-like (UPK3BL1), mRNA. [Source:RefSeq mRNA;Acc:NM_001168011]	25.5484371	0.3468303	6.20492255	0.00010761
ENSBTAG00000000376	P2RX3	purinergic receptor P2X 3 [Source:VGNC Symbol;Acc:VGNC:32519]	37.0572054	0.6936606	5.83671086	1.40E-05
ENSBTAG00000003030	KCNQ2	potassium voltage-gated channel subfamily Q member 2 [Source:VGNC Symbol;Acc:VGNC:30487]	9.85363751	0	5.79271357	0.003043
ENSBTAG00000006806	KRT17	keratin 17 [Source:VGNC Symbol;Acc:VGNC:50398]	56.5290977	1.22118738	5.46777552	1.27E-08
ENSBTAG00000012630	PAMR1	peptidase domain containing associated with muscle regeneration 1 [Source:VGNC Symbol;Acc:VGNC:32562]	10.1895288	0.30529685	4.87936486	0.01556863
ENSBTAG00000018395	KIAA1217	KIAA1217 [Source:VGNC Symbol;Acc:VGNC:30566]	18.4306952	0.61059369	4.85316467	0.00212362
ENSBTAG00000048591	THBD	thrombomodulin [Source:HGNC Symbol;Acc:HGNC:11784]	16.6750819	0.61059369	4.71007582	0.00360285
ENSBTAG00000008944	CRB1	Bos taurus crumbs 1, cell polarity complex component (CRB1), mRNA. [Source:RefSeq mRNA;Acc:NM_001192482]	171.441218	7.37485138	4.53543835	2.45E-27
ENSBTAG00000002661	ISLR2	immunoglobulin superfamily containing leucine rich repeat 2 [Source:VGNC Symbol;Acc:VGNC:30299]	284.796605	12.7310177	4.47289549	5.13E-41
ENSBTAG00000004261	SPON2	spondin 2 [Source:VGNC Symbol;Acc:VGNC:35228]	98.5821353	5.22738488	4.25252666	7.42E-15
ENSBTAG00000003777	TIE1	Bos taurus tyrosine kinase with immunoglobulin like and EGF like domains 1 (TIE1), mRNA. [Source:RefSeq mRNA;Acc:NM_173965]	11.9451421	0.65212714	4.2094648	0.01658889
ENSBTAG00000005404	MSC	musculin [Source:VGNC Symbol;Acc:VGNC:31691]	26.5104467	1.57839235	4.05093325	0.00034497
ENSBTAG00000050719	–	calcium-dependent phospholipase A2 PLA2G2D1 [Source:NCBI gene;Acc:494318]	10.864386	0.6936606	4.04658787	0.03146184
ENSBTAG00000054512	IGSF1	Bos taurus immunoglobulin superfamily member 1 (IGSF1), mRNA. [Source:RefSeq mRNA;Acc:NM_001105048]	9.91758328	0.66250877	3.93311066	0.03703225
ENSBTAG00000025634	FMN1	formin 1 [Source:HGNC Symbol;Acc:HGNC:3768]	17.0322943	1.30425429	3.72041477	0.00404415
ENSBTAG00000008832	CCL1	C–C motif chemokine ligand 1 [Source:VGNC Symbol;Acc:VGNC:26943]	114.442319	9.05707389	3.66458396	1.12E-16
ENSBTAG00000007421	CDH5	cadherin 5 [Source:NCBI gene;Acc:414735]	31.246492	2.52544167	3.61207206	8.13E-05
ENSBTAG00000037553	KRT33A	Bos taurus keratin 33A (KRT33A), mRNA. [Source:RefSeq mRNA;Acc:NM_001099099]	227.067215	18.905438	3.58304894	2.45E-27
ENSBTAG00000017060	ITGB2	integrin subunit beta 2 [Source:VGNC Symbol;Acc:VGNC:30327]	22.5558378	1.94599286	3.54144292	0.00091222
ENSBTAG00000002739	PDE1C	phosphodiesterase 1C [Source:VGNC Symbol;Acc:VGNC:32673]	15.0138893	1.34578774	−3.52040797	0.01313049
ENSBTAG00000006686	NPNT	nephronectin [Source:VGNC Symbol;Acc:VGNC:32209]	6.81535641	68.6539285	−3.3327037	2.22E-09
ENSBTAG00000042166	RF00410	–	1.68557075	17.2356305	−3.341479	0.01078732
ENSBTAG00000006855	TDRD1	tudor domain containing 1 [Source:VGNC Symbol;Acc:VGNC:35715]	3.05655381	32.6042682	−3.4157894	4.53E−05
ENSBTAG00000003398	KCNG1	potassium voltage-gated channel modifier subfamily G member 1 [Source:VGNC Symbol;Acc:VGNC:30442]	61.5126929	666.608932	−3.4385742	2.36E-44
ENSBTAG00000005370	TMTC1	Bos taurus transmembrane and tetratricopeptide repeat containing 1 (TMTC1), mRNA. [Source:RefSeq mRNA;Acc:NM_001192761]	4.40013653	57.4349605	−3.7038803	1.50E-08

Continued

Gene_id	Gene_name	Gene_description	Attenuated (average RPKM*)	Virulent (Average RPKM*)	log2FoldChange	padj
ENSBTAG00000042696	RF00071	small nucleolar RNA, C/D box 73A [Source:HGNC Symbol;Acc:HGNC:10235]	2.38781081	32.1493473	− 3.7487039	0.00147441
ENSBTAG00000005425	GATA4	GATA binding protein 4 [Source:VGNC Symbol;Acc:VGNC:29268]	0.67787935	9.54307417	− 3.8148273	0.04964
ENSBTAG00000053497	GNLY	granulysin [Source:NCBI gene;Acc:404173]	2.04887114	28.7995165	− 3.8210854	0.00013213
ENSBTAG00000009341	CCDC146	Bos taurus coiled-coil domain containing 146 (CCDC146), mRNA. [Source:RefSeq mRNA;Acc:NM_001205487]	1.01377939	14.6271358	− 3.8396071	0.0153719
ENSBTAG00000009302	RCAN2	Bos taurus regulator of calcineurin 2 (RCAN2), transcript variant 2, mRNA. [Source:RefSeq mRNA;Acc:NM_001015632]	1.01073103	15.3083605	− 3.9136064	0.00772622
ENSBTAG00000046107	TTBK1	tau tubulin kinase 1 [Source:HGNC Symbol;Acc:HGNC:19140]	1.69165875	26.4921376	− 3.965986	0.00012693
ENSBTAG00000010111	TCF7L1	transcription factor 7 like 1 [Source:NCBI gene;Acc:515303]	2.06104713	32.20969	− 3.9795809	7.21E-05
ENSBTAG00000012368	IL21	interleukin 21 [Source:VGNC Symbol;Acc:VGNC:30141]	1.34358271	21.6281861	− 3.9958212	0.00108001
ENSBTAG00000024675	CYSLTR1	Bos taurus cysteinyl leukotriene receptor 1 (CYSLTR1), mRNA. [Source:RefSeq mRNA;Acc:NM_001099726]	0.99855504	16.4111412	− 4.0180996	0.00870421
ENSBTAG00000001068	ZCWPW1	zinc finger CW-type and PWWP domain containing 1 [Source:VGNC Symbol;Acc:VGNC:37125]	0.68701571	11.4433525	− 4.0661589	0.02374012
ENSBTAG00000048565	BASP1	Bos taurus brain abundant membrane attached signal protein 1 (BASP1), mRNA. [Source:RefSeq mRNA;Acc:NM_174780]	1.36185543	23.6780022	− 4.120178	0.00037734
ENSBTAG00000015094	VNN1	Bos taurus vanin 1 (VNN1), mRNA. [Source:RefSeq mRNA;Acc:NM_001024556]	1.02900375	17.8296211	− 4.1234276	0.00314342
ENSBTAG00000007589	SMAD9	Bos taurus SMAD family member 9 (SMAD9), mRNA. [Source:RefSeq mRNA;Acc:NM_001076928]	0.33285168	8.39456512	− 4.5214841	0.03301325
ENSBTAG00000003791	LPAR3	Bos taurus lysophosphatidic acid receptor 3 (LPAR3), mRNA. [Source:RefSeq mRNA;Acc:NM_001192741]	0.34807603	18.2533247	− 5.6437392	0.0009192
ENSBTAG00000002135	CD69	Bos taurus CD69 molecule (CD69), mRNA. [Source:RefSeq mRNA;Acc:NM_174014]	0	12.6541583	− 6.0743754	0.00080407
ENSBTAG00000025659	ZNF618	zinc finger protein 618 [Source:VGNC Symbol;Acc:VGNC:37314]	0.33893968	34.9199228	− 6.5764645	2.81E-05

Table 1. Top 40 up- and down-regulated host genes in attenuated passage based on the log2FoldChange and padj value. *RPKM = reads Per Kilobase of transcript, per Million mapped reads.

We were wondering if our list of DEGs contain genes that are directly controlled by *T. annulata* infection and are down-regulated during the attenuation process. To identify this group of genes we merged the lists of genes upregulated upon infection with those downregulated in attenuated Beja. The list of genes significantly de-regulated by *T. annulata* between non-infected immortalized bovine B lymphocyte lines BL3 and BL20 compared to parasite infected TBL3 and TBL20 was previously published[33]. A comparison of our results with that study revealed that 11 and 13 genes were common with TBL3 and TBL20, respectively, whereas only two genes (ANXA and FAM161A) were common among the three datasets (Fig. 4, panel a). The other category of potentially interesting genes was those that were repressed by *T. annulata* infection (down-regulated in TBL3/TBL20) and upregulated in attenuated Beja. This category likely represents tumour suppressors and were more than pro-tumorigenic genes (only two genes) shared between TBL3, TBL20 and attenuated Beja (i.e., nine genes) (Fig. 4, panel b).

To date, comparative differential host cell gene expression of only one attenuated *T. annulata* (Anand, India) transformed macrophage, termed Ode has been reported[33,34]. For the purpose of finding common differentially expressed host genes between the Beja cell line studied herein and Ode that might be useful as attenuation biomarkers, a comparison of the differential transcriptome between virulent and attenuated passage of the two cell lines was conducted. The comparison showed only five common genes, three of which were up-regulated (CAPN3, CTNND1, IL4I1) and two were down-regulated in attenuated Beja passage (IL12A and TXNIP) (Fig. 4, panel C).

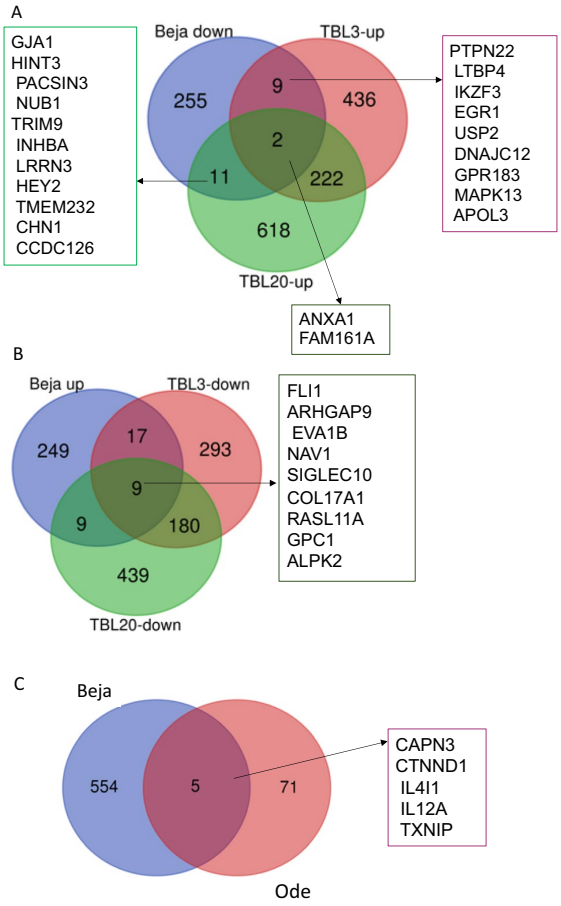

Figure 4. Venn diagrams showing the genes inversely differentially expressed in Beja high passage (attenuated) compared to TBL3, TBL20 and attenuated Ode. Panel A showing an inverse comparison of genes down-regulated in attenuated Beja vs genes up-regulated by infection; Panel B showing genes up-regulated in Beja vs genes down-regulated in TBL and panel C showing the common genes in attenuated passage between Beja and Ode cell lines.

Activator protein 1 (AP-1) is a transcription factor whose constitutive activity is essential for *T. annulata*-induced leukocyte transformation[10]. Down-regulated AP-1 activity has been demonstrated in Indian (Ode) and Tunisian (Jed4) cell lines [10,27,28]. We conducted a bioinformatic screen (PROMO) of our DEG list for the presence of potential AP-1 binding sites on their promoters (1000 base pairs upstream of the start codon). Among the 277 down-regulated genes and 284 up-regulated genes, a total of 30 and 39 genes possesses potential binding sites for AP-1 transcription factor, respectively (Supplementary Table 2).

Nuclear factor (NF-κB) is known to be regulator of *Theileria* transformed cell proliferation and survival[35]. In our DEGs, putative binding sites of NF-κB were found in the promoters of 13 up-regulated genes and 14 down-regulated genes (Supplementary Table 2).

Transcriptional differences in *T. annulata* parasite between virulent and attenuated passage of Beja strain

As it has been demonstrated that virulence and attenuation of *T. annulata* transformed leukocytes is a parasite encoded trait[25,26], the most interesting genes are those that were down-regulated during attenuation especially the ones with a signal peptide (SP) or transmembrane domain(s) (TMDs). These are potential virulence factors as they are likely to be secreted and transferred to either schizont membrane, host cytoplasm or host cell nucleus. These sets of genes hold the potential to interact with host cell signalling pathways that are associated with virulence of the infected host cell. Based on the p value, we selected the top 40 genes which were significantly up- or down-regulated. Using PiroplasmaDB, we found that five of them possessed signal peptides and ten had one to nine TMDs which suggest their involvement in host parasite interaction (Table 2).

Gene_name	Gene_ description	Attenuated (average RPKM*)	Virulent (average RPKM*)	log2FoldChange	*p* value	Computed GO Functions	# TM Domains	SignalP Peptide
Tap370b08. q2ca38.02c	Cytochrome C oxidase subunit III (COX3 homologue), putative	491.304754	165.981616	1.56559474	2.78E-07	Cytochrome-c oxidase activity;obsolete heme-copper terminal oxidase activity	7	N/A
TA06810	Hypothetical protein, conserved	67.7844536	29.4781952	1.20130623	0.00209882	N/A	0	MYSNRNITV-VLLLYITH-FVHS
Tap370b08. q2ca38.03c	Cytochrome B, putative	339.412059	160.394615	1.08141211	0.00243271	Electron transfer activity;oxidoreductase activity	9	N/A
TA07960	Ubiquinol-cytochrome c reductase complex, subunit, putative	90.3826372	45.0169167	1.0055784	0.00241293	Ubiquinol-cytochrome-c reductase activity	0	N/A
EPrG00000717687	rRNA	3746.44801	2147.23358	0.80304429	0.00017134	N/A	0	
TA11950	hypothetical P-, Q-rich protein family protein, putative	1148.83556	685.63827	0.74465277	0.00475935	N/A	1	MINNIKYLIFV-LIFRSCIFVASS
TA03075	Transcription initiation factor (TBP homologue), putative	216.967375	362.893299	−0.7420673	0.00151448	DNA binding	0	N/A
TA15770	Hypothetical protein	120.081482	202.018472	−0.7504735	0.00458963	nucleic acid binding;zinc ion Binding	0	N/A
TA09685	Hypothetical protein, conserved	355.15881	602.645021	−0.7628442	0.00106305	Nucleic acid binding;protein binding	0	N/A
TA05280	Hypothetical protein	144.195729	249.042043	−0.7883609	0.00372746	N/A	4	N/A
TA03875	RNA poly(A)-binding protein, putative	161.595485	280.590833	−0.796081	0.00261815	Nucleic acid binding	1	N/A
TA15120	Chromatin assembly protein, putative	120.319726	211.979633	−0.8170525	0.00287531	N/A	0	N/A
TA06515	Hypothetical protein, conserved	98.4987798	176.816262	−0.8440732	0.00160673	N/A	0	N/A
TA18745	Nicotinate-nucleotide adenylyltransferase-like protein, putative	70.273125	129.167696	−0.8782003	0.00450453	Catalytic activity;nucleotidyltransferase activity	0	N/A
TA13830	Hypothetical protein	53.9241059	99.6059114	−0.885301	0.00377777	N/A	0	N/A
TA03785	Proteasome subnit, putative	141.085401	264.287756	−0.9055409	0.00064132	Endopeptidase activity;threonine-type endopeptidase activity	0	N/A
TA08715	Bacterial histone-like protein, putative	192.899489	368.459133	−0.9336553	0.00225835	DNA binding	0	MFTYTNSFLL-LIIIICLTVES
TA14000	Hypothetical protein, conserved	161.171375	313.652327	−0.9605707	0.0005686	N/A	0	N/A
TA04835	Hypothetical protein, conserved	40.7638947	82.7821973	−1.0220286	0.00206278	N/A	1	N/A
TA09825	Hypothetical protein, conserved	27.3304986	58.0526749	−1.0868508	0.00322895	N/A	1	N/A
TA06365	Hypothetical protein	40.415858	91.4168075	−1.177538	0.00137322	N/A	0	N/A
TA15565	Hypothetical protein, conserved	40.2554646	92.4333837	−1.1992294	0.00083339	N/A	0	N/A
TA12655	Hypothetical protein	19.7224715	45.3836084	−1.202331	0.00413025	N/A	0	N/A
TA03040	Hypothetical protein, conserved	20.6161283	49.5589351	−1.2653718	0.00326846	Nucleic acid binding	0	N/A

Continued

Gene_name	Gene_ description	Attenuated (average RPKM*)	Virulent (average RPKM*)	log2FoldChange	*p* value	Computed GO Functions	# TM Domains	SignalP Peptide
TA02785	Hypothetical protein, conserved	17.277774	41.8650219	− 1.276828	0.00461403	N/A	0	MKIILILLIIN-FVIN
TA15135	Hypothetical protein, conserved	22.6745348	56.0820538	− 1.3064662	0.00295876	Protein binding	0	N/A
TA07115	40S Ribosomal S12-related protein, putative	41.1243984	102.839588	− 1.3223293	0.00330076	N/A	0	N/A
TA04840	60S Ribosomal L34 protein, putative	307.069625	769.990431	− 1.3262747	3.12E-08	Structural constituent of ribosome	0	N/A
TA04425	Ribosomal protein L18, putative	31.6375187	81.5085534	− 1.365315	0.00013327	N/A	0	N/A
TA08625	RNA polymerases I and III subunit (RPC19 homologue), putative	18.4423666	48.4360839	− 1.3930584	0.0015048	DNA binding;DNA-directed 5'-3' RNA polymerase activity;protein dimerization activity	0	N/A
TA07230	Hypothetical protein	46.3186425	124.313033	− 1.4243127	2.12E-05	N/A	1	MKIKILFIILI-INFIKC
TA08610	Prefoldin subunit, putative	26.7160243	78.5055381	− 1.5550891	0.0004934	Unfolded protein binding	0	N/A
TA20245	Hypothetical protein	18.989486	55.907725	− 1.5578468	0.00086278	N/A	0	N/A
TA04760	Hypothetical protein, conserved	27.8035159	90.4935596	− 1.7025478	1.13E-05	Transcription coregulator activity	0	N/A
TA20225	Hypothetical protein	22.2882439	73.9714359	− 1.7306853	5.22E-05	N/A	1	N/A
TA09695	30S Ribosomal protein S8, putative	18.2494387	60.6542957	− 1.7327577	2.17E-05	Structural constituent of ribosome	0	N/A
TA13935	Hypothetical protein	21.4356829	76.061673	− 1.8271553	1.95E-05	N/A	0	N/A
TA15915	Hypothetical protein, conserved	20.0711006	73.5057958	− 1.8727383	2.79E-05	N/A	0	N/A
TA18140	Hypothetical protein	8.81402991	39.582718	− 2.166997	9.03E-05	N/A	0	N/A
TA08280	Hypothetical protein	3.22449848	24.2049842	− 2.9081575	0.00017088	N/A	2	N/A

Table 2. Top 40 up-and down regulated genes in *Theileria annulata* virulent and attenuated strain based on *P* value. *RPKM = reads Per Kilobase of transcript, per Million mapped reads.

We focused our analyses on the significantly down-regulated genes containing SPs or/and TMDs and looked at their orthologs in *T. parva* or *T. orientalis* to examine if their function is known. However, these genes were either not annotated (unspecified product or uncharacterized protein) or are known as integral membrane proteins (Supplementary Table 3). Only one gene (TA03875) was predicted to contain a NLS fragment (PKRKNIPG-YNRRRTNNRT) located between position 116 and 133 aa (Supplementary Table 3).

Of the most significantly differentiated *Theileria* genes (based on the padj value), only three (TA04760, TA04840 and TA09695) were predicted to be involved in regulating different process such as gene expression, transcription, translation, biosynthesis, protein metabolic process and cellular metabolic process. It is interesting to note that these three parasite genes are hypothetical proteins that are associated with 29 common predicted pathways and are down-regulated in attenuated passages (Supplementary Fig. 3).

Using PiroplasmaDB, we looked at the orthologues of these genes in other Apicomplexa and their possible described function(s). TA04760 in *T. parva* is annotated as MED6 mediator sub complex component family protein and it regulates the transcription using polymerase II, thus its down-regulation following attenuation might affect expression of a subset of parasite genes that are potentially virulence factors. TA04840 and TA09695 are ribosomal proteins with similar functions and are involved in translation and structural constituent of the ribosome (Supplementary Table 4).

Furthermore, given the cancer-like phenotype induced by infection, we screened our dataset for genes that have a role in tumorigenesis. Interestingly, many of the transcripts that are differentially expressed (24 out of 40 DEGs) remain unannotated, despite publication of the *T. annulata* Ankara genome. As for the genes for which annotation is available, the roles of most of them in *T. annulata* pathogenesis or attenuation remain uncharacterised and only few are known to possess transcription coregulator activity (TA04760), nucleic acid and protein

binding (TA09685, TA15135, TA03040) and nucleic acid and zinc ion binding (TA15770). All these genes are down-regulated in attenuated passage.

For validation of Illumina RNA-seq determined levels of parasite gene expression by RT-qPCR, we selected 11 parasite genes which exhibited significant differential expression based on their padj values; nine of them were down regulated and two were up-regulated (supplementary Table 1). The comparative RNA seq results were corroborated by the qRT-PCR data (Fig. 5).

Discussion

The mechanisms by which the multinucleated schizont stage of *T. annulata* hijacks host cell intracellular signalling and transcription pathways to reversibly transform infected leukocytes are not fully understood. Similarly, the complex interplay between *T. annulata* and the bovine macrophage during the generation of attenuated cell-line vaccines is not unravelled either. The amount of host and parasite genetic information is increasing exponentially and so are the resources with which to investigate the complex host–pathogen interactions. One such resource, RNA-seq analysis, is suited for investigating complex host–pathogen interactions as it allows for the simultaneous analysis of the expression of thousands of genes. Here, we report on an RNA-seq analysis comparing host and parasite mRNA levels in a virulent and attenuated *T. annulata* cell line from Tunisia.

The analysis identified over 500 bovine host genes and 200 parasite genes that exhibited a statistically significant differential expression, implying that there are differences in transcriptional pathways between virulent and attenuated cell lines. Overall, there was good agreement between the RNA-seq data and the qRT-PCR results for the genes exhibiting cell line-specific differential expression that were selected for validation. A demonstration that the data set represents differences in gene expression profiles between virulent and attenuated cell lines was

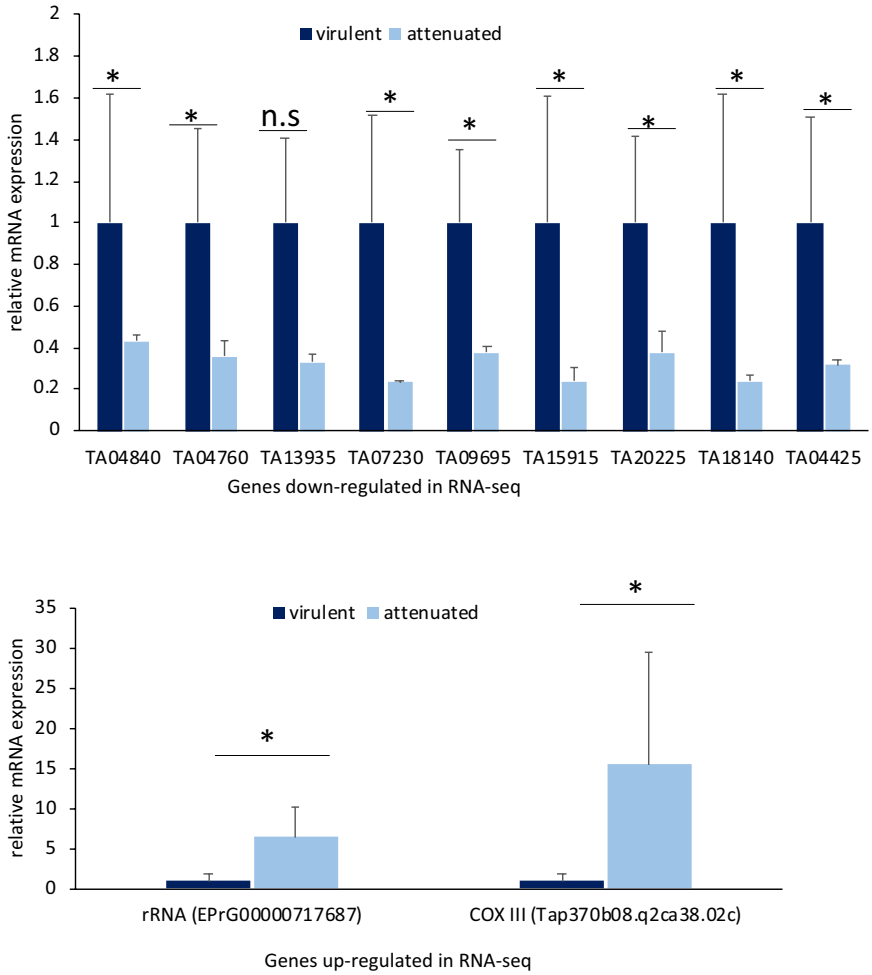

Figure 5. qRT-PCR validation of the selected up or down-regulated *Theileria* genes identified by RNA-seq. The y-axis shows the relative mRNA expression. *Theileria annulata* actin was used as a reference gene. Asterisks show significant difference in gene expression; (n.s) = non-significant.

provided by PCA where clustering of the samples with passage level can clearly be observed (Fig. 1), and PC1 explained 50.6% of variation in the data.

In order to include genes of putatively greater biological relevance, we refined the list of differentially expressed genes to include only those that exhibited a twofold or greater average difference in expression between the virulent and attenuated (Table 1). Based on the fold change value, the largest difference between virulent and attenuated cell line was observed for the host more than parasite genes. A total of 168 host genes showed a fold change value of 2 or more with significant padj values. Among these genes, 99 were up-regulated and 69 were down-regulated. Given the cancer-like transformation of infected macrophages by *T. annulata*, it is not surprising that a large proportion of the differentially expressed genes with fold change above the twofold cut-off, encode proteins that have been associated with tumours. The most striking host gene expression difference (exceeding sixfold) was observed for UPK3BL1 (*Bos taurus* uroplakin 3B-like) followed by P2RX3 (purinergic receptor), KCNQ2 (potassium voltage-gated channel subfamily Q member 2) and KRT17 (keratin 17) with a fold change of 5.8, 5.7 and 5.4, respectively.

UPK3BL1 is implicated in stabilizing and strengthening the urothelial cell layer of the bladder, it has been shown to be highly expressed in mesotheliomas and in the urothelial tumours of the urinary bladder[36]. P2RX3, has been reported to be involved in tumour formation and play an important role in malignant transformation of several different cell types and they are highly expressed in cancer[37,38]. The biological relevance of its high expression in attenuated *T. annulata* infected cell lines has not been investigated. KCNQ2 gene is known to encode for subunits of a potassium channel complex and to be involved in membrane polarisation, they are acting as a suppressors or activator of gastrointestinal cancer[39]. The most down-regulated gene was ZNF618 (zinc finger protein 618) followed by CD69 (*Bos taurus* CD69 molecule), LPAR3 (*Bos taurus* lysophosphatidic acid receptor 3) with a fold change of − 6.5, − 6 and − 5.6, respectively. CD69 is a glycoprotein type II known to regulate inflammation through T-cell migration and retention in tissues and has a crucial role in inducing the exhaustion of tumour-infiltrating T cells[40,41]. CD69 is also an AP-1 target gene[42,43], its role in *T. annulata* pathogenesis and attenuation might be important to investigate. As already mentioned, the genes that exhibited statistically significant passage differences in expression and are also supported by the highest fold difference in expression can be investigated as candidates underlying attenuation.

A comparison of Beja DEGs with attenuated Ode showed five common genes. Two of them were down-regulated (IL12A and TXNIP), which could be potential attenuation markers, and three were up-regulated (CAPN3, CTNND1, IL4I1). The up-regulated genes could be that they are not involved in the attenuation process but rather other mechanisms such as immunosuppression or inflammation. For example, IL4I1 (interleukin 4 induced 1) protein may play a role in immune system escape as it is expressed in tumour-associated macrophages and it was described as a metabolic immune checkpoint which promotes tumour progression[44]. While Calpain-3 (CAPN3) is an intracellular cysteine protease, it has been shown in human melanoma that a variant of this gene called hMp84 increases the intracellular production of ROS (Reactive Oxygen Species) leading to DNA damage[45] which has been also confirmed in *Theileria* to cause an oxidative stress by elevation of ROS and disruption of the redox balance which are required for parasite transformation[46]. This is interesting as it might explain why in attenuated macrophages H2O2 type of oxidative express accumulates in spite of lower JNK (an antioxidant) activity[47]. It should be noted that H2O2 levels in virulent and attenuated *T. annulata*-transfomed Ode macrophages inversely correlated with their Matrigel traversal capacity[47].

Parasite-dependent induction of key host cell transcription factors (AP-1, NF-kB, etc.) in *Theileria annulata* transformed cells causes massive reprogramming of the host cell transcriptome[33,48]. Thousands of host cell genes increase or decrease upon *Theileria annulata*-induced cell transformation. We conducted an inversed comparison with previously published *T. annulata* infected BL3 and BL20 cell lines differential transcriptome in order to find genes that have been induced by infection and are down-regulated in attenuated passage or vice-versa (Fig. 3). For example, MAPK13 (mitogen-activated protein kinase 13) has been found in gynaecological cancer to be preferentially expressed in cancer stem cells and it is implicated in the tumour-initiation[49]. It was induced by infection in TBL20 but down-regulated in attenuated Beja (log2 Fold Change = − 1.2; p adj = 0.0001). AnxA1 (annexin A1) is a phospholipid-binding protein that has been described to have either activator or suppressor role in different cancer types and stages[50]. In TBL3 and 20, this gene was up-regulated by infection. It is found to be significantly down-regulated in attenuated transcript (log2 Fold Change = − 1.08; p adj < 0.001). FAM161A (centrosomal protein) is known to be involved in development of retinal progenitors during embryogenesis and it is a part of microtubule-organizing centres in cultured cells and it has been demonstrated to be involved in the intracellular microtubule network[51,52], it was down-regulated in attenuated Beja (log2 Fold Change was − 1.005; p adj value = 0.01).

It has also been shown in breast cancer research that apolipoprotein modulates the cholesterol metabolism and stimulates the proliferation, migration, and tumour growth[53]. In our list Apolipoprotein 3 (APOL3) is induced by *Theileria* infection and down-regulated upon attenuation (log2 Fold Change was − 1.09; p adj value < 0.001) which suggest its implication in the attenuation process. Tumour protein 63 (p63) is known to enhance the migration, invasion, and tumour growth in human cancer[54]. This gene was down-regulated in attenuated passage (log2 Fold Change was − 1.09; p adj value < 0.001).

ARHGAP9 (Rho GTPase-activating protein 9), a tumour suppressing gene in bladder cancer[55] that has been reported to be suppressed by infection in TBL, was significantly up-regulated in the attenuated passage (log2 Fold Change = 1.6; p adj < 0.001) but other ARHGAP were significantly down-regulated such as ARHGAP15 (log2 Fold Change = − 1.05; p adj < 0.001) and ARHGAP29 (log2 Fold Change = − 1.32; p adj < 0.001). These genes could also be evaluated as candidates involved in the attenuation process.

Our results suggest that long term passage of infected cells primarily results in transcriptional down-regulation of some cytokines (e.g., IL6, IL12 and IL21) and genes that may be active in pathways controlling the interaction between the cytokines and their receptors. Remodelling the host cell transcriptome in this way by

attenuation appears consistent with the previous suggestion that upregulation of pro-inflammatory cytokines might play a role in pathology. This was on the basis of the observation that the clinical signs of *T. annulata* infection, particularly fever, cachexia, leucopoenia and anaemia are the same signs that follow experimental administration of pro-inflammatory cytokines[56].

A high proportion of the differentially expressed genes (51 host genes in total where 14 of them had fold changes between 2 and 6) are unannotated and the importance of these proteins is unclear at this time. However, identification of these genes may provide vital information pertaining to attenuation of *T. annulata* infected cell lines. It is also important to mention that besides the reduced expression of pro-inflammatory cytokines that we confirm, attenuation has also previously been associated with reduced expression of the matrix metalloproteinase MMP9[25], which is linked to the invasive capacity of virulent cell lines[57]. In *T. annulata*-transformed macrophages, MMP9 transcription is regulated by Activator protein 1 (AP-1)[27]. Down-regulated AP-1 activity has been demonstrated in Indian (Ode) and Tunisian (Jed4) cell lines[10,27,28]. However, our comparative RNA-seq transcription profiles did not reveal a downregulation of this candidate attenuation marker. Interestingly, MMP9 had very low level of read counts in our RNA-seq data. Therefore, differential expression of genes other than MMP9 could be associated with diminished virulence of attenuated Beja, such as those presented in Fig. 4 (for example MAPK13, ANXA, etc.…). Also, the fact that Beja cell line is less virulent than Jed4 at low passage should be taken into account as it might be that the expression level of AP-1 is low at virulent passage[58]. Although we did not measure AP-1 activity in virulent versus attenuated Beja, we found potential AP-1 binding sites on promoters of 30 genes that were down-regulated in attenuated Beja. The presence of potential AP-1 binding sites on down-regulated gene promoters and dampened AP-1 activity in attenuated cells suggest that reduced AP-1 activity might explain the attenuated phenotype of high passage Beja leukocytes. It has also been reported that NF-κB (nuclear factor kappaB) is activated in *Theileria* transformed cells via the IKK (IκB kinase) complex to regulate cell proliferation and protect it against apoptosis[35,59] but its role in the attenuation process is not yet demonstrated. In our DEGs list we found NF-κB binding sites in 13 up-regulated genes and 14 down-regulated genes promoters. The role of NF-κB in the attenuation process of *T. annulata* need to be investigated.

As regards to the parasite, it has previously been suggested that both altered gene expression and clonal selection of parasite populations may be involved in the loss of pathogenicity of *T. annulata* during continuous in vitro culture[60]. For the parasite transcript, only five down-regulated genes had a fold change more than − 2, all of which were hypothetical proteins. Availability of Beja genome sequence will likely make it possible to expand the annotation available for these genes, particularly given that analysis of antigen gene sequences, including TaSP, have shown that the Tunisian *T. annulata* differs from parasites from other locations including Turkey from where the reference genome is derived[61]. There was one up-regulated parasite gene that had a fold change of 3 (cytochrome C oxidase subunit III), while the most of the DEGs have a fold change value less than 2. Nonetheless, we below describe some of the parasite genes such as TA08610, TA03875, TA18945, TA20090 and TA09695 that likely have a role in *T. annulata* virulence.

TA08610 is a gene coding for the prefoldin subunit (one of the prefoldin complex), a cytoplasmic chaperone involved in protein folding with multiple roles in different tumours. The alpha subunit has a tumour-suppressing role and the beta subunit is involved in tumorigenesis[62,63]. This gene was significantly down-regulated in the attenuated cell line dataset (log2 Fold Change = − 1.5; $p < 0.001$). TA03875 codes for RNA poly(A)-binding protein which is a post-transcriptional regulator of gene expression controlling mRNA stability, polyadenylation, and other functions. An aberrant expression of this gene has previously been reported to be associated with the development of breast cancer[64,65], this gene was down-regulated in attenuated passage in the attenuated Beja passage (log2 Fold Change = − 0.9; $p < 0.001$). We also found that this gene contains a nucleus localization signal (NLS) which suggest its possible transport into the host nucleus, however this needs to be confirmed since previous studies showed that some genes such as TaMISHIP *(T. annulata* proline-rich microtubule and SH3 domain-interacting protein) presented NLS but they were not detected in the host nucleus[66]. Additionally, we checked some of the previously characterised *T. annulata* effector proteins (TaSP, TashATs, TaHSP90, TaPIN1, Ta-p104; reviewed by Tajeri and Langsley (2021)[67] for their possible differential expression, all of these genes were unchanged in our list.

It is known that protozoa execute a strict regulation on the expression of genes that are involved in their pathogenicity[68]. In *Theileria* transcripts, we found three hypothetical proteins (TA04840, TA04760 and TA09695) that have been reported to be involved in gene expression and in translation. TA04760 is involved in the regulation of various process such as cellular process, biological process, transcription process, regulation of gene expression and RNA biosynthetic processes. These genes were down-regulated in the attenuated passage. The role of the mentioned hypothetical proteins needs to be characterized in order to confirm their implication in the attenuation process and if they are involved in regulation of the host gene expression. If this is confirmed, it might be helpful for the control of the *T. annulata* by manipulating gene expression as it was described in *Plasmodium falciparum* by using Peptide Nucleic Acids (PNAs) for gene silencing which induced down-regulation of stably expressed transgene and endogenous essential gene led to reduction of plasmodium viability[69].

In conclusion, our comparative analysis catalogued for the first time differentially expressed host and parasite genes between virulent and attenuated Tunisian cell lines (Beja). A major finding of the study is that both the bovine host and *Theileria* display changes in gene expression following attenuation. Besides the use of statistical significance and average fold change to prioritize candidates, recent advances in cancer also enabled description of additional genes with known roles in different tumour and their potential role in *T. annulata* pathogenesis. In particular, the number of host (e.g., ZNF618, LPAR3, APOL3, CD69) and parasite (TA03875, TA04840, TA04760 and TA09695) differentially expressed genes listed herein could be further validated using previously described methods such as the Matrigel chamber assay[33], Enzyme-Linked Immunosorbent Assay (ELISA)[70], immunoblotting and immunofluorescence[71], in order to find potential attenuation markers. Such studies would contribute to a better understanding of the mechanism driving attenuation, reduce the time needed to establish

an attenuated cell line and contribute to the application of 3Rs principle in *Theileria* research by reducing the number of animals required for the validation of attenuation.

Materials and methods

Theileria annulata cell line and culture

The *T. annulata*-infected cell line used in this study is a stock of the Beja strain (also referred to as CL1 in literature). The strain was originally isolated in 1989 from an infected five-year-old crossbred cow with an acute form of TT and it was used to vaccinate cattle in Tunisia against tropical theileriosis[24,72]. Phenotyping of the Beja strain, up to passage 300 using flow cytometry and specific monoclonal antibodies, revealed a dominance of the myeloid lineage (macrophages/monocytes). Furthermore, the markers associated to B cells were not entirely absent relative to the parental lines, which tend to indicate that a minor population of infected B cells (2–8%) may persist during the process of attenuation[58]. The cell line was frozen in liquid nitrogen and was resuscitated and propagated in RPMI supplemented with 10% FBS (RPMI-FBS), 2 mM L-alanyl-l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. The culture flasks (three replicates for each passage) were incubated at 37 °C in a 5% CO_2-in-air atmosphere.

RNA extraction

RNA was extracted from three replicates of both virulent (passage 26) and attenuated (passage 296) cells using a Direct-Zol RNA miniprep plus kit (Zymo Research Europe GmbH, Freiburg, Germany) according to the manufacturer's guidelines. The transcriptome of the *T. annulata* virulent and attenuated Beja cell line from Tunisia was subsequently determined using Illumina RNA sequencing.

RNA quantification, qualification and library preparation for transcriptome sequencing

Three purified RNA replicates from each passage with concentrations from 168 to 361 ng/µl were sequenced at Novogene laboratories (Cambridge Science Park, Cambridge, CB4 0FW, United Kingdom). Messenger RNA was purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand cDNA was synthesized using random hexamer primers, followed by second strand cDNA synthesis using dUTP for directional library or dTTP for non-directional library. The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms. The clustering of index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated. Raw data were first processed through in-house perl scripts where clean reads were obtained by removing reads containing adapter, reads containing poly-N and low-quality reads. The RNA seq raw data has been submitted to the SRA database (sequence read archive) under the accession number PRJNA957332.

Mapping to the reference genome and quantification of gene expression levels

Reference genome annotation files were downloaded for the *T. annulata* Ankara cell line (PRJNA16308) and *Bos taurus* (PRJNA391427). Index of the reference genome was built using Hisat2 v2.0.5 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.0.5. The mapped reads of each sample were assembled by StringTie (v1.3.3b)[73] in a reference-based approach. Feature Counts v1.5.0-p3 was used to count the reads numbers mapped to each gene after which the FPKM (number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced) of each gene was calculated based on the length of the gene and reads count mapped to this gene.

Differential expression analysis, GO and KEGG enrichment analysis of differentially expressed genes

Differential expression analysis of the two groups was performed using the DESeq2 R package (1.20.0). The resulting *P*-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate[74]. In general, genes with p value ≤ 0.05 were assigned as differentially expressed. A more stringent criteria of an absolute fold change of 2 or more with p adj value ≤ 0.05 was initially applied to both the host and parasite genes. The list of differentially expressed parasite genes was subsequently extended to include genes that exhibited significant differential expression based on the p value only, but additional possessed important features such as transmembrane domains and/or signal peptides.

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the clusterProfiler R package, in which gene length bias was corrected. GO terms with corrected p-values less than 0.05 were considered significantly enriched by differentially expressed genes. Cluster Profiler R package was used to test the statistical enrichment of differential expression genes in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways[75].

Additional bioinformatic screen: in silico search for differentially expressed *T. annulata* proteins with signal peptides, transmembrane domains or nuclear localization signals and prediction of AP-1 binding sites on promoters from differentially expressed bovine genes

PiroplasmaDB (https://piroplasmadb.org/piro/app/) was used to further characterize *Theileria* DEGs including their assignment to GO functions and predictions of the presence of a signal peptide (SP) and transmembrane domains (TMDs). In order to find nuclear localization signals (NLS) we used the novopro lab online tool (https://www.novoprolabs.com/tools/nls-signal-prediction). We also conducted a bioinformatic screen of our host DEGs

list for the presence of AP-1 (activator protein 1) and NF-kappa B potential binding sites using the PROMO website (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). A promoter region was considered 1000 bp upstream of the start codon and retrieved from Ensembl website (https://www.ensembl.org/biomart/martview/151e6b41a7a359e264c5aa1d96328fa7).

Validation of RNA-seq results by qRT-PCR

The differential gene expression was validated by quantitative reverse transcription PCR (qRT-PCR). cDNA synthesis was conducted using the ProtoScript® II First Strand cDNA Synthesis Kit (New England BioLabs Inc, Ipswich, US) according to manufacturer´s instructions from the same RNA samples used for the RNA-seq. The specific primer pairs for 11 differentially expressed *T. annulata* genes were designed using Geneious software[76] (Supplementary Table 1). The *Theileria* actin gene (TA13410) was used as a reference gene. The qPCR reactions were performed using Luna® Universal qPCR Master Mix (New England BioLabs Inc) according to the manufacturer's protocol. The $2^{-\Delta\Delta CT}$ methodology was used to estimate the relative gene expression levels[77].

Data availability

RNA seq raw reads have been uploaded to Sequence Read Archive (SRA) repository under the BioProject Number PRJNA957332.

Received: 30 May 2023; Accepted: 19 October 2023
Published online: 24 October 2023

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Acknowledgements
Khawla Elati was supported by a doctoral scholarship from the German Academic Exchange Service (DAAD). Isaiah Obara was supported by DFG Grant OB 490/2-1. Ard Nijhof was funded by the German Federal Ministry of Education and Research (BMBF, Grant Number 01KI1720) as part of the Junior Research Group "Tick-borne Zoonoses." Open Access Funding provided by Freie Universität Berlin.

Author contributions
A.N., K.E., and M.D. Conceived the experiments, A.N. Funding acquisition, K.E. Conducted the experiments, K.E., S.T. and I.O. analysed the results, K.E. wrote the original draft, I.O., S.T., A.N., M.M., M.D. and E.Z. Reviewed the manuscript. All authors contributed to the manuscript and approved the submitted version.

Funding
Open Access funding enabled and organized by Projekt DEAL.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-45458-9.

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Chapter 4:

In vitro infection of bovine erythrocytes with *Theileria annulata* merozoites as a key step in completing the *T. annulata* life cycle *in vitro*

The content of this chapter was published as an open access research article in *Scientific Reports.*

Elati, K., Tajeri, S., Mugo, R.M. *et al.* In vitro infection of bovine erythrocytes with *Theileria annulata* merozoites as a key step in completing the *T. annulata* life cycle in vitro. *Sci Rep* **14**, 3647 (2024). doi: 10.1038/s41598-024-54327-y

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In vitro infection of bovine erythrocytes with *Theileria annulata* merozoites as a key step in completing the *T. annulata* life cycle in vitro

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Theileria annulata **is a protozoan parasite with a complex life cycle involving a bovine host and a tick vector. It is transmitted by *Hyalomma* ticks and is the causative agent of tropical theileriosis, a debilitating and often fatal disease in southern Europe, northern Africa and large parts of Asia. Understanding the biology of different life cycle stages is critical for the control of tropical theileriosis and requires the use of experimental animals which poses an ethical concern. We present for the first time the in vitro infection of red blood cells (RBCs) with *T. annulata* differentiated schizonts. The Ankara cell line of *T. annulata* was cultured at 41 °C for nine days to induce merogony and subsequently incubated with purified RBCs for one to three days. Percentage of parasitized erythrocyte (PPE) over the short culture period was estimated by Giemsa staining (0.007–0.01%), Flow cytometry activated sorting (FACS) (0.02–1.1%) and observation of FACS sorted cells by confocal microscopy (0.05–0.4%). There was a significant difference in the PPE between FACS and the two other techniques (one-way ANOVA followed by Tukey test, P = 0.004) but no significant difference was observed between the confocal imaging and Giemsa staining methods (ANOVA one-way followed by Tukey test, P = 0.06). Importantly, all three complementary methods confirmed the invasion of RBCs by *T. annulata* merozoites in vitro. Although the experimental conditions will require further optimization to increase the PPE, the in vitro infection of RBCs by *T. annulata* merozoites is pivotal in paving the way for the eventual completion of the *T. annulata* life cycle in vitro when combined with artificial tick feeding.**

Keywords *Theileria annulata*, In vitro infection, Merozoites, Bovine erythrocytes

Cattle theilerioses, including diseases caused by *Theileria annulata* and *Theileria parva*, are tick-transmitted and cause serious impediments to livestock productivity in Africa, Southern Europe and Asia, resulting in a large economic burden. Tropical theileriosis caused by *T. annulata* occurs in Northern Africa, Southern Europe and large part of Asia where *Hyalomma* vector ticks occur[1], while East Coast fever, caused by *T. parva*, is transmitted by *Rhipicephalus appendiculatus* ticks distributed in large parts of eastern, central and southern Africa[2–4]. For *T. parva*, the losses were estimated to be 300 million US$, with a mortality of 1.1 million cattle annually only in sub-Saharan Africa[5,6]. For *T. annulata*, the total losses were for instance estimated to reach 598,133 US $ for 2 years in endemic regions in Turkey only[7].

Both protozoan parasites have similar life cycles involving a mammalian host and a tick vector. For *T. annulata*, *Hyalomma* ticks infect cattle by releasing sporozoites with their saliva during feeding on bovine hosts. The

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sporozoites infect leukocytes and after differentiation to macroschizonts induce uncontrolled proliferation of the infected cell, resulting in a cancer-like phenotype. The macroschizonts undergo merogony and produce a large number of microschizonts and merozoites, which upon rupture of the infected cell are released into the circulation and actively invade erythrocytes where they develop further into piroplasms. Ticks become infected with *Theileria* when they imbibe infected erythrocytes during a blood meal[8,9].

Studies involving different life stages of *T. annulata* (schizonts, piroplasms and sporozoites) and parasite-vector interactions often require the use of experimental animals, which is of ethical concern. Artificial tick feeding systems (ATFSs) based on silicone membranes or animal-derived membranes have been successfully adapted for the tick vectors of *T. annulata*, such as *Hyalomma dromedarii, H. anatolicum*[10], *H. lusitanicum*[11], *H. excavatum* and *H. marginatum*[12]. *Theileria* transmission has also been studied in vitro using naturally infected blood. *Hyalomma anatolicum* nymphs were for instance naturally infected with *Theileria lestoquardi*-infected blood in vitro using an ATFS[13] and *Rhipicephalus appendiculatus* adults that were infected with *T. parva* as nymphs by acquisition feeding on an infected calf were demonstrated to secrete infective *T. parva* sporozoites with their saliva into an ATFS[14]. Both examples depended on the use of experimental animals to obtain the piroplasm stage infective to ticks.

There has been only one reported attempt to infect erythrocytes in vitro with *T. annulata* schizonts[15]. In that experiment, assessment of invasion was solely based on the examination of Giemsa-stained blood smears, which lacks sensitivity as piroplasms cannot always be clearly distinguished from artefacts to confirm successful invasion. Additional methods to confirm invasion are therefore required, as for instance established for *Plasmodium falciparum*, where successful invasion of RBCs with *P. falciparum* in vitro has been validated using additional cytoplasmic and nuclear dyes[16–18].

In the present work, we confirmed infection of RBCs with *T. annulata* merozoites generated in vitro using a combination of flow cytometry and confocal microscopy, in addition to the classical Giemsa staining method. We recently demonstrated that it is possible to successfully feed all stages of the *T. annulata* vectors *H. dromedarii*, *H. excavatum* and *H. scupense* in vitro on silicone-based membranes (Elati et al., 2023, submitted). If this could be combined with the feeding of RBCs infected with *T. annulata* merozoites in vitro, it would bring the objective of completing the full lifecycle of *T. annulata* in vitro one step closer.

Results

The infection of RBCs with *T. annulata* merozoites or schizonts was confirmed by three techniques that showed different percentages of infection. Flow cytometry results with SYBR green I and DDAO staining showed that RBCs incubated with *T. annulata* schizonts-infected leukocytes have higher PPE than cells incubated with merozoites. The microschizonts-infected PPE decreased from 1.22% at day 1 to 0.61% at day 3 (chi-square test, $P < 0.0001$). While the cells infected with merozoites had a lower infection rate from the first to the third day, ranging from 0.02 to 0.04% (chi-square test, $P = 0.1$) (Fig. 1).

The sorted cells observed by confocal microscopy showed clear infection of RBCs with *T. annulata* piroplasms (Fig. 2) but with lower infection rates compared to FACS. Free parasites and uninfected RBCs were also observed. The PPE varied between 0.05% and 0.4% for cells incubated with microschizonts-infected leukocytes (Chi-square test, $P = 0.01$) while no infected cells were observed for RBCs incubated with merozoites (Fig. 4).

The microscopic observation of Giemsa-stained thin smears from the same culture on different days confirm the infection of RBCs with *T. annulata* piroplasms (Fig. 3) but the PPE also varied between 0.007 and 0.01% for merozoite-infected cells with (Chi-square test, $P = 1$) and between 0.008 and 0.01% for cells infected with microschizonts (Chi-square test, $P = 0.5$), lower than the value observed with FACS and confocal microscopy (Fig. 4).

The PPE varied significantly between FACS and both confocal microscopy and Giemsa staining techniques for cells incubated with merozoites or microschizonts-infected leukocytes (ANOVA one-way followed by Tukey test, $P = 0.004$). While no significant difference was observed between confocal imaging and Giemsa staining (ANOVA one-way followed by Tukey test, $P = 0.06$).

Discussion

The present work aimed to infect RBCs with *T. annulata* in vitro, and evaluate the invasion using three complimentary techniques, without the need for experimental animals.

In this study, we followed four key steps to infect and validate the invasion of erythrocytes with *T. annulata* in vitro. These were: (i) induction of merogony from an initially established schizont-transformed leukocyte culture, (ii) collection of blood samples and isolation of erythrocytes, (iii) infection of erythrocytes by mixing of the differentiated *T. annulata* parasites with the isolated RBCs and subsequent incubation at 37 °C and (iv) validation of invasion using three complementary methods allowing for a calculation of the PPE over time.

Our choice of techniques for confirming invasion of erythrocytes was informed by findings from previous experiments assessing invasion of erythrocytes by *Plasmodium* spp. by combining different methods such as Giemsa staining, immunofluorescence microscopy, flow cytometry, PCR-based methods and confocal microscopic imaging[17,19,20]. It is important to emphasise that a combination of methods was shown to be essential for confirmation of pathogen invasion into erythrocytes[20].

Giemsa staining, FACS and confocal microscopy all confirmed the invasion of *T. annulata* merozoites into RBCs over a short culture period, albeit with low parasitaemias. The lowest PPE was recorded for the Giemsa-stained samples (0.007–0.01%) followed by confocal microscopy (0.05–0.4%). The highest PPE was recorded by FACS (1.2%) for cells incubated with schizont-transformed leukocytes on the first day of culture. FACS is a sensitive and powerful technique that allows for the detection of low parasitaemia levels, which is particularly helpful when the number of infected erythrocytes is low. The sensitivity of FACS depends on the fluorescent labels used and the sensitivity of the flow cytometer[20,21]. One possible explanation for the relatively high PPE

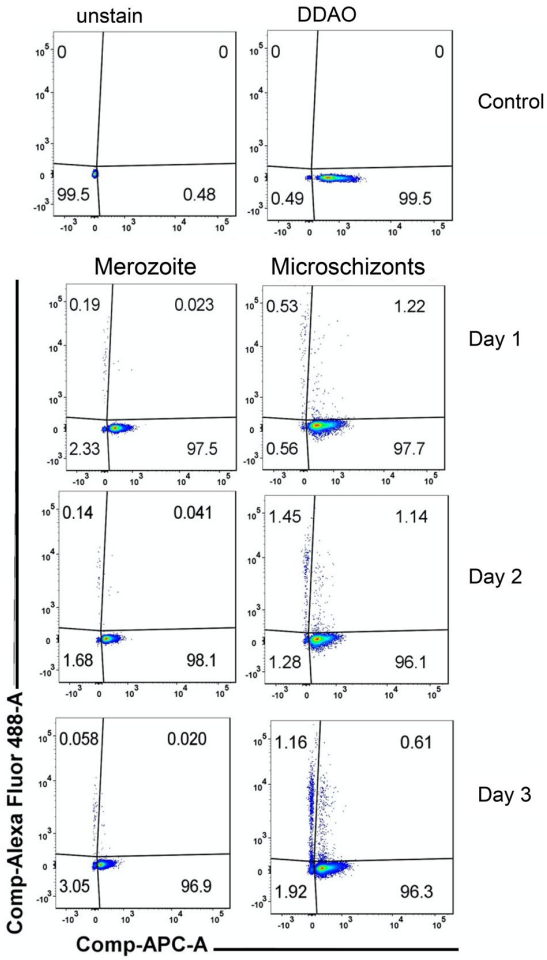

Figure 1. Infection of RBCs by *T. annulata*, flow-cytometry dot-plots (APC-DDA versus Alexa Fluor 488A-SYBR Green) showing the percentage of infected erythrocytes (PPE) incubated with *T. annulata* merozoites (left) or microschizont-infected leukocytes (right) for one, two or three days of cultivation. Unstained cells and RBCs stained only with DDAO (single stain) served as control.

estimated by FACS is that it potentially includes the parasites present on the surface of the cells, which are cleaved during cell sorting and this may explain both uninfected RBCs and free parasites were observed by confocal microscopy after sorting.

There are some limitations that must be taken into account when using FACS for this purpose. The first factor to consider is sample preparation. FACS requires that the cells are in a single-cell suspension for analysis. Achieving a single-cell suspension from whole blood samples can be challenging especially when erythrocytes are infected with the parasite. The presence of cell debris, clumped cells, or host cell aggregates may interfere with accurate analysis and lead to incorrect assessment of parasitaemia. Moreover, loading a large amount of sample requires a lot of time for analysis, which is why only ten microliters of culture were used for FACS sorting in this study.

In addition, flow cytometry instruments and reagents are expensive, specialized training is required to operate the equipment and accurate analysis of the data and the whole process is time-consuming.

Despite these limitations, flow cytometry was shown to be a valuable tool for assessing infected erythrocytes. Combining flow cytometry with other complementary techniques can improve accuracy and provide more comprehensive information about the dynamics of parasite infection in erythrocytes.

Giemsa staining is usually a technique used in acute phases of infection but has limited use during the carrier state of infection when low parasitaemia are present, due to the risk of obtaining false negative results or underestimating parasitaemia[22]. As for the confocal microscopy, although we saw clear evidence for the infection of RBCs with *T. annulata,* we were unable to fix the sorted cells on glass slides, mainly due to photobleaching. Fixing the cells and improving the quality of the captured images will be a priority for future research.

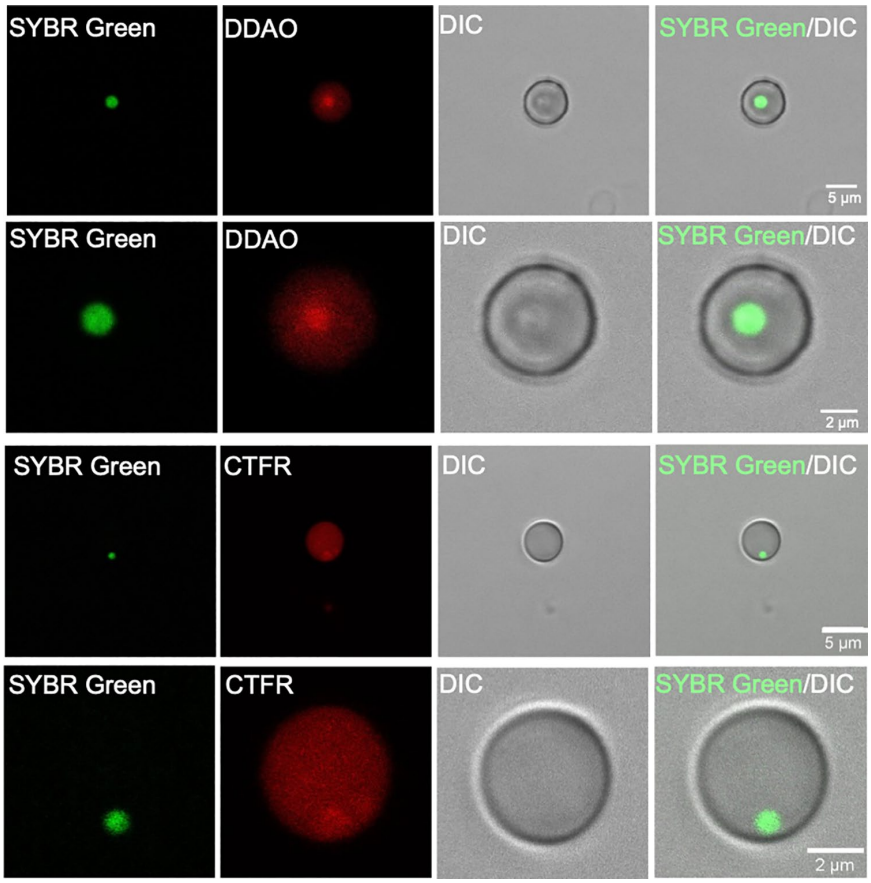

Figure 2. Bovine red blood cells infected with *T. annulata* in vitro and observed with confocal microscopy using DDAO or CTFR (red) for staining of the erythrocytes and SYBR green I (green) for DNA staining of piroplasms. *DIC* differential interference contrast microscopy.

It is possible that the low parasitaemia observed in this study could be attributed to the type of medium and serum used. For example, it has previously been shown for *Theileria uilenbergi* that the use of HL-1 medium supported the growth of the intraerythrocytic parasite for longer periods in culture[23]. Other studies showed that equine piroplasms grew better in M199 supplemented with 40% FBS where the heat inactivation of the serum negatively affected the growth and survival of the culture, thus suggesting an essential role of the complement system in the continuous culture of erythrocytic stages of *Theileria equi* (formerly referred to as *Babesia equi*)[24].

The complement system was also required for successful invasion of RBCs by *Babesia rodhaini*[25]. The addition of other supplement such as bovine serum albumin (BSA), lipids-cholesterol-rich mixture, insulin, transferrin, selenite, and putrescine could help improve the PPE and have been shown to replace the use of FBS in *Plasmodium* and *Babesia* cultures[26,27]. The purine compounds are also crucial for the growth of the parasite due to their inability to synthesise it, which justifies the use of hypoxanthine[23,24].

To improve schizont to merozoite differentiation yield, other methods based on chemical compounds such as apicidin[28] and colchicine[29] could be used. It was also reported that merogony could be induced using apicomplexan-specific histone deacetylase inhibitor FR235222, similar to Apicidin, which could be combined with elevated temperatures (41 °C) suggesting that two merogony inducing stimuli might act synergistically to generate higher merozoite numbers or more infectious merozoites[30].

Infection of RBCs with *Theileria* has been attempted for six strains of *T. parva* using microschizonts and free merozoites incubated at 37 °C in normal atmosphere (5% CO_2) and RMPI supplemented with 20% FBS but no penetration of merozoites to RBCs was observed in Giemsa-stained smears[31]. A single study showed the possible infection of bovine, ovine and caprine erythrocytes in vitro with *T. annulata* macroschizonts also used Giemsa staining, where the first piroplasm form was observed at day 19 for bovine RBCs, with a parasitaemia level varying from 0.01% to 0.03%[15]. There are several ways in which the present findings extend previous research. Foremost, we use a combination of three complementary methods to confirm the invasion of RBCs by *T. annulata* merozoites in vitro. As mentioned, this is an important step towards completion of the life cycle in vitro as techniques like artificial tick feeding can be utilized to feed infected erythrocytes to tick vector. The

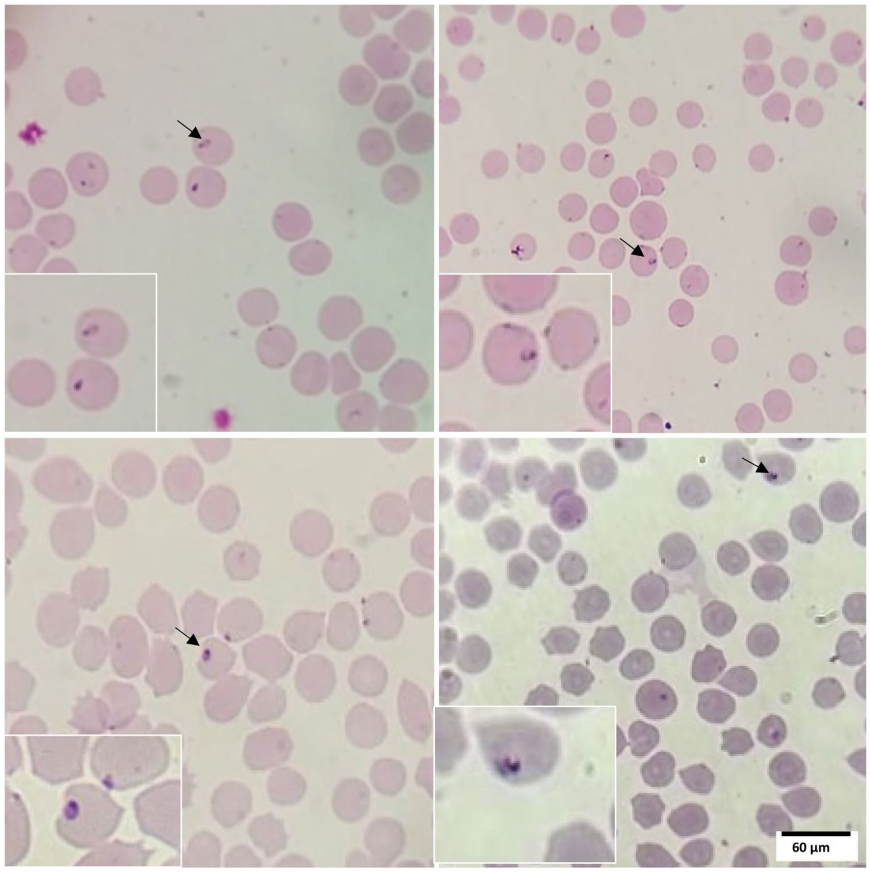

Figure 3. Giemsa-stained blood smear prepared from in vitro co-cultures of bovine RBCs and *T. annulata* merozoites/microschizonts. The piroplasm-like forms of the parasite are clearly visible within the infected RBCs.

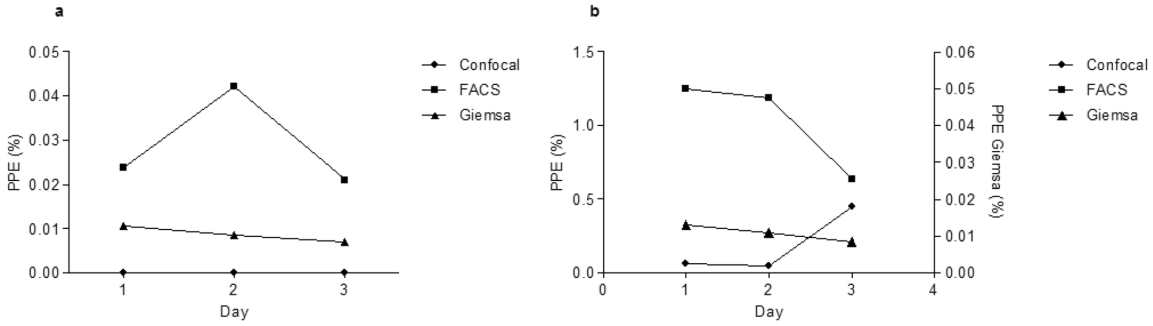

Figure 4. Variation of percentage of parasitized erythrocytes (PPE) incubated with merozoites (**a**) or with microschizont-infected leukocytes, (**b**) over 3 days of culture.

other key methodological improvement in the generation of infected erythrocytes relates to the duration of incubation before successful infection of erythrocytes. Herein, piroplasms were observed from the first day of culture as opposed to the 19 days previously reported[15]. This is likely attributable to the fact that we incubated the RBCs with cells already differentiated into microschizonts and free merozoites. Also, important to note is that we used different culture conditions (reduced oxygen levels) while the previous two studies[15,31] used normal culture conditions with CO_2. One other important difference was in the composition of the media, as we used hypoxanthine which is necessary for the growth of the parasite.

Although several studies have been conducted on the mechanism used by *T. annulata* sporozoite to invade and transform bovine leukocytes[32,33], the process of invasion of RBCs by *T. annulata* merozoites has not yet been investigated. For the closely related *T. parva*, it has been demonstrated that the mechanism of entry of merozoites into RBCs is similar to that of sporozoite invasion[32,34]. There are far more studies on *Plasmodium* invasion of RBCs and their in vitro culture which is facilitated by the fact that re-invasion of the parasite into new RBCs takes only 1–2 min[35]. Furthermore, the structure of infected RBCs is altered to improve their survival resulting in greater rigidity, adhesion and permeability of the cell membrane[36]. The validated infection of RBCs in vitro and the establishment of a continuous culture of the piroplasm stage of *T. annulata* will serve as starting point for conducting further studies to understand the invasion process of *T. annulata* merozoites.

We present for the first time a validation of infection of bovine RBCs with *T. annulata* merozoites in vitro and confirmed the invasion using three complimentary techniques. The current findings set the stage for additional experiments aimed at increasing the number of parasitized RBCs and maintaining a continuous culture of the intra-erythrocytic stages of the parasite. Ultimately, these infected erythrocytes will be an important material for the infection of ticks thus completing the life cycle of *T. annulata* in vitro while at the same time adhering to the 3Rs principle in humane animal research by reducing the use of animals for this purpose. This model could also be applied to other pathogens such as *T. parva* and *T. lestoquardi* which have similar life cycles. Studying in vitro infection of erythrocytes with *T. annulata* can help researchers to understand not only the molecular mechanisms that control the interaction between the parasite and host cells but also the invasion process.

Materials and methods

Parasite culture and purification of microschizonts and merozoites

The *T. annulata* Ankara cell line at passage 4 established as previously described[37] was propagated in a 75 cm^2 culture flask containing 50 ml RPMI 1640 medium supplemented with 10%(v/v) heat inactivated foetal bovine serum (FBS). The medium was buffered with 20 mM HEPES (*N*[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid]) and supplemented with 2 mM L-alanyl-lglutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin. The culture was placed at 41 °C for nine days in order to induce merogony and obtain a sufficient number of merozoites[38]. The culture was diluted (1:1) at day 2. At day 9, merozoites were isolated by differential centrifugation[38] from an initial number of schizonts estimated to be 1.45×10^6 cells/ml. The cells were first centrifuged at 120*g* for 5 min, resulting in a pellet that mainly contained undifferentiated cells infected with macroschizonts. The supernatant was then centrifuged at 1000*g* for 15 min, the pellet that was formed during this step mainly contained microschizonts. A third spin of the supernatant at 4300*g* for 15 min was performed to obtain the merozoites-enriched pellet. The pellets from the second and the third centrifugation step containing microschizonts and free merozoites were separately used to infect RBCs as described below.

Non-parasitized erythrocytes for initiation of cultures

Blood was collected in tubes containing heparin as an anticoagulant from a piroplasm-free bovine calf and centrifuged at 800×*g* for 10 min. Plasma and buffy coat were discarded and the cell pellet containing the RBCs was washed 3–4 times with sterile modified Vega Y Martinez solution (mVYM)[24] or 10 ml of phosphate-buffered saline (PBS) without calcium and centrifuged at 800×*g* for 10 min. After the final wash, the supernatant was removed and the cell pellet was used in initiating cultures.

Sample preparation: parasite and red blood cells staining

Cells from the washed pellet were stained and used in initiating cultures. Staining procedures of erythrocytes with dichloro dimethyl acridin one succinimidyl ester (DDAO-SE) or Cell trace far-red (CTFR) (Thermo Fisher Scientific, United Kingdom) were adapted from previously described methods for *P. falciparum*[17,18]. The erythrocytes were stained with 20 mM DDAO-SE or 5 mM CTFR in complete RPMI medium and incubated under dark condition at 37 °C while shaking (150 r/min) for 2 h. The cells were subsequently pelleted by centrifugation at 2000 r/min (800×*g*) and incubated in RPMI complete medium for 10 min at room temperature to remove excess dyes. Finally, the pellets were washed three times at 2000 r/min (800×*g*) for 3 min with PBS. The resulting pellets were suspended in complete RPMI medium and stored at 4 °C until use. For the initial experiments both CFTR or DDAO were used, for later experiments only DDAO was used. Both stains had similar staining effectiveness when observed with confocal microscopy.

The parasite DNA was labelled using DNA-specific dye 1:1000 SYBR Green I (same sample volume) (Lonza, Rockland, ME, USA) and incubated for one hour at 37 °C in a shaking incubator. The cells were then washed once with PBS and assessment of invasion was done by flow cytometry[18]. The invasion rate was calculated as the percentage of RBCs positive for both the cytoplasmic dye DDAO and SYBR Green I.

Initiation and maintenance of cultures

RPMI 1640 (Gibco) supplemented with 20 mM HEPES (*N*-2 hydroxymethylpiperazine *N*1-2 ethanosulfonic acid) as buffer, 20% of foetal bovine serum (FBS), with 2 mM L-alanyl-l-glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin, 500 µl L-cysteine hydrochloride (200 mM), 250 µl of bathocuproine sulfonate (8 mM) and 200 µl of hypoxanthine (100 mM) was used for the culture[23,24,39,40].

The stained RBCs (10 µl containing approximately 15×10^6 RBCs) were placed in a 96-well plate after sedimentation. Purified and labelled merozoites in 190 µl of RPMI 20% FBS medium was subsequently added to the well and carefully mixed with the RBCs. The mixture was incubated at 37 °C in a humidified 5% CO_2, 2% O_2, and 93% N_2 atmosphere for 24 h, 48 h and 72 h. The medium was changed every 24 h for cells. Uninfected erythrocytes served as a negative control.

Assessment of cell viability
The fixable viability dye eFluor 506 (eBioscience, UK) was used as an exclusion dye for dead cells. The stock solution was diluted 1:1000. For 10^7 cells, 100 µl of dye (10 µl per 1×10^6 cells in suspension) was mixed thoroughly followed by incubation for 15 min at 4 °C under protection from light[41]. Finally, the cells were washed twice with PBS and analysed using flow cytometry.

Fluorescence-activated cell sorting of parasitised red blood cell
Stained RBCs were analysed using a BD FACSAria III (BD Biosciences, Germany) cell sorter. Total erythrocytes were gated using forward scatter area and side scatter area. RBCs singlets were then gated using the forward scatter height versus the forward scatter area followed by dead cells exclusion. Further, parasitised RBCs were identified using DDAO/CTFR (APC fluorophore) and SYBRGreen I (Alexa Fluor 488). Cells were sorted in ibidi microdishes (35 mm high) (ibidi GmbH, Gräfelfing, Germany). FlowJo software version 10 (Ashland, Becton, Dickinson and Company, 2023) was used for flow cytometry data analyses.

Giemsa staining and microscopic examination
Thin blood smears were prepared from the culture mixture on slides on different days and were stained for 40 min in 5% Giemsa solution (Merck, Darmstadt, Germany). Due to the low number of sorted cells, fixation using methanol and staining with Giemsa was not feasible as the cells were washed off during fixation. Smears were observed under a light microscope at × 1000 magnification to detect *T. annulata*-infected RBCs in 150 fields examined. The percentage of parasitized erythrocytes (PPE) was calculated as follows:

$$\mathrm{PPE}(\%) = 100 \times \left(\frac{\text{number of infected erythrocytes}}{\text{number of examined erythrocytes}}\right)$$

Confocal microscopy and image acquisition
The microdishes with the sorted cells were viewed using immersion oil (×63) on a Leica DMI 8 confocal microscope and images were acquired using LAS X (version 4.5.0.25531) software. Laser power was adjusted to 498 for Alexa 488 and to 638 for APC corresponding to SYBR green and DDAO, respectively. All acquired images were processed using Fiji ImageJ 2 version 2.9/1.53t[42].

Statistical analyses
The chi-square test for trend was used to compare the infection rate over time using an online version of Epitools (http://epitools.ausvet.com.au). A one-way ANOVA test was used to compare the difference of PPE between the three different techniques using GraphPad Prism 5 (GraphPad Software, San Diego California, United States). For both tests, a 5% threshold value was considered statistically significant.

Ethical approval
Permission to collect bovine blood from the calf was granted by the Landesamt für Gesundheit und Soziales, Berlin, Germany (LAGeSo) under registration number H0387/17. Blood was collected by a trained veterinarian and the animal was kept in a stable at the Institute for Parasitology and Tropical Veterinary Medicine of Freie Universitaet Berlin.

Data availability
All data generated or analysed during this study are included in this published article.

Received: 21 November 2023; Accepted: 11 February 2024
Published online: 13 February 2024

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Acknowledgements
The authors thank Dr Andreas Nerlich and Mrs Yvonne Weber for their technical assistance.

Author contributions
K.E.: conceptualization, methodology, investigation, formal analysis, visualization, writing—original draft preparation, S.T.: conceptualization, review and editing, R.M.M.: formal analysis, review and editing, I.O.: review and editing, M.A.D.: conceptualization, supervision, review and editing, E.Z. conceptualization, supervision, review and editing, A.M.N.: conceptualization, supervision, project administration, review and editing. All authors read and approved the final version of the manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL. Khawla Elati was supported by a doctoral scholarship from the German Academic Exchange Service (DAAD). Ard Nijhof was funded by the German Federal Ministry of Education and Research (BMBF, grant number 01KI1720) as part of the Junior Research Group "Tick-borne Zoonoses". Isaiah Obara was supported by DFG grant OB 490/2-1. Open Access Funding was provided by Freie Universität Berlin.

Competing interests
The authors declare no competing interests.

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Chapter 5:

In vitro feeding of all life stages of two-host *Hyalomma excavatum* and *Hyalomma scupense* and three-host *Hyalomma dromedarii* ticks

The content of this chapter was published as an open access research article in *Scientific Reports.*

Elati, K., Benyedem, H., Fukatsu, K. *et al.* In vitro feeding of all life stages of two-host *Hyalomma excavatum* and *Hyalomma scupense* and three-host *Hyalomma dromedarii* ticks. *Sci Rep* 14, 444 (2024). doi: 10.1038/s41598-023-51052-w

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In vitro feeding of all life stages of two-host *Hyalomma excavatum* and *Hyalomma scupense* and three-host *Hyalomma dromedarii* ticks

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Ticks are blood-sucking ectoparasites and can transmit various pathogens of medical and veterinary relevance. The life cycle of ticks can be completed under laboratory conditions on experimental animals, but the artificial feeding of ticks has attracted increased interest as an alternative method. This study represents the first report on the successful in vitro feeding of all life stages of two-host tick species, *Hyalomma scupense* and *Hyalomma excavatum*, and the three-host tick *Hyalomma dromedarii*. The attachment and engorgement rates of adults were 84% (21/25) and 76% (19/25) for *H. scupense* females. For adult *H. excavatum* and *H. dromedarii*, 70% (21/30) and 34.4% (11/32) of the females attached and all attached females successfully fed to repletion. The oviposition rates of the artificially fed females were 36.4%, 57.1% and 63.1% for *H. dromedarii*, *H. excavatum* and *H. scupense*, respectively, with a reproductive efficiency index varying between 44.3 and 60.7%. For the larvae, the attachment and engorgement rates were 44.2% (313/708) and 42.8% (303/708) for *H. dromedarii*, 70.5% (129/183) and 56.8% (104/183) for *H. excavatum* and 92.6% (113/122) and 55.7% (68/122) for *H. scupense*. The attachment and engorgement rates for the nymphs were 90.2% (129/143) and 47.6% (68/143) for *H. dromedarii*, 66.7% (34/51) and 41.2% (21/51) for *H. excavatum*, and 44.1% (30/68) and 36.8% (25/68) for *H. scupense*. Molting rates of the immature stages varied between 71.3% (216/303) and 100% (68/68) for the larvae and between 61.9% (13/21) and 96% (24/25) for the nymphs. The successful in vitro feeding of all stages of the three *Hyalomma* species makes this method a valuable tool for tick research, with potential applications in studies on the pathogens transmitted by these tick species such as *Theileria annulata*.

Ticks are hematophagous ectoparasites that may act as vectors for a variety of pathogens infecting humans and animals worldwide. This includes several species of the *Hyalomma* genus that are associated with the transmission of Crimean-Congo Hemorrhagic Fever virus in humans[1,2] and *Theileria annulata*, the causal agent of tropical theileriosis in livestock[3].

The duration of tick life cycle is strongly affected by environmental factors, e.g. temperature and relative humidity and by the availability of hosts[4]. *Hyalomma scupense*, *H. dromedarii* and *H. excavatum* have different life cycles. In Tunisia, *H. scupense* was found to behave as a two-host tick species with peak activity of the adults feeding on cattle in summer[5], whereas in other regions, a cold-adapted ecotype of *H. scupense* was found to have a one-host life cycle[6]. *Hyalomma scupense* collected in China were reported to behave as one- and two-host tick

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under laboratory conditions[7]. *Hyalomma excavatum* can have a two-or three host life cycle tick depending on the availability of hosts. In North Africa, it is active during the whole year with a peak in spring. Larvae and nymphs of this species feed on rodents and the adults infest various hosts such as cattle, small ruminants and equids[8]. *Hyalomma dromedarii* can behave as a one-, two-, or three-host tick[8–10], whereby the immature stages can feed on camels, but also on rodents and birds. The activity of this ticks species does not exhibit a seasonal variation and it can be found throughout the year on animals[11,12].

Studies on the biology of these ticks frequently require laboratory tick colonies that are reared and maintained on animals[29]. In recent years, artificial tick feeding systems (ATFS) have been developed and adapted for different ixodid ticks such as *Amblyomma hebraeum*, *Dermacentor reticulatus, Ixodes ricinus, Rhipicephalus appendiculatus* as well as nymphs and adult *Hyalomma* species such as *H. dromedarii* and *H. anatolicum*[13–19]. The development and optimization of methods to feed hard ticks artificially in the laboratory not only contributes to the 3Rs principle (to Reduce, Replace and Refine animal experiments) for humane animal research[20], but also provides researchers with a versatile tool to study various aspects of tick biology, pathogen transmission and drug discovery under controlled laboratory conditions[13,21].

However, extensive optimization of the artificial feeding method is still required. There is for instance a lack of studies examining the artificial feeding of juvenile stages of one- and two-host tick species where ticks molt on the host to the subsequent life stage. In addition, the success rate reported for the in vitro feeding of ixodid ticks varies considerably and the addition of antibiotics to the blood meal to prevent microbial contamination may exert a negative effect on tick endosymbionts and tick fecundity[22,23]. In this study, we targeted the first point by feeding all life stages of *H. dromedarii, H. excavatum* and *H. scupense* in vitro.

Results

Feeding of *Hyalomma* adults

In vitro feeding

All life stages of *H. excavatum*, *H. dromedarii* and *H. scupense* were successfully fed in vitro (Fig. 1). Results of the in vitro feeding of adult *Hyalomma* ticks are summarized in Table 1. Briefly, *H. excavatum* females (fed in winter, January) achieved a 70% (21/30) attachment rate where all of the attached ticks (21/21) engorged. The mean weight of engorged females was 539.8 ± 163 mg. Twelve of the 21 engorged females (57.1%) laid eggs (6665 ± 2009 eggs), with a mean REI of 51.8 ± 11.9%, where 11 of the females (91.6%) produced viable larvae. The percentage of hatched larvae varied between 90 and 100% (except for one tick where the hatching rate was 30%) resulting in a mean of 6217 ± 1031 larvae (Table 1).

Hyalomma dromedarii fed in winter (January) reached an engorgement rate of 34.4% (11/32 females). The mean weight of engorged detached females was 276 ± 105 mg. Only four out of 11 females (36.4%) produced eggs, with a mean number of 4036 ± 2967 with a REI of 60%. Most of the eggs hatched successfully (99.5%) (Table 1).

As adult *H. scupense* ticks are under natural conditions in Tunisia (from where the tick colony originates) active in summer, we tried to examine this seasonal behaviour in vitro by conducting one feeding experiment in winter (January) and one in summer (June). This seasonal difference was not evaluated for the other two tick species as their activity does not exhibit seasonal variation. *Hyalomma scupense* adults that fed in winter showed lower engorgement rates compared to those fed in summer. Despite the high attachment rate observed in winter (18 out of 20 ticks, 90%), only five ticks (5/20, 25%) partially engorged and detached with a low mean detachment weight of 36.8 ± 22.5 mg. Whereas in summer, the attachment and engorgement rates were 84% (21/25)

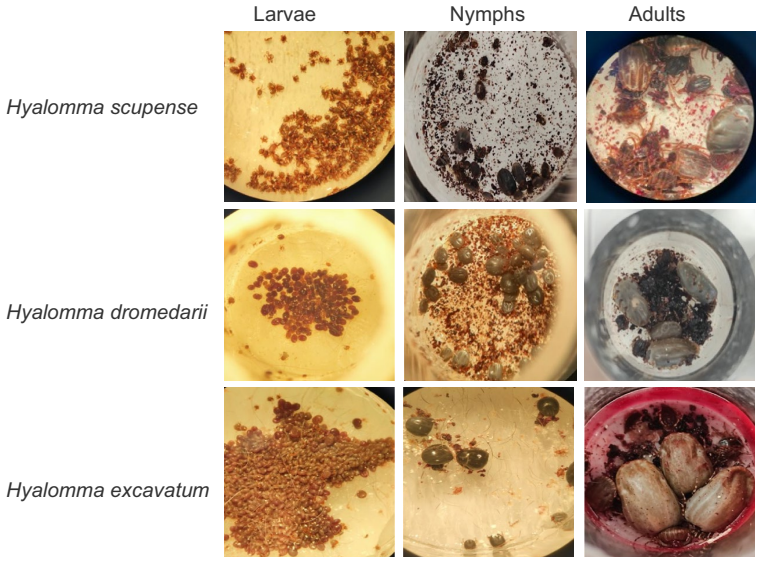

Figure 1. Attachment and engorgement of different *Hyalomma* life stages in vitro.

	H. dromedarii	*H. excavatum*	*H. scupense W*	*H. scupense S*
Attachment rate (%)	11/32 (34.4%)	21/30 (70%)	18/20 (90%)	21/25 (84%)
Engorgement rate (%)	11/32 (34.4%)	21/30 (70%)	5/20 (25%)	19/25 (76%)
Mean feeding duration (days ± SD, [range])	9 ± 1.7 [6–12]	7.6 ± 1.6 [6–10]	9.2 ± 2.5 [7–12]	11.5 ± 2.2 [7–14]
Mean detachment weight (mg ± SD, [range])	276 ± 105 [67–448]	539.8 ± 163 [200–768]	36.8 ± 22.5 [16–63.3]	298 ± 65 [160–378]
Oviposition rate (%)	4/11 (36.4%)	12/21 (57.1%)	0	12/19 (63.1%)
Mean duration of pre-oviposition (days ± SD, [range])	12.6 ± 2.5 [10–15]	58.2 ± 29 [20–96]	–	7.2 ± 1.3 [6–10]
Mean egg mass (mg)	204.25 ± 150 [1–338]	337.3 ± 101 [223–508]	–	134.4 ± 45 [58–213.5]
Mean reproduction efficiency index (%) (Mean ± SD [range])	60.7 ± 40 [0.2–83]	51.8 ± 11.9 [35–68]		44.3 ± 7.3 [29–59]
Mean number of eggs produced ± SD [range]	4036 ± 2967 [20–6679]	6665 ± 2009 [4406–10,038]	–	2656 ± 902 [1146–4218]
Mean oviposition-hatching (days ± SD, [range])	39.7 ± 13 [30–59]	34.4 ± 14 [15–54]	–	43.11 ± 5.8 [36–55]
Female producing fertile eggs (%)	4/3 (100%)	11/12 (91.6%)	–	9/12 (75%)
Mean hatching rate (% ± SD, [range])	99.5 ± 0.005% [99–100%]	84.1 ± 27% [30–100%]	–	54.4 ± 39% [5–95%]
Mean number of larvae produced (± SD, [range])	4010 ± 2949 [20–6612]	6217.5 ± 1031 [5493–7706]	–	1239 ± 1036 [111–3058]

Table 1. In vitro feeding of adult *Hyalomma* species.

and 76% (19/25), respectively, with a mean detachment weight of 298.0 ± 65.4 mg. None of the females fed during the first experiment laid eggs, whereas females from the second in vitro experiment had an oviposition rate of 63.2% (12/19) and produced a mean number of eggs of 2656 ± 902 with a mean REI of 44.3 ± 7.3% (Table 1). Out of the 12 females laid eggs, nine were able to produce viable larvae (75%) with a hatching rate estimated to 54%.

In vivo feeding results and comparison with in vitro feeding of Hyalomma adults

The engorgement rates of *H. excavatum* and *H. dromedarii* females fed on rabbits ("in vivo", fed in winter and in summer, respectively) were 84% (10/12) and 70% (14/20), higher than their counterparts fed in vitro (Z-test, $P = 0.35$ for *H. excavatum* and $P = 0.01$ for *H. dromedarii*). For *H. scupense* fed in winter, the engorgement rate was low for both conditions but still higher in vitro 25% (5/20) than on calf 10% (1/10) (Z-test, $P = 0.33$) (Fig. 2a), but none of the in vitro fed ticks laid eggs whereas the one female that fed to repletion on a calf did. *Hyalomma scupense* fed in summer had engorgement rates of 76% (19/25) in vitro and 86.7% (13/15) when fed on a calf

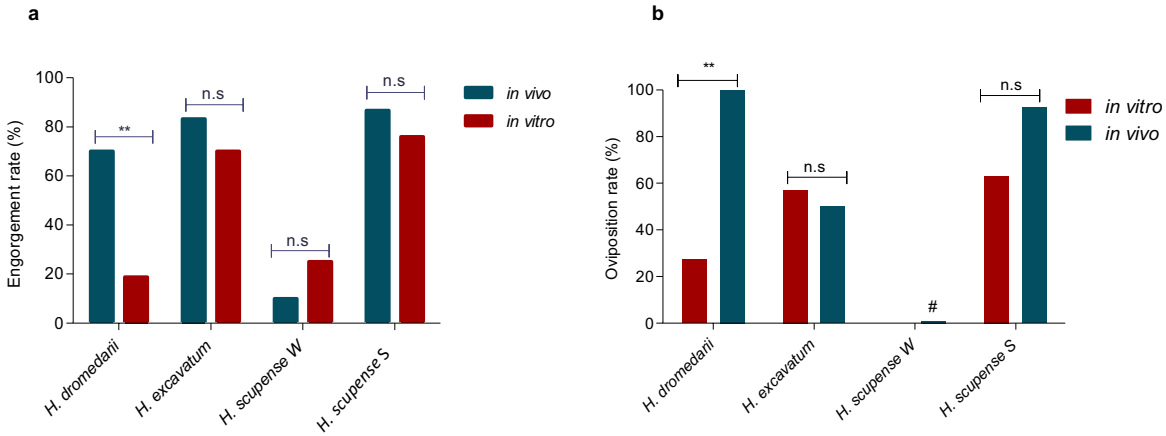

Figure 2. Engorgement (**a**) and oviposition (**b**) rates of adult *Hyalomma* fed in vivo and in vitro. Asterisks indicate level of significance using Z-test ($P < 0.01$ (**), n.s = non-significant, # for *Hyalomma scupense* fed in winter, only one engorged tick laid eggs and the oviposition rate was not calculated. *H. scupense* S = *Hyalomma scupense* fed in summer (S); *H. scupense* W = *Hyalomma scupense* fed in winter (W). Engorgement rate (%) = (number of engorged females/numbers of initially placed ticks) *100; Oviposition rate (%) = (number of females laying eggs/number of engorged females) *100.

(Z-test, $P=0.4$), respectively. The oviposition rate was with 92.3% (12/13) also higher in vivo than in vitro (63.2%, 12/19; Z-test, $P=0.06$) (Fig. 2b).

Adults of *H. dromedarii* and *H. excavatum* took longer to engorge on a rabbit (10.4 ± 1.8 days and 9.6 ± 2.5 days, respectively) than in vitro (9.1 ± 1.6 days and 7.6 ± 1.6 days, respectively) (Mann Whitney test, $P=0.03$ for both species), whereas *H. scupense* females fed longer in vitro (11.5 ± 2.2 days) compared to *H. scupense* females fed on a calf (9.1 ± 0.95 days) (Mann Whitney test, $P=0.001$; Fig. 3).

The weight of engorged females was significantly higher for all *Hyalomma* ticks fed on animals compared to those fed in vitro (One-way ANOVA and Tukey test, $P<0.0001$). The highest weight of engorged females was found for *H. excavatum* fed on an animal (903 ± 114 mg) and in vitro (540 ± 167 mg) (Fig. 4a). There were no significant differences in egg mass produced between ticks fed in vivo and in vitro for any of the species (Fig. 4c). The REI was higher in vitro than in vivo for *H. dromedarii* (60.7 and 49.5%, respectively) and *H. excavatum* (51.8 and 44.6%), but not for *H. scupense* (in vivo 52.1%, in vitro 44.3%).

The longest preoviposition period was observed for *H. excavatum* fed in vivo (86 ± 41 days) and in vitro (58 ± 29 days) with no significant difference between the two feeding conditions (Mann Whitney test, $P=0.1$). The shortest preoviposition duration was recorded for *H. scupense* fed in vitro (7.3 ± 1.4 days) and in vivo (7.8 ± 1.2 days, Mann Whitney test, $P=0.2$). *Hyalomma dromedarii* females fed in vivo (8 ± 1.4 days) had significantly shorter preoviposition period than *H. dromedarii* fed in vitro (12.6 ± 2.5 days) (Mann Whitney test, $P=0.01$) (Fig. 4b).

The mean oviposition-hatching duration (the period from the start of oviposition until the hatching of the first larvae) was relatively similar between the feeding conditions (Fig. 4d).

Feeding of larvae and nymphs

In vitro feeding of Hyalomma immatures

Results of the in vitro feeding of immature *Hyalomma* species are shown in Table 2. *Hyalomma scupense* acted as a two-host tick in vitro, i.e., the larvae molted to nymphs on the membrane (Supplementary Fig. 1) similar to ticks fed on rabbits. This contrasted with *H. dromedarii*, that acted as a three-host tick in vitro, but as a two-host tick when fed on rabbits. *Hyalomma excavatum* behaved as two-host tick when fed in vitro or on a rabbit, but as a three-host tick when the larvae were fed on a gerbil.

A high attachment rate was observed for *H. scupense* larvae, with 113 out of 122 attached ticks (92.6%). Sixty-eight larvae out of 122 (55.7%) engorged. All engorged larvae successfully molted to the nymphal stage on the membrane. Out of these 68 nymphs, 25 fed to repletion (36.8%). Twenty-four nymphs (96%) molted to the adult stage (11 males and 13 females) in a mean of 34.8 ± 6.1 days. The mean weight of engorged nymphs was 13.4 ± 3.6 mg, whereas the mean weight of unfed adults was 6.7 ± 1.7 mg.

For *H. excavatum*, 129 out of 183 larvae attached (70.5%) and 104 engorged (56.8%). Ninety-four larvae molted (90.4%) on the membrane to the nymphal stage. An attachment rate of 66.7% (34/51), engorgement rate of 41.2% (21/51) with a mean detachment weight of 10.4 ± 2.8 mg was recorded for the nymphs. Out of 21 engorged nymphs, 13 (61.9%) molted to the adult stage (five females and eight males) in 17.9 ± 1.9 days. The average weight of unfed adults was 6 ± 2 mg.

For *H. dromedarii*, an attachment and engorgement rate of 44.2% (313/708) and 42.8% (303/708) was recorded for the larvae. Of the engorged larvae, 71.3% (216/303) molted to the nymphal stage. For the nymphs, the attachment rate was 90.2% (129/143) and the engorgement rate was 47.6% (68/143). The nymphal weight varied between 6.1 and 19.9 mg. Fifty-four (79.4%) of the nymphs molted to adults (22 females and 32 males) in a mean

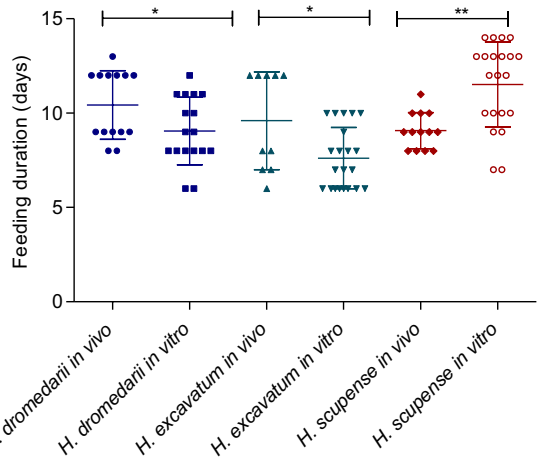

Figure 3. Feeding duration of engorged females *Hyalomma* fed in vivo and in vitro. Asterisks indicate level of significance using Mann Whitney test ($P<0.05$ (*) and ($P<0.01$ (**).

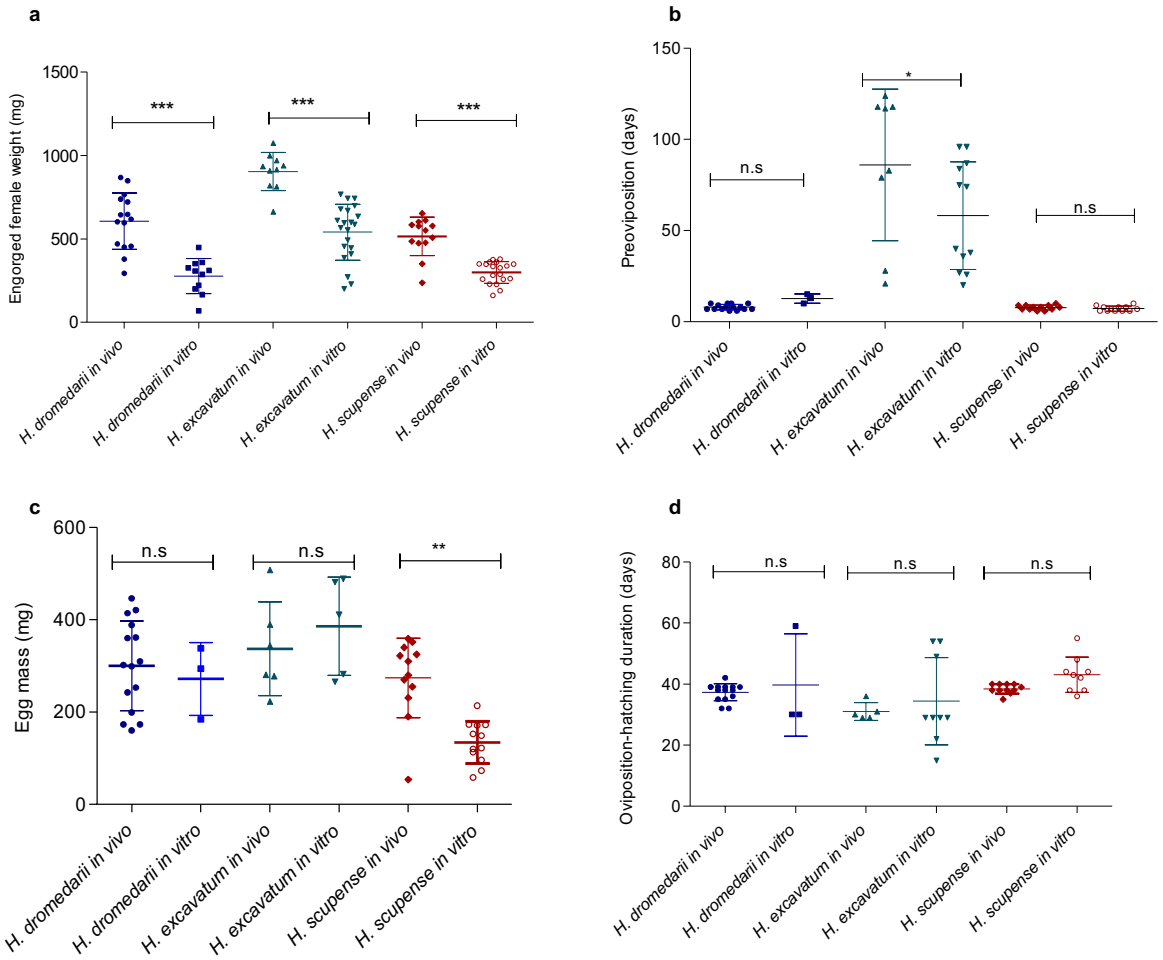

Figure 4. Variation of engorged female weight (**a**), duration of preoviposition (**b**), egg mass (**c**) and oviposition-hatching duration (**d**). Asterisks indicate level of significance using Mann Whitney test ($P<0.05$ (*), ($P<0.01$ (**), $P<0.0001$ (***), n.s = non-significant.

	Immature stages	*H. dromedarii*	*H. excavatum*	*H. scupense*
Attachment rate (%)	Larvae	313/708 (44.2%)	129/183 (70.5%)	113/122 (92.6%)
	Nymphs	129/143 (90.2%)	34/51 (66.7%)	30/68 (44.1%)
Engorgement rate (%)	Larvae	303/708 (42.8%)	104/183 (56.8%)	68/122 (55.7%)
	Nymphs	68/143 (47.6%)	21/51 (41.2%)	25/68 (36.8%)
Mean feeding duration (days ± SD, [range])	Larvae	6.2 ± 1.7 [4–9]	9.3 ± 2.5 [6–13]*	16.5 ± 3.5 [14–19]*
	Nymphs	10.3 ± 2 [7–16]	7.9 ± 1 [7–13]	9.6 ± 2.6 [4–14]
Average detachment weight (mg ± SD, [range])	Larvae	NA	NA	NA
	Nymphs	12.7 ± 3.1 [6.1–19.9]	10.4 ± 2.8 [5–18.3]	13.4 ± 3.6 [6–19]
Molting rate (%)	Larvae	216/303 (71.3%)	94/104 (90.4%)	68/68 (100%)
	Nymphs	54/68 (79.4%)	13/21 (61.9%)	24/25 (96%)
Molting duration (days ± SD, [range])	Larvae	14.6 ± 2.6 [11–18]	–	–
	Nymphs	29.7 ± 7.5 [15–63]	17.9 ± 1.9 [15–20]	34.8 ± 6.1 [23–49]

Table 2. In vitro feeding of *Hyalomma* immature stages. **H. excavatum* and *H. scupense* engorged larvae molt to nymphs while attached to the membrane, so the feeding and molting durations were given together.

of 29.7 ± 7.5 days (Table 2). The attachment, engorgement and molting proportions varied significantly between the three species for larvae and nymphs (Chi-square test, $P < 0.0001$).

In vivo feeding results and comparison with in vitro feeding of immatures of Hyalomma ticks

During the in vitro feeding, *H. excavatum* and *H. scupense* behaved as two-host ticks and *H. dromedarii* acted as a three-host tick. When fed on rabbits, all three *Hyalomma* species acted as two-host ticks. *Hyalomma excavatum* acted as a three-host when larvae were fed on gerbils.

Due to the different feeding behaviour, the only possible comparison that could be done was on the duration of feeding for *H. dromedarii* larvae, which was not significantly different between both conditions (Mann Whitney test, $P = 0.06$) (Fig. 5a). For the nymphs, the feeding duration varied significantly between the two feeding conditions for *H. excavatum* (Mann Whitney test, $P < 0.01$) and *H. scupense* (Mann Whitney test, $P < 0.0001$), but no significant difference was observed for *H. dromedarii* (Mann Whitney test, $P = 0.2$) (Fig. 5b).

As for the females, the weight of engorged nymphs fed on animals was significantly higher than that of nymphs fed in vitro for all the three *Hyalomma* species (One-way ANOVA followed by Tukey test and confirmed by Mann Whitney test, $P < 0.0001$) (Fig. 6).

There was a significant positive correlation between engorged *H. scupense* nymphs in vitro and the weight of their counterpart unfed adults (Pearson $r = 0.73$, $P < 0.0001$) (Fig. 7a). The same but stronger correlation was observed for unfed adult *H. excavatum* fed in vitro (Pearson $r = 0.92$, $P < 0.0001$) (Fig. 7b).

The weight of unfed *H. scupense* adults obtained from in vivo feeding was significantly higher than those fed in vitro (One-way ANOVA and Tukey test, $P < 0.0001$) (Fig. 8a). The same trend was also reported for unfed *H. excavatum* adults (Mann Whitney test, $P < 0.0001$) (Fig. 8b). For both species fed in the two feeding conditions,

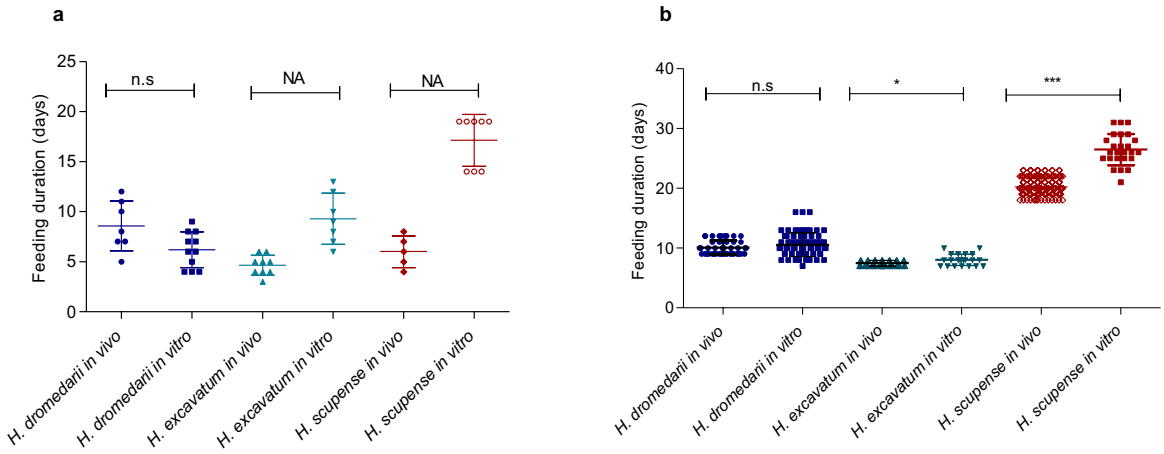

Figure 5. Mean feeding duration of *Hyalomma* larvae (**a**) and nymphs (**b**) fed in vitro and in vivo. NA: For *Hyalomma excavatum* and *H. scupense* larvae, statistics between in vivo and in vitro feeding condition was not conducted as ticks acted different (*H. excavatum* and *H. scupense* engorged larvae molt to nymphs while attached to the membrane, so the feeding and molting durations were given together), therefore we indicated as not available (NA). Asterisks indicate level of significance using Mann Whitney test ($P < 0.05$ (*), $P < 0.0001$ (***), n.s = non-significant.

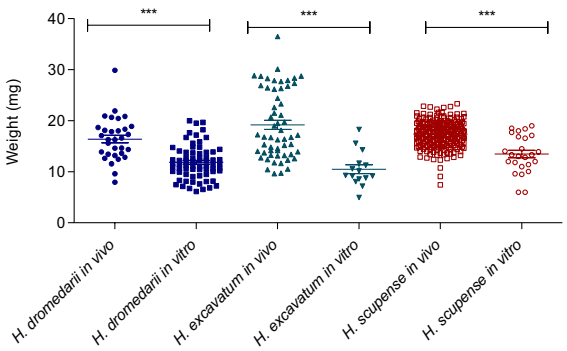

Figure 6. Variation of weight of *Hyalomma* nymphs fed in vivo and in vitro. Asterisks indicate level of significance using Mann Whitney test $P < 0.0001$ (***).

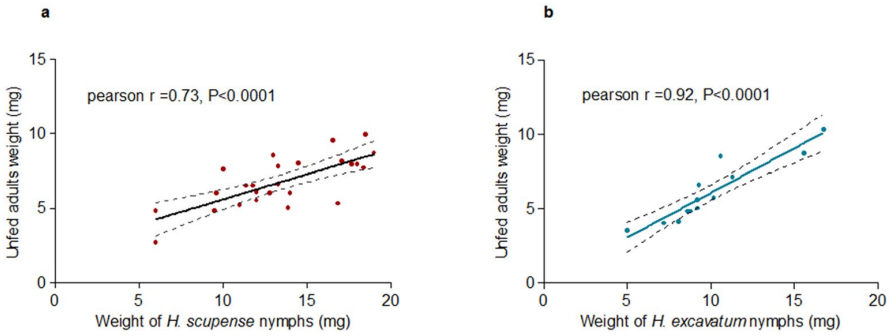

Figure 7. Correlation between the weight of engorged *H. scupense* (**a**) and *H. excavatum* (**b**) fed in vitro and the weight of unfed adults after molting.

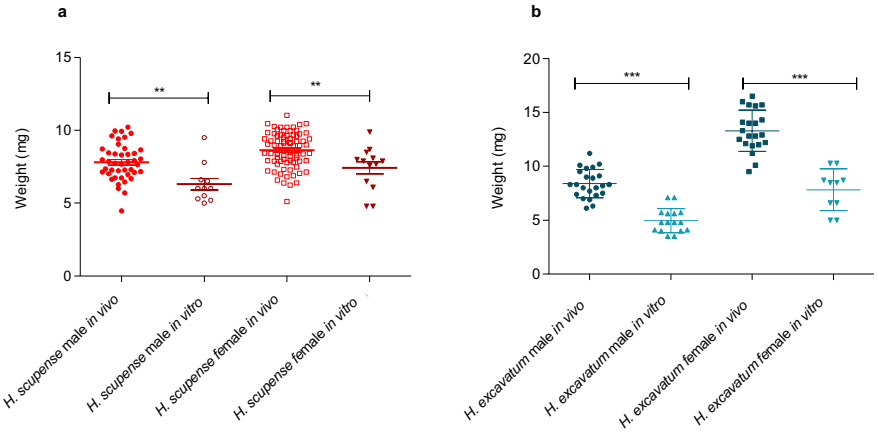

Figure 8. Difference between weight of unfed adults of *Hyalomma scupense* (**a**) and *Hyalomma excavatum* (**b**) obtained from nymphs fed in vivo and those fed in vitro. Asterisks indicate level of significance using Mann Whitney test ($P<0.01$ (**) and $P<0.0001$ (***).

unfed females had higher weight than unfed males (One-way ANOVA and Tukey test, $P<0.0001$) except for *H. scupense* fed in vitro where the difference was not significant (Mann Whitney test, $P=0.06$).

Discussion

The use of artificial feeding systems to feed ticks in a laboratory setting instead of relying on vertebrate hosts has been used to study various aspects of tick biology, including host-tick interactions, tick feeding behaviour, and the transmission of tick-borne pathogens[24–26]. Feeding all life stages on membranes was previously reported for the three-host ticks *A. hebraeum*[15], *A. variegatum*[27] and *I. ricinus*[16].

A number of studies describing the in vitro feeding of *Hyalomma* ticks have been previously published. This includes *H. dromedarii*, *H. anatolicum*[17,18], *H. lusitanicum*[28], *H. excavatum* and *H. marginatum*[29]. However, these studies were limited to the nymphal or adult stage. In this paper, we present the first successful in vitro feeding of all life stages of *H. dromedarii, H. scupense* and *H. excavatum* on silicone membranes.

Feeding of *Hyalomma* adults

The highest engorgement rate was observed for adult *H. scupense* (76%) followed by *H. excavatum* (70%) and *H. dromedarii* (34.4%) with a mean feeding duration of 9, 7.6 and 11.5 days. These results were comparable to previous reports on the artificial feeding of *H. lusitanicum* which had an engorgement rate of 40.2% in approximately 11 days[28]. Similar results were previously reported by Tajeri and Razmi, (2011) for *H. dromedarii* (55%) and *H. anatolicum* (75%) with a feeding duration that varied between 11 and 16 days. The mean feeding duration found in this study for *H. dromedarii* (9.1 days) and *H. excavatum* (7.6 days) was shorter than the feeding durations of their counterparts fed on rabbits, which lasted 10.4 ± 1.8 days for *H. dromedarii* and 9.6 ± 2.5 days for *H. excavatum*. This is surprising as in vitro feeding usually lasts longer than the feeding on animals[16,30,31]. This could perhaps be attributed to the final feeding phase, which might have been quicker in vitro due to mechanical

limitations caused by the confined space of the feeding units in combination with ticks clustering on the membrane in the feeding units, forcing the ticks detach earlier[25,31] or perhaps a feeding stimulant factor(s) that was missing in the in vitro system. It would be interesting to observe how these ticks would behave in feeding units with a larger diameter or lower tick density.

A recent publication on the in vitro feeding *H. excavatum* adults, which originated from the same tick colony as used for this study, and *H. marginatum* showed much lower engorgement rates of 8.3 and 7.9%, respectively, with a longer feeding period of over 20 days[29]. Several factors might have led to this variation in feeding success such as tick fitness at time of feeding, feeding preferences and behaviour, membrane composition and thickness and specific environmental conditions such as temperature, relative humidity, CO_2 levels, attachment stimuli, and dark/light condition[22,32].

It has previously been suggested for *H. lusitanicum* that season could affect the tick feeding success (31.6%)[28]. Although it was limited to two experiments only, we did make similar observations for adult *H. scupense*, where we observed a higher engorgement rate in summer (June) (76%) compared to ticks fed in winter (January) (25%). This corroborates with the *H. scupense* adult activity in North Africa as these are mainly found on animals in summer[5,33]. However, more extensive experiments should be conducted to confirm whether the in vitro feeding success of *H. scupense* is truly season dependent.

Another important factor that can influence tick feeding efficacy is the feeding system itself. In a feeding system recently used for *R. appendiculatus*, the chambers were inverted (upside down) based on the natural tendency of ticks to crawl up towards a host[34,35]. Such an inverted system where the blood is placed above the membrane might also be useful for studies working on the artificial infection of ticks with intra-erythrocytic pathogens such as *Babesia* and *Theileria* spp.[32].

In the present study, the highest mean detachment weight was recorded for *H. excavatum* (539.8 ± 167 mg) followed by *H. dromedarii* (305 ± 102 mg) and *H. scupense* S (298.01 ± 65.4 mg). The average weight of *H. excavatum* in this study is higher than the weight previously reported for *H. excavatum* fed in vitro (260.6 mg), where tick feeding did not take place inside an incubator but in a water bath to maintain a constant blood temperature. In general, the average weight of the engorged *Hyalomma* females was significantly higher for ticks fed in vivo than those fed in vitro ($P < 0.0001$), also leading to higher egg mass production. This finding is in line with previous report for *H. lusitanicum* fed in vivo[36] and in vitro[28] where the average weight were 543 mg and 274.65 mg, respectively. Higher average weights were also recorded for *I. ricinus* fed in vivo (231 ± 72.3 mg) compared to the ones fed in vitro (136 ± 44.9 mg)[16]. The lower engorgement weights of all fed life stages, resulting in smaller ticks and decreased egg production, is a consistent finding in the in vitro feeding of ixodid ticks when compared to feeding on natural hosts. This, in combination with the necessity to use antibiotics with potential negative effects on tick endosymbionts and tick fecundity remains a major stumbling block for the complete maintenance of tick laboratory colonies using artificial feeding alone. It will be essential to conduct long-term studies to examine whether *Hyalomma* tick colonies could also be maintained by in vitro feeding over several generations without detrimental side effects on the tick biology and physiology.

Despite the lower detachment weight, the percentage of engorged females that laid eggs was 50, 57.1 and 63.1% for *H. dromedarii, H. excavatum* and *H. scupense*, respectively. This was lower than their equivalents fed on animals, where 100%, 50% and 92.3% of the females laid eggs. The low oviposition rates observed in artificial feeding in comparison to in vivo feeding might be due to the elimination of endosymbionts or antibiotic toxicity. In general, the mean REI was around 50% for all three species under both feeding conditions (Table 1), except for *H. dromedarii* fed in vitro where the mean RE was 60%. This is higher than its counterparts fed on rabbit (49.5%) but in line with previous reports on *H. dromedarii* where high REI of up to 72% were reported[4]. This variation could be explained by host-differences, it was for instance shown that *H. anatolicum* had a higher REI when fed on a rabbit compared to sheep and goats[37], but also by the tick strain, quantity of blood ingested, humidity, temperature and possible disturbance during oviposition[38].

Feeding of immature stages

The engorgement rate of larvae in vitro was 42.8%, 56.8% and 55.7% for *H. dromedarii*, *H. excavatum* and *H. scupense*, respectively. These proportions are in line with those obtained for *I. ricinus* fed in vitro (55%) and in vivo (41%)[16]. An engorgement rate of 71% was obtained for *R. australis* larvae using a different feeding system, where most of the larvae attached after 48 h which is similar to the observations made in this study[39]. The encouraging engorgement rate in our study may be associated to the presence of attachment stimuli (hair extract and rabbit hair). The thickness and type of membrane appear to be crucial factors for the larval and nymphal feeding success. As *Hyalomma* juvenile ticks have a relatively short hypostome, varying from 54 to 119 µm for the larvae and 156–268 µm for the nymphs[9,29], they require membranes thin enough to pierce through and feed while avoiding leakage. This problem might explain why immatures stages of other *Hyalomma* species could reportedly not feed or fed with low attachment rates[28,29]. In addition, the age of the ticks and the season in which they are fed were also suggested to influence the efficacy and the success of the tick feeding[16].

Hyalomma anatolicum nymphs were previously fed on mouse skin membrane with an engorgement rate reached 89% with a feeding duration varied from 7 to 8 days in a water bath set to 37 °C[17]. The high engorgement rate reported in that study is related to the use of animal skins, which do not require external stimuli to simulate attachment and more closely mimic natural feeding conditions. However, the use of animal skin may cause increased contamination when maintained at 37 °C, especially when used for one- or two-host tick species which have a longer feeding duration, as well as bioethical concerns[21,32].

In our study, the feeding-molting duration was between 9.3 ± 2.5 and 16.5 ± 3.5 days for *H. excavatum* and *H. scupense*, respectively. The longer feeding duration, especially when the tick are not attached synchronously (Fig. 3) as previously reported[18,28], increase the risk of contamination inside the feeding unit. For *H. scupense*,

partially engorged larvae were moved manually to another feeding unit where not all of them reattached, which explains why only 55.7% of the initially placed larvae engorged. The engorged larvae molted to nymphs on the membrane (Supplementary Fig. 1). The freshly molted nymphs were transferred to a new feeding unit where the engorgement rate was only 36.8% (Table 2). The fungal contamination inside the chamber was the main factor that affected the engorgement rate of the immature stage of both two-host ticks (*H. excavatum* and *H. scupense)*. To prevent this from happening, we tried using nystatin cream to clean the inner side of the membrane and the addition of Whatman filter paper and bags with silica gel to absorb the humidity inside the feeding unit. These precautions appeared to help in delaying the contamination until most of the nymphs detached, but did not completely prevent contamination from occurring. In general, the transfer of immature ticks to clean feeding units and other precautions to limit contamination makes the feeding laborious, and future studies may focus on optimising these steps in order to improve the engorgement rate for one- and two-host tick species in vitro.

The average weight of nymphs fed in vitro was 12.7 ± 3.1, 10.4 ± 2.8 and 13.4 ± 3.6 mg for *H. dromedarii*, *H. excavatum* and *H. scupense*, respectively. As for the females, the nymphal weight was significantly higher for ticks fed on animals compared to in vitro fed ticks and showed a significant difference between the three *Hyalomma* species (Mann Whitney test, $P < 0.0001$). Lower detachment weights of all stages fed in vitro were also recorded for *I. ricinus*[16]. Although the weights were lower, larvae and nymphs fed in vitro had weights that allowed them to successfully molt to the next stage. We also observed that incomplete feeding of nymphs lead to their death or resulted in smaller adults, similar to previous reports[40,41]. We note a significant positive correlation between engorged *H. scupense* and *H. excavatum* nymphs fed in vitro and the weight of their counterpart unfed adults where the weight of unfed female is higher than males. These observations were in line with previous reports on *I. ricinus*, *I. scapularis* and *Dermacentor variabilis*[16,42] which showed that weight of nymphs that become female imbibe more blood than those became male.

The reported in vitro feeding method was successfully used to feed all *H. excavatum*, *H. dromedarii* and *H. scupense* life stages. While there are some similarities in their response to in vitro feeding, some variations specific to the species existed which require more investigation and understanding these differences will improve our knowledge of tick biology, tick-borne disease transmission, and improve the development of control measures. The ATFS could also contribute to the 3Rs by reducing the use of laboratory animals required to maintain tick colonies, although the impact of in vitro feeding on tick microbiota due to the use of antibiotics requires further investigation.

Materials and methods

Maintenance of tick colonies

Colonies of *Hyalomma scupense* and *H. dromedarii* used in this study were originally obtained from the National School of Veterinary Medicine of Sidi Thabet, Tunisia and the *H. excavatum* ticks originated from a colony of the Aydın Adnan Menderes University, Türkiye. The colonies were maintained at the Institute of Parasitology and Tropical Veterinary Medicine of the Freie Universität Berlin, whereby the juvenile life stages were fed on gerbils or the ears of rabbits. The adults were fed on the ears of rabbits (for *H. dromedarii* and *H. excavatum*) or calves (for *H. scupense*) in linen bags. Ethical approval for the feeding of ticks on experimental animals was granted by the Landesamt für Gesundheit und Soziales, Berlin, Germany (LAGeSo) under Registration Number H0387/17 and 0144/22.

Collected ticks were stored in an exsiccator at 27 °C or at room temperature with approx. 90% relative humidity (RH). Data from the feeding of ticks on experimental animals for tick colony maintenance were recorded for comparison purpose with in vitro feeding results.

In vitro feeding of *Hyalomma* ticks

The methods for the in vitro feeding of all *Hyalomma* life stages were adapted from previously published protocols[16,28]. Ticks were fed on a silicone membrane based on a matrix of goldbeater's skin (for larvae and nymphs) or lens cleaning paper (for adults) with thicknesses of 40–50 µm for larvae, 50–70 µm for nymphs and 80–120 µm for adults. The feeding units were placed in a 6-, or 12-well cell culture plate, depending on the unit's diameter and placed in an incubator (ICH110C, Memmert, Schwabach, Germany) at 27 °C, 70% RH and 5% CO_2 in complete darkness. The culture plates were placed on a heating plate (hot plate 062, Labotect, Göttingen, Germany) set at 42 °C to keep the blood warm. Bovine hair extract and rabbit hair was added as an attachment stimulus on top of the silicone membranes approx. 45 min before ticks were introduced into the units. Bovine hair extract was prepared as described by Militzer et al.[16]. Briefly, approximately 50 g of fresh bovine hair was immersed three times for two hours each in different ratios of chloroform–methanol mixture (2:1, 1:1 and 1:2). The first two immersions took place at room temperature, the final step at 45 °C. Extracts from each immersion step were collected and combined, vacuum filtered and concentrated by roto-evaporation. Finally, the bovine hair extract was dissolved in a 1:2 chloroform–methanol mixture, aliquoted to small working solutions and stored at − 20 °C until use. One hundred µl of bovine hair extract was used for feeding units with a diameter of 30 mm and 50 µL for feeding units with a diameter of 20 mm. The feeding units were closed using a mite plug made from polyurethane foam (K-TK e.K., Retzstadt, Germany) to allow for gas exchange while preventing the ticks from escaping. Defibrinated aseptic bovine blood was purchased from Xebios Diagnostic (Düsseldorf, Germany) and supplemented with 0.1 M adenosine triphosphate (ATP, Carl Roth, Karlsruhe, Germany), vitamin B[43], 2 mg/mL glucose and 5 µg/mL gentamicin prior to each blood change, which took place at 14:10 h intervals. The vitamin B was added based on previous observations by Militzer et al.[16] suggesting that this may result in better engorgement and fecundity rates. To avoid contamination of the feeding unit, the underside of the membrane was washed with 1% nystatin diluted in PBS, followed by 0.9% NaCl at each blood change.

Once ticks were attached, the rabbit hair used to stimulate attachment was removed, as well as any dead ticks and faeces produced by the ticks during feeding to keep the feeding unit as clean as possible. The inner side of the feeding unit was initially cleaned with 1% nystatin in PBS when fungal contamination was observed. As this increased the humidity inside the feeding units and caused droplets to form that wetted the membrane and made it unsuitable for ticks to feed, we subsequently switched to the treatment of fungal contamination with a nystatin cream (Nystalocal, Pierre Fabre, Freiburg) and also added a Whatman filter paper to cover the attached ticks and bags with silica gel to absorb excess humidity to the feeding unit. In cases where this treatment was not successful, ticks were manually removed, washed in 0.9% NaCl and dried with tissue paper before being transferred to a newly prepared feeding unit. In each ø 30 mm feeding unit, ten male and ten to 12 female ticks were placed. For the in vitro feeding of the immature stages, a maximum of approx. 75 nymphs or 150 larvae were placed in each ø 30 mm unit. One group was fed for each life stage, with the exception of the *H. scupense* adults for which one group was fed in summer and one in winter.

Biological parameters

The rates of attached and engorged ticks were recorded for all life stages, as well as additional parameters such as detachment weight, molting rates and reproductive parameters including oviposition duration, weight of egg mass and oviposition-hatching duration. The number of eggs for each species was estimated by dividing the weight of the egg batch with the weight of a single egg, whereby the average egg weight was 0.058 ± 0.009 mg for *H. dromedarii*, 0.057 ± 0.001 mg for *H. scupense* and 0.053 ± 0.008 mg for *H. excavatum* as measured on an analytic scale. The percentage of hatched larvae was estimated visually under binocular stereoscope by two persons[44]. The following parameters were calculated:

$$\text{Engorgement rate } (\%) = \left(\text{number of engorged females/numbers of initially placed ticks}\right) * 100$$

$$\text{Reproductive efficiency Index (REI) } (\%) = (\text{egg mass/female engorgement weight}) * 100$$

Statistical analyses

Statistical analyses and graphs were generated using GraphPad Prism 5 windows (GraphPad Software, San Diego California, United States). Chi-square test was used to compare the feeding proportions between the three species and Z-test was used to compare two proportions using an online version of epitools (http://epitools.ausvet.com.au). One-way ANOVA followed by Tukey-test was used to assess the difference in feeding parameters such as feeding duration, weight, pre-oviposition and oviposition-hatching duration between the tick species in vivo and in vitro (between more than two groups). The Mann–Whitney test was used to analyse the variation of some parameters between two groups and to confirm the results of one-way ANOVA test. Pearson correlation and linear regression were performed to assess the relationship between the following parameters: female weight and egg mass produced, engorged nymphs' weight and their corresponding unfed adult weight. For all test used, a 5% threshold value was considered as statistically significant.

Data availability

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

Received: 6 November 2023; Accepted: 29 December 2023
Published online: 03 January 2024

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Acknowledgements
Khawla Elati was supported by a doctoral scholarship from the German Academic Exchange Service (DAAD). Ard Nijhof was funded by the German Federal Ministry of Education and Research (BMBF, Grant Number 01KI1720) as part of the Junior Research Group "Tick-borne Zoonoses." The authors thank Dr Shahin Tajeri, Nina Militzer and Sophia Pinecki for their technical assistance. We acknowledge support by the Open Access Publication initiative of Freie Universität Berlin.

Author contributions
K.E.: conceptualization, methodology, investigation, formal analysis, visualization, writing—original draft preparation. H.B., M.M., S.B., H.B.B., T.K.: resources, review and editing. P.H.K., K.F.: investigation. M.A.D.: conceptualization, supervision, review and editing. A.M.N.: conceptualization, supervision, project administration, writing—review and editing. All authors read and approved the final version of the manuscript.

Funding
Open Access funding enabled and organized by Projekt DEAL.

Competing interests
The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-023-51052-w.

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Chapter 6:
General discussion and Conclusion

The tick-borne disease Tropical Theileriosis (TT) is a serious impediment to livestock production that mainly affects smallholders in three continents, where up to 250 million cattle are at risk (Gharbi et al. 2020; Onar 1989).
As the control of TT is mainly based on classical tick control options, which often have some limits and may have a negative impact on the environment, the use of cell culture-based attenuated live vaccines holds promise as a more effective and environmentally friendly preventive option. Although these vaccines were shown to confer a good immunity against homologous challenges, the protection against heterologous challenges is limited and requires further improvement. In addition, the application of live vaccines in the field is hampered by delivery and biosafety constraints (Darghouth 2008).

Therefore, there is a need to develop and improve the efficacy of vaccines against *T. annulata* and to conduct further studies to better understand the mechanism of tick-pathogen interactions in greater detail, which may lead to a better design and implementation of new and effective tick vector and parasite control measures.
However, such studies require the use of experimental animals and animal-derived products not only to maintain tick colonies, but also to cultivate *T. annulata in vitro* and to improve our knowledge of the mechanism of attenuation of the strain. These issues make the ultimate objective of this thesis to develop new tools to study different aspects of *T. annulata* control, while adhering to the 3Rs principle by reducing the number of experimental animals required for: (i) the maintenance of tick colonies, (ii) the cultivation of *T. annulata* schizonts, (iii) the generation of infected red blood cells *in vitro* to eventually produce *T. annulata*-infected tick material, and (iv) assessing the attenuation status of a cell line.
Parts of the applied 3Rs methods focusing on tick and *Theileria* research, can be extrapolated to other vector-borne pathogens with similar life cycles.

The four studies performed here focused on developing and improving methods to study *T. annulata* by (i) establishing a *T. annulata* cell line using serum-free culture medium, (ii) comparing virulent and attenuated passages of *T. annulata* using RNA-seq to catalogue putative attenuation markers, (iii) inducing merogony in *T. annulata* cell lines and obtaining piroplasm-containing erythrocytes that will later be useful to infect ticks *in vitro*, and (iv) successfully feeding all life stages of *H. scupense, H. excavatum* and *H. dromedarii in vitro*. All of these studies are crucial steps in studying and reproducing the life cycle of *T. annulata in vitro* and will serve as source material for further studies on *Theileria*, while replacing or reducing the use of experimental animals.

As highlighted in Chapter 2, the cultivation of the *T. annulata*-infected cell line is usually performed in RPMI 1640 containing FBS. The use of FBS has intrinsic scientific, ethical and biosafety concerns due to its cost, batch-to-batch variability and risk of contamination. These challenges justify the need to test the use of serum-free media as an alternative for cultivating other protozoan parasites such as *Plasmodium falciparum* (Basco 2023), *Toxoplasma gondii*, *Neospora caninum* (De Meerschman et al. 2002) and *Babesia* spp. (Grande et al. 1997; Zweygarth et al. 1995). In this study, we have performed three different experiments and confirmed that *T. annulata* transformed cells can be established in a serum-free medium (ISF-1). As detailed in Chapter 2, our results showed that freshly isolated cells proliferate better in serum-free media than cells adapted to RPMI-FBS. However, the effect of culturing *T. annulata* in serum-free media on the genetic make-up of the cell line and on the process and rate of strain attenuation compared to medium containing FBS is not known and requires further studies. It has been shown that the optimal composition of serum-free media is cell line-specific and needs to be optimised (Tan et al. 2015). Furthermore, it has been reported that the success of serum-free culture is driven by a molecular background, this has for instance been documented for glial tumour cells cultured in serum-free medium, which showed specific expression of extracellular matrix related genes (Balvers et al. 2013).
In this context, a comparative transcriptomic or proteomic analysis between cell lines cultured under different conditions could be performed to identify differences between the two culture conditions and further validate the use of SF as an alternative. If successful, a serum-free medium would be an attractive alternative to media supplemented with FBS and could play a role in research and development of vaccines against *T. annulata*, avoiding the influence of serum supplementation. However, cultivation of *Theileria* spp. in SFM is not straightforward. Zweygarth et al. (2020) for instance showed the successful adaptation of *T. annulata* cell lines in SFM, however, out of four *Theileria parva* strains, only one could be propagated in ISF-1 with no significant difference in generation doubling time compared to HL-1 10% FBS, the classic medium used to propagate *T. parva*. The same authors showed the failure of another *Theileria* spp isolated from a roan antelope (*Hippotragus equinus*) to successfully grow in SFM.

To attenuate *T. annulata* cell lines, long-term cultivation is required and this process may take between two and three years, depending on the cell line. The mechanism driving the attenuation in *T. annulata* is still not fully understood and the attenuation phenotype is usually assessed *in vivo,* as explained in Chapter 3. The RNA-seq analysis we conducted allowed us to catalogue differentially expressed host and parasite genes that could be investigated further to confirm their potential use as attenuation biomarkers. This could for instance be done using techniques such as Matrigel chamber assay (Rchiad et al. 2020) and immunoblotting and

immunofluorescence (Larcombe et al. 2022) that have previously been used to assess the dissemination ability of *Theileria* cells and investigate the role of proteins in *Theileria* pathogenesis. It will also be important to test multiple cell lines from different geographic regions as some changes may be isolate specific. The validation of genes as effective and reliable markers of attenuation could not only lead to a better understanding of the attenuation process but also reduce the time required to establish an attenuated cell line and contribute to the application of the 3Rs in *Theileria* research by reducing the number of animals required for validating the attenuation.

Another crucial step in completing the *T. annulata* life cycle *in vitro* is the *in vitro* feeding of *Hyalomma* ticks on silicone membranes, which has been attracting attention for years as an alternative to feed ticks on blood without use of experimental animals. We report the successful feeding of all life stages of *H. excavatum*, *H. dromedarii* and *H. scupense* in Chapter 5. This is the first time that this has been reported for juvenile stages of a two-host tick species on silicone membranes. Several problems were encountered when feeding *Hyalomma* ticks in vitro. This included issues such as the amount of labour associated with regular blood meal changes and contamination-related problems as ticks fed for a long period for up to several weeks, which for instance reduced the number of ticks that successfully fed to repletion, their engorgement weight and fecundity.

This low fertility could be explained by an early detachment of ticks *in vitro* compared to those fed *in vivo*, which could affect the mating phase which is crucial for the production of fertile eggs. It has also been reported that supplementation with exogenous haem, an iron-containing molecule that is involved in different biological process, improves tick reproduction (Perner et al. 2016).

Hyalomma ticks, like other ixodid ticks, are known to harbour endosymbionts that are involved in various mechanisms that affect the biology and ecology of the tick. In *Hyalomma marginatum*, *Midichloria* and *Francisella* are obligate nutritional endosymbionts (syntheses of vitamin B) that are thought to have a mutualistic interaction and play a role in tick fitness (Buysse et al. 2021). Another study on field-collected *H. excavatum, H. scupense* and *H. marginatum* detected three endosymbionts, *Francisella, Midichloria* and *Rickettsia*, with *Rickettsia* symbionts playing a role in tick physiology, fitness and pathogen transmission (Benyedem et al. 2022).

Another reason for the low fertility may be the elimination of endosymbionts due to the antibiotics used, so reducing the use of gentamycin or replacing it with another compound with lesser toxicity to endosymbionts might be helpful in improving the tick fertility.

Changes in the microbiome of ticks fed *in vivo* and *in vitro* have recently been reported for *Ixodes ricinus* and were suggested to affect the fecundity of ticks (Militzer et al. 2023). This

would also need to be verified for *Hyalomma* ticks fed consecutively for at least one generation *in vitro* and compared to ticks fed on animals as well.
Since tick endosymbionts are thought to play an important role in tick biology, changes in the symbiont richness could affect tick fitness and biology and will therefore have implications for studies of tick biology, tick-pathogen interaction, pathogen transmission and control.

Despite the advantages of *in vitro* feeding, such as the possibility to feed and observe ticks in a controlled environment, reduced costs compared to the housing and use of experimental animals, standardisation of the tick feeding assays when testing acaricidal products, in addition to its contribution to the 3Rs, it does not mimic the natural feeding of ticks as an interaction with the host immune system is lacking and the feeding and reproductive parameters are in general not as good as those of ticks fed on animals (Bonnet and Liu 2012). Such variation should be taken into account when trying to establish and maintain tick colonies using *in vitro* feeding and conducting molecular studies.
The success of artificially feeding the juvenile life stages of *Hyalomma* is encouraging, as it would facilitate the infection of these ticks with *T. annulata*-infected blood. This has previously been done for *Hyalomma anatolicum* nymphs which were infected *in vitro* with *Theileria lestoquardi* (Tajeri et al. 2016), but not yet for *T. annulata*. Such studies still depend on the use of experimental animals infected with *T. annulata* to obtain erythrocytes containing piroplasms infectious to ticks. Therefore, the *in vitro* infection of RBCs with *T. annulata* merozoites is a crucial step for the eventual completion of the *T. annulata* life cycle *in vitro,* when combined with artificial tick feeding.
As detailed in Chapter 4, we have reported the successful infection of RBCs with *T. annulata* merozoites *in vitro*, but the culture conditions require further optimization to further increase the parasitaemia and to maintain a continuous culture of the piroplasm stage that can be used to infect ticks *in vitro*. Different factors could be tested separately or combined in order to increase the parasitaemia. For example, a combination of methods, such as the addition of Apicidin to cell cultures together with an elevated cultivation temperature of 41°C to induce merogony could be helpful to increase merozoites yield (Tajeri et al. 2022). In addition, the use of different media and amount of FBS could be optimized, as it has been reported that HL-1 medium support the growth of intraerythrocytic stages of *T. uilenbergi* better than RPMI (Miranda et al. 2006). Another study showed that *Theileria equi* grew better in M199 supplemented with 40% normal FBS as the heat inactivation of the serum negatively affected the growth and survival of the culture, which suggest an essential role of the complement system in the continuous culture of erythrocytic stages. Furthermore, the addition of other supplements such as bovine serum albumin (BSA), lipids-cholesterol-rich mixture, insulin, transferrin, could help improve the parasitaemia and have been shown to be useful in

replacing the use of FBS in *Plasmodium* and *Babesia* cultures (Ofulla et al. 1993; Rojas-Martínez et al. 2018).

The results of the studies reported in this thesis may be helpful to future studies on the production of culture-derived *Theileria* vaccines, improve our understanding of the attenuation process and provide tools to study the life cycle of *T. annulata*, including its interaction with the tick vector *in vitro*. It will also contribute to the 3Rs of humane animal research by providing a viable alternative to the use of laboratory animals and animal-derived products in tick and *Theileria* research.

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Appendices

Appendix 1: Supplementary material publication 2

Supplementary table 1. Details of the quantitative RT-PCR primers used and designed in this study

Gene Id/description	Sequence name & direction	Product size (bp)	Sequence (5'-3')
EPrG00000717687 (rRNA)	EPr687-F	150	AGTGACGCGCATGAATGGAT
	EPr687-R		TCAAGTCGTTTCACAAAGCCGG
ribosomal protein L18, putative	TA04425-F	159	ACACATGATGTACTTTGTTTTGTTTCT
	TA04425-R		TGCTGCTTTTATTGTACCACCT
hypothetical protein, conserved	TA04760-F	150	CAAGGATCAATTTTTATGTGTCCACCA
	TA04760-R		TGGTTTCGGATTCCAATCATATCCA
60S ribosomal L34 protein, putative	TA04840-F	149	CTGGTGCTCGTCTTGTTCTTCA
	TA04840-R		TGGTCTTGATACTGTACGATTTCTATG
hypothetical protein	TA07230-F	150	AGGAAGAATTAAAAACAACACCAATGA
	TA07230-R		TGAGTTTGGTTTTAGTTTGTTGTGGA
30S ribosomal protein S8, putative	TA09695-F	150	AAAACCTAGTCGTGATATTTGGGT
	TA09695-R		GCAATGAGCATTTTACCACCAA
hypothetical protein	TA13935-F	150	TGGAGAATTTGAAGATTTGGGTGTG
	TA13935-R		GTAGCAAATTTATAAAGTGCTCCTACA
hypothetical protein, conserved	TA15915-F	150	TGGTAGAGTATGGAAAGGAACATCA
	TA15915-R		CCTTAGCTTCCTTTTTGATTTCTTCCA
hypothetical protein	TA18140-F	184	TGGATATTATTTGGAAGAAAAAGATG
	TA18140-R		TTCACCCAAACTTTCAATATCA
hypothetical protein	TA20225-F	183	TGGTCAAATTGGTTAACATTTGAAGA
	TA20225-R		ACAACTTGCTATACCTGCTGT
Tap370b08.q2ca38.02c (cytochrome C oxidase subunit III	Tap370-F	150	TCAAGGTGATGAATACTCATTGGT
	Tap370-R		CCACAATTTTCAGAAGCAAAGGC
T. annulata actin ii, putative (TA13410), partial mRNA	TA13410-F	150	GACATTAAGGAGCGGTGCTG
	TA13410-R		AGTAGTGCCGTCTGGGAGTTT

Supplementary table 2: Differentially expressed genes (DEGs) with a potential AP-1 (activator protein 1) and NFkB (nuclear factor kappaB) binding sites

	Gene stable ID	Gene description	Gene name	Status in attenuated Beja
AP-1	ENSBTAG00000000381	sphingosine-1-phosphate receptor 4 [Source:VGNC Symbol;Acc:VGNC:34255]	S1PR4	Up
	ENSBTAG00000005313	EPH receptor B3 [Source:VGNC Symbol;Acc:VGNC:28539]	EPHB3	Up
	ENSBTAG00000006232	WD repeat domain 86 [Source:VGNC Symbol;Acc:VGNC:53682]	WDR86	Up
	ENSBTAG00000006806	keratin 17 [Source:VGNC Symbol;Acc:VGNC:50398]	KRT17	Up
	ENSBTAG00000008331	transmembrane protein 54 [Source:VGNC Symbol;Acc:VGNC:36097]	TMEM54	Up
	ENSBTAG00000008832	C-C motif chemokine ligand 1 [Source:VGNC Symbol;Acc:VGNC:26943]	CCL1	Up
	ENSBTAG00000008868	calpain 3 [Source:VGNC Symbol;Acc:VGNC:26747]	CAPN3	Up
	ENSBTAG00000010270			Up
	ENSBTAG00000010371	ChaC glutathione specific gamma-glutamylcyclotransferase 1 [Source:VGNC Symbol;Acc:VGNC:27262]	CHAC1	Up
	ENSBTAG00000010416	Ras and Rab interactor 3 [Source:VGNC Symbol;Acc:VGNC:53950]	RIN3	Up
	ENSBTAG00000012219	chondroitin sulfate proteoglycan 4 [Source:VGNC Symbol;Acc:VGNC:57158]	CSPG4	Up
	ENSBTAG00000012682	unc-13 homolog A [Source:VGNC Symbol;Acc:VGNC:36664]	UNC13A	Up
	ENSBTAG00000012834	arylsulfatase family member I [Source:VGNC Symbol;Acc:VGNC:26177]	ARSI	Up
	ENSBTAG00000013736	prominin 1 [Source:VGNC Symbol;Acc:VGNC:33364]	PROM1	Up
	ENSBTAG00000014358	eva-1 homolog B [Source:VGNC Symbol;Acc:VGNC:52770]	EVA1B	Up
	ENSBTAG00000014612	dedicator of cytokinesis 2 [Source:VGNC Symbol;Acc:VGNC:28157]	DOCK2	Up
	ENSBTAG00000014855	microtubule associated monooxygenase, calponin and LIM domain containing 2 [Source:VGNC Symbol;Acc:VGNC:31458]	MICAL2	Up
	ENSBTAG00000014915	ETS variant transcription factor 5 [Source:VGNC Symbol;Acc:VGNC:28631]	ETV5	Up
	ENSBTAG00000016881	alanyl aminopeptidase, membrane [Source:VGNC Symbol;Acc:VGNC:25960]	ANPEP	Up
	ENSBTAG00000020674	lymphotoxin beta [Source:VGNC Symbol;Acc:VGNC:31068]	LTB	Up
	ENSBTAG00000023073	family with sequence similarity 89 member A [Source:VGNC Symbol;Acc:VGNC:28842]	FAM89A	Up
	ENSBTAG00000025634	formin 1 [Source:HGNC Symbol;Acc:HGNC:3768]	FMN1	Up
	ENSBTAG00000038240			Up

ENSBTAG00000048682	zinc finger protein 548 [Source:NCBI gene (formerly Entrezgene);Acc:504913]	ZNF548	Up
ENSBTAG00000050361			Up
ENSBTAG00000050719	calcium-dependent phospholipase A2 PLA2G2D1 [Source:NCBI gene (formerly Entrezgene);Acc:494318]	PLA2G2D1	Up
ENSBTAG00000052473			Up
ENSBTAG00000052516	cytidine deaminase [Source:VGNC Symbol;Acc:VGNC:27055]	CDA	Up
ENSBTAG00000001839	OCIA domain containing 2 [Source:VGNC Symbol;Acc:VGNC:32396]	OCIAD2	Down
ENSBTAG00000004126	myeloid leukemia factor 1 [Source:NCBI gene (formerly Entrezgene);Acc:533379]	MLF1	Down
ENSBTAG00000004971	GRAM domain containing 1C [Source:VGNC Symbol;Acc:VGNC:29624]	GRAMD1C	Down
ENSBTAG00000007388	zinc finger CCCH-type containing 12D [Source:VGNC Symbol;Acc:VGNC:37100]	ZC3H12D	Down
ENSBTAG00000007704	ELOVL fatty acid elongase 7 [Source:VGNC Symbol;Acc:VGNC:28453]	ELOVL7	Down
ENSBTAG00000009080	integrin subunit beta 6 [Source:VGNC Symbol;Acc:VGNC:30331]	ITGB6	Down
ENSBTAG00000009294	delta 4-desaturase, sphingolipid 2 [Source:VGNC Symbol;Acc:VGNC:27991]	DEGS2	Down
ENSBTAG00000010103	tripartite motif containing 9 [Source:VGNC Symbol;Acc:VGNC:36353]	TRIM9	Down
ENSBTAG00000013010	coiled-coil domain containing 126 [Source:VGNC Symbol;Acc:VGNC:26842]	CCDC126	Down
ENSBTAG00000013284	serum/glucocorticoid regulated kinase family, member 3 [Source:NCBI gene (formerly Entrezgene);Acc:504480]	SGK3	Down
ENSBTAG00000014972	prostaglandin E receptor 4 [Source:VGNC Symbol;Acc:VGNC:33504]	PTGER4	Down
ENSBTAG00000018043	lecithin-cholesterol acyltransferase [Source:VGNC Symbol;Acc:VGNC:30809]	LCAT	Down
ENSBTAG00000018059	CD80 molecule [Source:VGNC Symbol;Acc:VGNC:27048]	CD80	Down
ENSBTAG00000018463	vimentin [Source:VGNC Symbol;Acc:VGNC:36796]	VIM	Down
ENSBTAG00000021336	kinesin family member 5A [Source:VGNC Symbol;Acc:VGNC:30605]	KIF5A	Down
ENSBTAG00000025219			Down
ENSBTAG00000030198	transmembrane protein 232 [Source:VGNC Symbol;Acc:VGNC:36046]	TMEM232	Down
ENSBTAG00000031814	serine dehydratase [Source:VGNC Symbol;Acc:VGNC:34399]	SDS	Down
ENSBTAG00000039154			Down
ENSBTAG00000040290	glutathione S-transferase omega 2 [Source:VGNC Symbol;Acc:VGNC:29687]	GSTO2	Down
ENSBTAG00000045581			Down

	ENSBTAG00000046666	tetratricopeptide repeat domain 9B [Source:VGNC Symbol;Acc:VGNC:36482]	TTC9B	Down
	ENSBTAG00000052924	sodium/potassium transporting ATPase interacting 2 [Source:HGNC Symbol;Acc:HGNC:16443]	NKAIN2	Down
NFkB	ENSBTAG00000001186	SH2 domain containing 4A	SH2D4A	Up
	ENSBTAG00000000917	bone morphogenetic protein 1	BMP1	Up
	ENSBTAG00000000625	Bos taurus SMAD family member 6 (SMAD6)	SMAD6	Up
	ENSBTAG00000000460	Bos taurus synaptotagmin like 2 (SYTL2	SYTL2	Up
	ENSBTAG00000000376	purinergic receptor P2X 3	P2RX3	Up
	ENSBTAG00000001146	human immunodeficiency virus type I enhancer binding protein 2	HIVEP2	Up
	ENSBTAG00000000016	Bos taurus lymphotoxin alpha (LTA)	LTA	Up
	ENSBTAG00000000828	calpain 6	CAPN6	Up
	ENSBTAG00000001004	Bos taurus endothelial cell adhesion molecule	ESAM	Up
	ENSBTAG00000000381	sphingosine-1-phosphate receptor 4	S1PR4	Up
	ENSBTAG00000001468	sterile alpha motif domain containing 4A	SAMD4A	Up
	ENSBTAG00000001301	leucine rich repeat containing 32	LRRC32	Up
	ENSBTAG00000001864	nuclear receptor subfamily 4 group A member 3	NR4A3	Up
	ENSBTAG00000001401	Bos taurus solute carrier family 45 member 1 (SLC45A1)	SLC45A1	Down
	ENSBTAG00000001335	Bos taurus growth hormone receptor (GHR)	GHR	Down
	ENSBTAG00000000667	apolipoprotein L, 3	APOL3	Down
	ENSBTAG00000000541	Bos taurus NK2 homeobox 1 (NKX2-1)	NKX2-1	Down
	ENSBTAG00000000240	Bos taurus A-kinase anchoring protein 7 (AKAP7)	AKAP7	Down
	ENSBTAG00000002135	Bos taurus CD69 molecule (CD69)	CD69	Down
	ENSBTAG00000008028	Bos taurus chimerin 1 (CHN1), transcript variant 2, mRNA	CHN1	Down
	ENSBTAG00000011003	Bos taurus IKAROS family zinc finger 3 (IKZF3	IKZF3	Down

ENSBTAG00000009126	Y-box binding protein 2	YBX2	Down
ENSBTAG00000012007	Bos taurus suppressor of cytokine signaling 2 (SOCS2)	SOCS2	Down
ENSBTAG00000008996	SFI1 centrin binding protein	SFI1	Down
ENSBTAG00000008062	Bos taurus doublesex and mab-3 related transcription factor 2 (DMRT2)	DMRT2	Down
ENSBTAG00000008084	Bos taurus zinc finger protein 382 (ZNF382)	ZNF382	Down
ENSBTAG00000009576	Bos taurus protein kinase C and casein kinase substrate in neurons 3 (PACSIN3)	PACSIN3	Down

Supplementary table 3: *Theileria annulata* genes with SPs and/or TMD with their orthologs genes in *Theileria parva* and *Theileria orientalis*

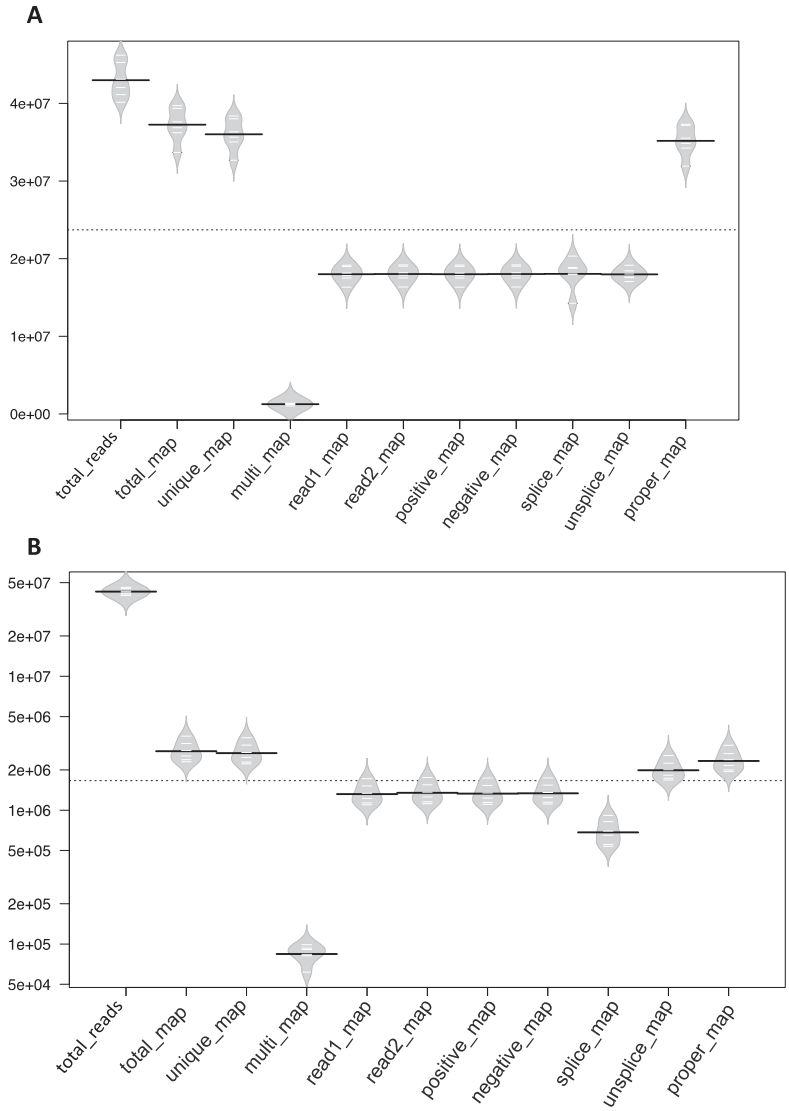
***Theileria annulata* Gene ID**	**Product Description**	**TM Domains**	**SignalP Peptide**	**Ortholog count**	**Computed GO Functions**	**NLS prediction**	**Ortholog in *T. parva***	**Product Description**	**Computed GO Functions**	**Ortholog in Orientalis**	**Product Description**	**Computed GO Functions**
TA02785	hypothetical protein, conserved	0	MKIILILLLIINFVIN	13	N/A	N/A	TpMuguga_01g02150	unspecified product	NA	MACJ_000566	unspecified product	NA
TA03875	RNA poly(A)-binding protein, putative	1	N/A	12	nucleic acid binding	PKRKNIPGYNRRRTNNRT	TpMuguga_03g00088	Polyadenylate-binding protein 2	nucleic acid binding	TOT_030000775	predicted protein	nucleic acid binding, RNA binding
TA04835	hypothetical protein, conserved	1	N/A	13	N/A	N/A	TpMuguga_03g00487	putative integral membrane protein	NA	TOT_030000364	conserved hypothetical protein	NA
TA05280	hypothetical protein	4	N/A	8	N/A	N/A	TpMuguga_03g00125	putative integral membrane protein		TOT_030000760	conserved hypothetical protein	integral component of membrane
TA06810	hypothetical protein, conserved	0	MYSNRNITVVLLLYITHFVHS	14	N/A	N/A	TpMuguga_01g00744	unspecified product	NA	TOT_010000701	conserved hypothetical protein	NA
TA07230	hypothetical protein	1	MKIKILFIILIINFIKC	7	N/A	N/A	TpMuguga_04g00068	putative integral membrane protein	NA	TOT_040000825	conserved hypothetical protein	integral component of membrane

TA08280	hypothetical protein	2	N/A	8	N/A	N/A	TpMuguga_04g00092	putative integral membrane protein	NA	TOT_040000803	conserved hypothetical protein	integral component of membrane
TA08715	bacterial histone-like protein, putative	0	MFTYTNSFLLLIIIICLTVES	14	DNA binding	N/A	TpMuguga_04g00110	Bacterial DNA-binding family protein	DNA binding	TOT_040000787	uncharacterized protein	DNA binding
TA09825	hypothetical protein, conserved	1	N/A	13	N/A	N/A	TpMuguga_04g00817	putative integral membrane protein	NA	TOT_040000111	conserved hypothetical protein	integral component of membrane
TA11950	hypothetical P-, Q-rich protein family protein, putative	1	MINNIKYLIFVLIFRSCIFVASS	332	N/A	N/A	TpMuguga_02g00216	unspecified product	NA	TOT_030000702	uncharacterized protein	NA
TA20225	hypothetical protein	1	N/A	3	N/A	N/A	TpMuguga_01g00574	putative integral membrane protein	NA	MACK_000497	unspecified product	NA

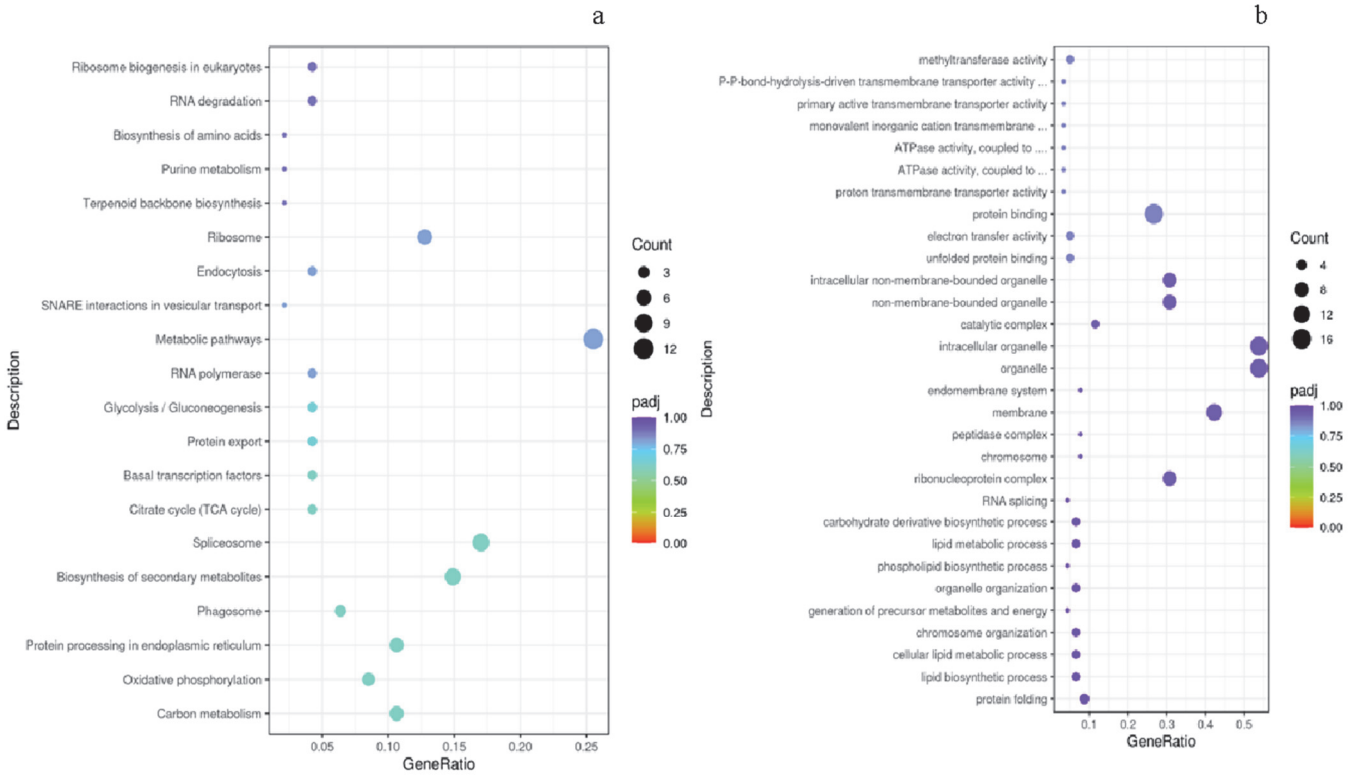
Supplementary table 4: Orthologs genes of *Theileria annulata* annotated in *Theileria parva* and their functions

Gene ID in *Theileria annulata*	Ortholog gene in *Theileria parva*	Ortholog gene description	Function (GO term)
TA04760	TpMuguga_03g00476	MED6 mediator sub complex component family protein	• regulation of transcription by RNA polymerase II • transcription coregulator activity
TA04840	TpMuguga_03g00488	60S ribosomal L34 protein, putative	• translation • ribosome • structural constituent of ribosome
TA09695	TpMuguga_02g00064	Ribosomal protein S8 family protein	• translation • ribosome • structural constituent of ribosome

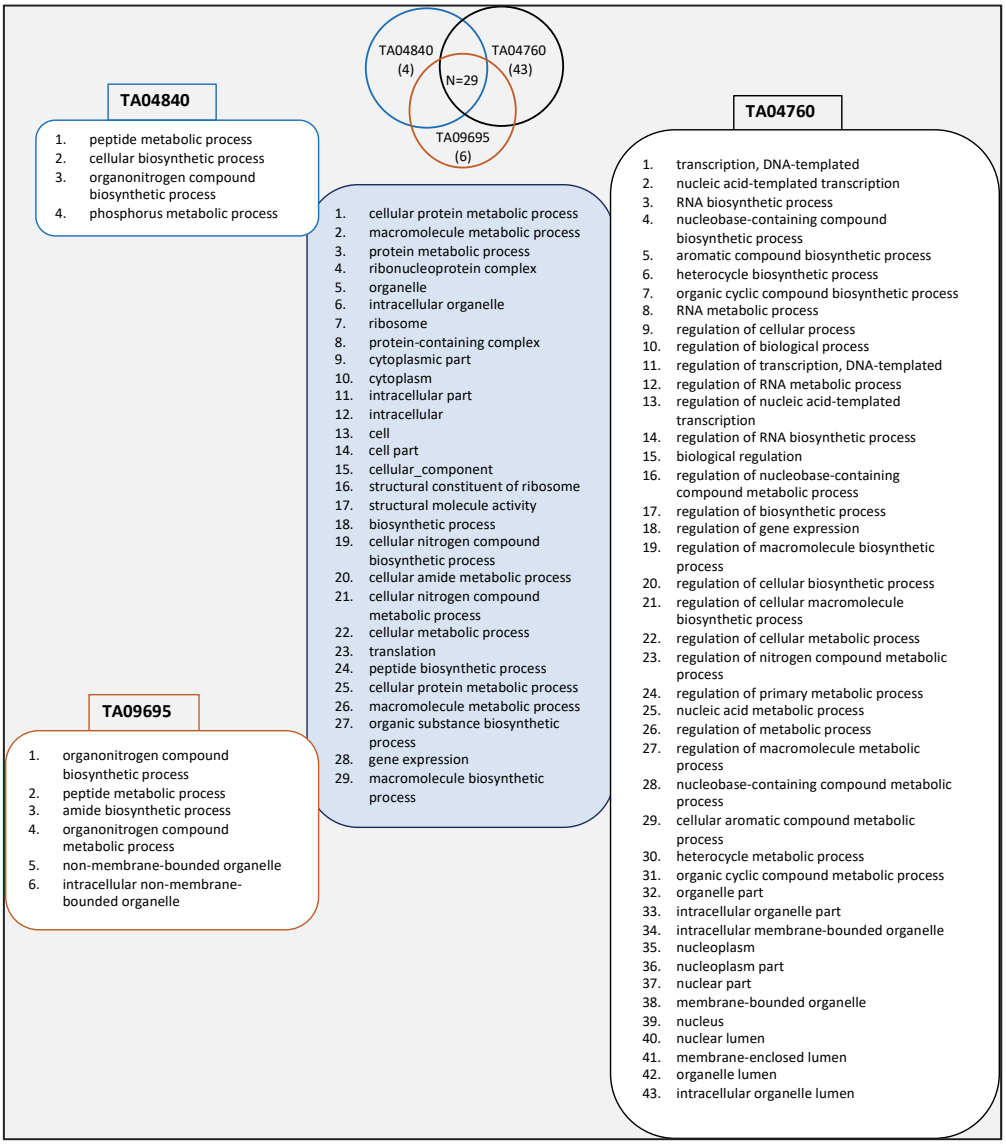
Supplementary Figure 1: Host (A) and parasite (B) mapped read count distribution. Black lines show the means; white lines represent individual data points; polygons represent density of the data. **total_reads:** Total clean reads used for analysis, **total_map**: Number and percentage of reads aligned to the genome, **total mapping rate**: (mapped reads)/(total reads)*100, **unique_map**: Number and percentage of reads aligned to the unique position of the reference genome (for subsequent quantitative data analysis), **unique mapping rate**: (uniquely mapped reads)/(total reads)*100, **Multi_map**: number and percentage of reads aligned to multiple locations in the reference genome, **multiple mapping rate**: (multiple mapped reads)/(total reads)*100, **read1_map**: Number and percentage of read1 aligned to the reference genome, **read2_map**: Number and percentage of read2 aligned to the reference genome, **positive_map**: Number and percentage of reads aligned to the positive chain of the reference genome, **negative_map**: Number and percentage of reads aligned to the negative chain of the reference genome, **splice_map**: Number of spliced reads on the genome and its percentage, **unsplice_map**: Number of complete reads aligned to genome and its percentage, **proper_map**: Number of paired read1 and read2 aligned to the genome and its percentage.



Supplementary Figure 2. KEGG (a) and GO (b) enrichment GO Enrichment Analysis Scatter Plot. The abscissa in the graph (a) is the ratio of the number of differential genes on the KEGG pathway to the total number of differential genes, and the ordinate is the KEGG pathway. The abscissa in the graph (b) is the ratio of the differential gene number to the total number of differential genes on the GO Term, and the ordinate is GO Term.



Supplementary Figure 3. Unique and shared GO enrichment pathways for the most differentially expressed genes in *Theileria annulata* transcript TA04840, TA04760 and TA09695 described as hypothetical proteins

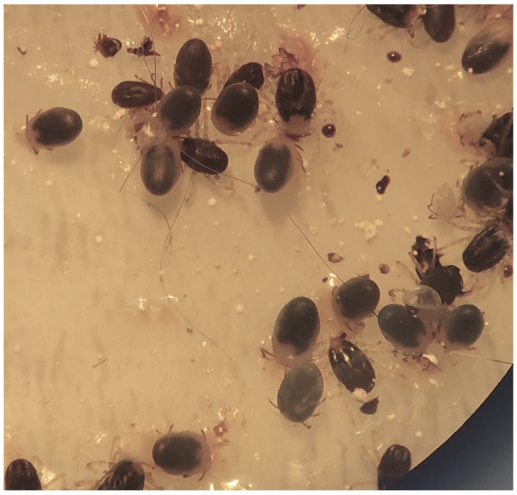


Appendix 2: Supplementary material publication 4

Supplementary table 1: Vitamin B component and concentrations

Vitamin B component	Final concentration (in blood meal)	Solvent
thiamine	100µg/mL	0.9% NaCl
riboflavin	20µg/mL	0.9% NaCl
nicotinic acid	100µg/mL	0.9% NaCl
panthotenic acid	100µg/mL	0.9% NaCl
pyridoxine	100µg/mL	0.9% NaCl
biotin	1µg/mL	0.9% NaCl
folic acid	30µg/mL	1N NaHCO3
cyanocobalamin	1µg/mL	0.9% NaCl
chlorine chloride	185µg/mL	0.9% NaCl
myo-inositol	118µg/mL	0.9% NaCl

Supplementary figure 1. Molting of *Hyalomma scupense* engorged larvae on the membrane



Summary

Improvement and development of methods to propagate *Theileria annulata in vitro* and to study the *T. annulata* life cycle based on cell culture techniques and an artificial tick feeding system

Tropical bovine theileriosis (TT, *Theileria annulata* infection) is a protozoan disease that affects cattle in the Mediterranean, Middle East and Asia. In countries where it occurs, the disease is mainly transmitted by ticks of the genus *Hyalomma*. Despite the availability of various control strategies, TT is still a major problem for the livestock industry in several countries. Progress in developing and improving control options (mainly vaccination) against *T. annulata* and its vectors is limited by several factors, including limited knowledge of the attenuation mechanism of the cell lines used as live vaccines, the need for experimental animals to maintain tick colonies, to produce infected tick challenge material and to assess the attenuation status. Another point of ethical concern is the use of Foetal Bovine Serum (FBS) in the production of the *T. annulata* cell culture vaccine. This reliance on the use of animal models and products, which is associated with high costs and logistical, biosafety and ethical constraints, frames the research approaches described in this thesis.

In the four main chapters, different aspects in studying the life cycle of *T. annulata in vitro* while adhering to the 3Rs principle are described. This includes (i) establishing a cell line using serum-free culture medium, replacing the use of FBS, (ii) comparing virulent and attenuated passages of *T. annulata* using RNA-seq to improve our understanding of virulence factors and the attenuation process and catalogue potential attenuation markers, (iii) inducing merogony in *T. annulata* cell lines and obtain piroplasm-containing erythrocytes that could later be used to infect ticks *in vitro,* and (iv) exploit recent advances in artificial tick feeding methods to artificially feed all life stages of two-host ticks *Hyalomma scupense* and *H. excavatum* and the three-host tick *Hyalomma dromedarii*. The successful feeding of all stages of *Hyalomma* ticks *in vitro* combined with the generation of *T. annulata* infected erythrocytes *in vitro,* may lead to the closure of the *T. annulata* life cycle *in vitro* without the use of experimental animals.

The results of these studies are encouraging, although they do require optimization, and are expected to contribute to the production of culture-derived *Theileria* vaccines, improve our understanding of the attenuation process and provide valuable tools to study the *Theileria* life cycle*,* including its interaction with vector ticks *in vitro*. It will also contribute to the 3Rs principle for humane animal research by providing a viable alternative to the use of laboratory animals in *Theileria* research. These findings could also be extrapolated to other pathogens with similar life cycles.

Zusammenfassung

Verbesserung und Entwicklung von Methoden zur Kultivierung von *Theileria annulata in vitro* und zur Untersuchung des Lebenszyklus von *T. annulata* auf der Grundlage von Zellkulturtechniken und einem künstlichen Zeckenfütterungssystem

Die tropische Rindertheileriose (TT, *Theileria annulata*-Infektion) ist eine Protozoenerkrankung, die Rinder im mediterranen Raum, im Mittleren Osten und in Asien vorkommt. In den betroffenen Ländern wird die Krankheit hauptsächlich durch Zecken der Genus *Hyalomma* übertragen. Obwohl verschiedene Bekämpfungsstrategien verfügbar sind, ist die TT noch immer ein bedeutendes ökonomisches Problem für die Tierproduktion. Die Fortschritte bei der Entwicklung und Verbesserung von Kontrollmöglichkeiten (hauptsächlich von Impfungen) gegen *T. annulata* und ihrer Vektoren werden durch mehrere Faktoren eingeschränkt, wie der begrenzten Kenntnis des Attenuierungsmechanismus der für die Lebendimpfstoffe verwendeten Zelllinien, der erforderlichen Nutzung von Versuchstieren für die Aufrechterhaltung von Zeckenkolonien, der Produktion infizierter Zecken für Infektionsversuche und der Bewertung des Attenuierungsstatus. Die ethisch bedenkliche Verwendung von fötalem Rinderserum (FBS) bei der Herstellung des *T. annulata*-Zellkulturimpfstoffs, die Abhängigkeit von Tiermodellen und -produkten verbunden mit hohen Kosten, logistischen und biosicherheits-relevanten Problemen beinhaltet den thematischen Forschungsansatz der vorliegenden Thesis.

In den vier Hauptkapiteln werden verschiedene Aspekte der Untersuchung des Lebenszyklus von *T. annulata in vitro* unter Einhaltung des 3R-Prinzips einer humanen Tierforschung beschrieben. Dazu gehören (i) die Etablierung einer Zelllinie unter Ersetzung von FBS durch ein serumfreies Kulturmedium, (ii) der Vergleich virulenter und abgeschwächter Passagen von *T. annulata* genutzer RNA-seq, um das Verständnis von Virulenzfaktoren und des Attenuierungsprozesses zu verbessern und um potenzielle Attenuierungsmarker zu katalogisieren, (iii) die Induktion von Merogonie in *T. annulata*-Zelllinien zu stimulieren und piroplasmahaltige Erythrozyten zu gewinnen zur späteren Nutzung für eine *in vitro*-Infektion von Zecken und (iv) die Nutzung jüngster methodischer Fortschritte bei der künstlichen Zeckenfütterung, um alle Lebensstadien der zweiwirtigen Zecken *Hyalomma scupense* und *H. excavatum* sowie der dreiwirtigen Zecke *H. dromedarii* künstlich zu füttern. Die erfolgreiche Fütterung aller Stadien von *Hyalomma*-Zecken *in vitro* sowie der Herstellung von mit *T. annulata* infizierten Erythrozyten *in vitro* kann dazu führen, dass der Lebenszyklus von *T. annulata in vitro* ohne den Einsatz von Versuchstieren geschlossen werden kann.

Die Ergebnisse dieser Studien sind ermutigend, auch wenn sie noch optimiert werden müssen. Sie sind ein Beitrag zur angestrebten Herstellung von *Theileria*-Vaccinen aus Kulturen, verbessern das Verständnis des Attenuierungsprozesses und unterstützen Untersuchungen zum Lebenszyklus von *Theileria* spp. einschließlich ihrer in vitro Interaktion mit Vektorzecken. Die Ergebnisse tragen zum 3R Prinzip humaner Tierforschung bei, indem sie eine praktikable Alternative zur Verwendung von Labortieren in der *Theileria*-Forschung bieten. Diese Erkenntnisse könnten auch auf andere Krankheitserreger mit gleichen Lebenszyklen extrapoliert werden.

List of publications

1. **Elati, K**., Daly, N., Dhibi, M., Laaribi, H., Rekik, M., Gharbi, M., (2024a). Repeated Cross-Sectional Survey of Ectoparasites in Sheep from Central Tunisia: Does Low Prevalence Indicate Good Hygiene or Resistance to Ectoparasites? Animals 14, 801. https://doi.org/10.3390/ani14050801

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3. **Elati, K.,** Benyedem, H., Fukatsu, K., Hoffmann-Köhler, P., Mhadhbi, M., Bakırcı, S., Bilgiç, H.B., Karagenç, T., Darghouth, M.A., Nijhof, A.M., (2024c). In vitro feeding of all life stages of two-host *Hyalomma excavatum* and *Hyalomma scupense* and three-host *Hyalomma dromedarii* ticks. Sci Rep 14, 444. https://doi.org/10.1038/s41598-023-51052-w

4. Benyedem, H., Hajji, T., Romdhane, R., Obara, I., Mhadhbi, M., Sebai, E., **Elati, K**., Chaari, S., Rekik, M., Darghouth, M.A., (2023). Genetic diversity of *Hyalomma marginatum* in Tunisia is not influenced by the bio-climate. Parasitol Res. https://doi.org/10.1007/s00436-023-07990-5

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6. **Elati, K**., Tajeri, S., Obara, I., Mhadhbi, M., Zweygarth, E., Darghouth, M.A., Nijhof, A.M., (2023b). Dual RNA-seq to catalogue host and parasite gene expression changes associated with virulence of *T. annulata*-transformed bovine leukocytes: towards identification of attenuation biomarkers. Sci Rep 13, 18202. https://doi.org/10.1038/s41598-023-45458-9

7. Obara, I., Nijhof, A., Atimnedi, P., Mijele, D., Nanteza, A., **Elati, K**., Bishop, R., 2023. The antigen recognition portion of African buffalo class I MHC is highly polymorphic, consistent with a complex pathogen challenge environment, and the 3' region suggests distinct haplotype configurations. Immunogenetics 75, 115–132. https://doi.org/10.1007/s00251-022-01287-0

8. **Elati, K.**, Khbou, M.K., Kahl, O., Mwacharo, J.M., El Shamaa, K., Rekik, M., Darghouth, M.A., Gharbi, M., (2022a). Preliminary study on the seasonal questing of *Ixodes ricinus* group ticks in Ain Draham forest (north-western Tunisia) with analyses of their phylogenetic diversity. Veterinary Parasitology: Regional Studies and Reports 36, 100786. https://doi.org/10.1016/j.vprsr.2022.100786

9. **Elati, K.**, Nijhof, A.M., Mwamuye, M.M., Ameen, V., Mhadhbi, M., Darghouth, M.A., Obara, I., (2022b). Sequence polymorphisms in a *Theileria annulata* surface protein (TaSP) known to augment the immunity induced by live attenuated cell line vaccine. Transbound Emerg Dis 69, 3350–3359. https://doi.org/10.1111/tbed.14687

10. **Elati, K.**, Zweygarth, E., Mhadhbi, M., Darghouth, M.A., Nijhof, A.M., (2022c). Cultivation, cryopreservation and resuscitation of *Theileria annulata* transformed cells in serum-free media. Front Vet Sci 9, 1055022. https://doi.org/10.3389/fvets.2022.1055022

11. Bouaicha, F., Eisenbarth, A., **Elati, K**., Schulz, A., Ben Smida, B., Bouajila, M., Sassi, L., Rekik, M., Groschup, M.H., Khamassi Khbou, M., 2021. Epidemiological investigation of Crimean-Congo haemorrhagic fever virus infection among the one-humped camels (*Camelus dromedarius)* in southern Tunisia. Ticks and Tick-borne Diseases 12, 101601. https://doi.org/10.1016/j.ttbdis.2020.101601

12. **Elati, K**., Bouaicha, F., Dhibi, M., Ben Smida, B., Mhadhbi, M., Obara, I., Amairia, S., Bouajila, M., Rischkowsky, B., Rekik, M., Gharbi, M., (2021). Phenology and phylogeny of *Hyalomma* spp. ticks infesting one-humped camels (*Camelus dromedarius*) in the Tunisian Saharan bioclimatic zone. Parasite 28, 44. https://doi.org/10.1051/parasite/2021038

13. Gharbi, M., Darghouth, M.A., **Elati, K.**, AL-Hosary, A.A.T., Ayadi, O., Salih, D.A., Hussein, A.M.E., Mhadhbi, M., Khbou, M.K., Hassan, S.M., Obara, I., Ahmed, L.S., Ahmed, J., (2020). Current status of tropical theileriosis in Northern Africa: A review of recent epidemiological investigations and implications for control. Transboundary and Emerging Diseases 67, 8–25. https://doi.org/10.1111/tbed.13312

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Abstract presentations

1. **Khawla Elati,** Faten Bouaicha, Mokhtar Dhibi, Boubaker Ben Smida, Moez Mhadhbi, Isaiah Obara, Safa Amairia, Mohsen Bouajila, Mourad Rekik, Mohamed Gharbi. Phenology and phylogeny of *Hyalomma* ticks infesting camels in the Tunisian Saharan bioclimatic zone. 5. Süddeutscher Zeckenkongress, Hohenheim, Germany, 2-4 March 2020 (Poster).
2. **Khawla Elati**, Isaiah Obara, Ameen Vahel, Moez Mhadhbi, Peter-Henning Clausen, Mohamed A. Darghouth & Ard M. Nijhof. Sequence polymorphisms in the *T. annulata* surface protein (TaSP) known to augment the immunity induced by live attenuated cell line vaccines, 29th Annual Meeting of the German Society for Parasitology, Bonn, Germany, 15-17 March 2021 (Poster).
3. **Khawla Elati**, Faten Bouaicha, Mokhtar Dhibi, Boubaker Ben Smida, Moez Mhadhbi, Isaiah Obara, Safa Amairia, Mohsen Bouajila, Barbara Rischkowsky, Mourad Rekik, Mohamed Gharbi. Ticks infesting one-humped camels (*Camelus dromedarius*) in the Tunisian Saharan bioclimatic zone and investigation of infection with Crimean-Congo haemorrhagic fever virus. Tagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten“, Berlin, Germany, 23-25 May 2022 (oral presentation).
4. **Khawla Elati**, Erich Zweygarth, Moez Mhadhbi, Mohamed Aziz Darghouth, Ard Menzo Nijhof. Cultivation, freezing and thawing of Theileria annulata schizont-infected lymphocytes in serum-free media: could the classic RPMI medium supplemented with Foetal Calf Serum be replaced? Tagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten“, Berlin, Germany, 23-25 May 2022 (poster).
5. **Khawla Elati,** Shahin Tajeri, Isaiah Obara, Med Aziz Darghouth, Ard Nijhof. RNA-seq identifies parasite genes potentially associated with virulence in *Theileria annulata* transformed macrophages. ApicoWplexa, Bern, Switzerland, 5-7 October 2022 (poster).
6. **Khawla Elati**, Erich Zweygarth, Mohamed Aziz Darghouth, Ard Menzo Nijhof. Cultivation of *Theileria annulata* transformed cells in serum-free media. ApicoWplexa, Bern, Switzerland, 5-7 October 2022 (poster).

7. **Khawla Elati**, Erich Zweygarth, Peggy Hoffmann-Köhler, Mohamed Aziz Darghouth & Ard Nijhof. Alternatives to the use of experimental animals in studies focusing on *Theileria annulata.* 15th International Symposium on Ticks and Tickborne Diseases, Weimar, Germany, 24–26 March 2023 (oral presentation).
8. **Khawla Elati,** Hayet Benyedem, Kohsuke Fukatsu, Peggy Hoffmann-Köhler, Moez Mhadhbi, Serkan Bakırcı, Hüseyin Bilgin Bilgiç, Tülin Karagenç, Mohamed Aziz Darghouth, Ard M. Nijhof*. In vitro* feeding of all life stages of two-host *Hyalomma excavatum* and *Hyalomma scupense* and three-host *Hyalomma dromedarii* ticks. 7. Süddeutscher Zeckenkongress, Hohenheim, Germany, 26-28 February 2024 (Poster).
9. **Khawla Elati,** Shahin Tajeri, Erich Zweygarth, Peggy Hoffmann-Köhler, Mohamed Aziz Darghouth & Ard Nijhof. Artificial tick feeding of *Hyalomma* ticks and *in vitro* infection of erythrocytes with *Theileria annulata* merozoites: valuable tools in completing the *T. annulata* life cycle *in vitro.* 7. Süddeutscher Zeckenkongress, Hohenheim, Germany, 26-28 February 2024 (oral presentation).

Awards/prizes

- **2nd poster prize:** Cultivation, freezing and thawing of *Theileria annulata* schizont-infected lymphocytes in serum-free media: could the classic RPMI medium supplemented with Foetal Calf Serum be replaced? Tagung der DVG-Fachgruppe „Parasitologie und parasitäre Krankheiten, Berlin, Germany, 23-25 May 2022.
- **1st poster prize:** RNA-seq identifies parasite genes potentially associated with virulence in *Theileria annulata* transformed macrophages. ApicoWplexa 2022 in Bern, Switzerland, 5-7 October 2022.
- **3rd oral presentation:** Alternatives to the use of experimental animals in studies focusing on *Theileria annulata.* 15th International Symposium on Ticks and Tickborne Diseases, Weimar, Germany, 24–26 March 2023**.**
- **Paper of the month award:** Dual RNA-seq to catalogue host and parasite gene expression changes associated with virulence of *T. annulata*-transformed bovine leukocytes: towards identification of attenuation biomarkers (2023). Department of Veterinary Medicine, Freie Universität Berlin, November 2023.

Intellectual content disclosure

Prof. Dr. Ard Nijhof, Prof. Dr. Mohamed Aziz Darghouth and Dr. Erich Zweygarth were all involved in the study's conceptualisation, supervision, reviewing and editing the manuscripts and the thesis. Khawla Elati designed the experiments and was responsible for the generation, analysis and interpretation of the data and writing the original draft of all the papers. The funding of the different experiments conducted was provided by Prof. Dr. Ard Nijhof. All co-authors revised and approved the publication of the articles. The review article that constitutes the content of chapter 1 was included in the thesis in agreement with the co-authors. Khawla Elati contributed significantly to the review of literature and writing the original draft of the review.

Acknowledgment

I would like to take this opportunity to express my appreciation and gratitude to the people and the organisations that have supported my doctoral studies. First of all, I would like to acknowledge the generous financial support provided by the German Academic Exchange Service (DAAD) during the four and a half years of my Ph.D. and the Freie Universität of Berlin for funding the open access publication of the research articles.

I am very grateful to Prof. Dr. Ard Nijhof for his unlimited and kind support, patience and understanding throughout the application process and the whole period of my PhD study. Thank you for the good working atmosphere, you are a true leader and scientist and you inspire me to do more.

Many thanks to Prof. Dr. Clausen for supporting my application and accepting to be my institutional host before I start working with Prof. Ard Nijhof.

I'm very grateful to Dr Erich Zweygarth for his kind guidance and support in my studies and personal matters.

Many thanks to Prof. Mohamed Aziz Darghouth for his support and advice during my studies, I appreciate it. Thanks to all the staff of the Laboratory of Parasitology in Tunisia, from whom I have learnt a lot. In particular, I can't forget the support and love I have received from my friends and brothers Moez Mhadhbi and Mokhtar Dhibi over the years, thank you for everything. Also, many thanks to Prof. Mohamed Gharbi, with whom I made the first steps of research, learned how to write a scientific article and much more.

While working in Berlin, I was fortunate and happy to have friendly colleagues such as Isaiah Obara, Bastian Gerst, Shahin Tajeri, Micky Mwamuye, David Omondi, Sophia Pinecki, Nina Militzer, Vincent Duru and Kousuke Fukatsu. Special thanks to Prof. Dr. Georg von Samson-Himmelstjerna for his encouragement. Thanks to Mrs Christine Gaede for her availability and kind assistance during the entire duration of the thesis.

My deepest gratitude goes to Barbara Mehlitz. Your friendship, generosity and support will always be engraved in my heart. You have made my life easy; no words can express my love and respect to you. Thanks to the other members of my German family Mike, Dieter, Roswitha and my little brother Paul. To my German sister Theresa Firsching, thank you for everything and I'm glad to have you.

I'm also grateful to Dr. Olaf Kahl for giving me the chance to work for tick-radar, I learned a lot from this collaboration on another project, thank you for your kind support and friendship.

My special thanks and heartfelt gratitude to my parents, Nejia and Khmaies, and my dear brothers, Aymen, Mohamed Salah and Achref. Your sacrifice, support and love mean the world to me. To my wonderful husband Achref, I'm very grateful for your love, support and patience during my difficult time. Without you, this achievement wouldn't be possible.

Conflict of Interest

In the context of this work, there are no conflicts of interest due to contributions from third parties.

Funding Sources

Khawla Elati was supported by a doctoral scholarship from the German Academic Exchange Service (DAAD).

Declaration of independence

I hereby certify that I have prepared this thesis independently and I have used only the sources and aids indicated. I confirm that this thesis is my original research work under the supervision and guidance of Prof. Dr Ard Nijhof, Prof. Dr Mohamed Aziz Darghouth and Dr Erich Zweygarth. I declare that this work has not been submitted for any award or other academic degree from any other institution or university.

Berlin, den 30.08.2024
Khawla Elati

Druck: Mensch und Buch Verlag Berlin