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Original article

Vector competence of *Rhipicephalus sanguineus* sensu stricto for *Anaplasma platys*

Alyssa N. Snellgrove[a,*], Inna Krapiunaya[a], Shelby L. Ford[a], Hannah M. Stanley[a], Alexandra G. Wickson[b], Kris L. Hartzer[a], Michael L. Levin[a]

[a] Rickettsial Zoonoses Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA
[b] Division of Foodborne, Waterborne & Environmental Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

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ABSTRACT

Anaplasma platys is a Gram-negative, obligate intracellular bacteria that causes canine infectious cyclic thrombocytopenia in dogs. The brown dog tick *Rhipicephalus sanguineus* sensu lato is presumed to be the vector of *A. platys* based on the overlap in distribution of *R. sanguineus* and *A. platys* infections, detection of *A. platys* DNA in both flat and engorged field-collected *R. sanguineus*, and the fact that dogs are primary hosts of both brown dog ticks and *A. platys*. However, the only study evaluating the vector competence of *R. sanguineus* for *A. platys* under controlled laboratory conditions reported an apparent inability of ticks to acquire or maintain the pathogen. In 2016, engorged female *Rhipicephalus sanguineus* sensu stricto ticks were collected off dogs to start a laboratory tick colony. After one generation of colony maintenance on tick-naïve and pathogen-free New Zealand White rabbits, a rabbit used to feed F1 adults seroconverted to *Anaplasma phagocytophilum* antigen. PCR and subsequent DNA sequencing identified the presence of *A. platys* in both the adult ticks fed on this rabbit and their resulting F2 progenies. Retrospective testing of all previous (P and F1) life stages of this colony demonstrated that the infection originated from one field-collected *A. platys*-infected female whose progeny was propagated in the laboratory and produced the PCR-positive F1 adults. Over the following (F2-F4) generations, the prevalence of *A. platys* in this colony reached 90–100 % indicating highly efficient transovarial and horizontal transmission, as well as transstadial maintenance, of this pathogen by *R. sanguineus* s.s.

1. Introduction

Anaplasmataceae (order: Rickettsiales) is a family of Gram-negative, obligate intracellular bacteria that includes the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia*. Currently there are seven recognized *Anaplasma* species: *A. bovis*, *A. caudatum*, *A. centrale*, *A. marginale*, *A. ovis*, *A. phagocytophilum*, and *A. platys*, with several additional *Candidatus* taxa being described in different parts of the world (Peng et al., 2018). Bacteria from this genus can cause clinical and subclinical infections in a variety of vertebrate hosts. *Anaplasma platys* (formerly *Ehrlichia platys)* was first described in 1978 as the causative agent of infectious canine cyclic thrombocytopenia (ICCT or CICT) (Dumler et al., 2001; Harvey et al., 1978). *Anaplasma platys* is a canine pathogen that infects host platelets and has a world-wide distribution that includes North, Central, and South America, Mediterranean Europe, Asia, Africa, and Australia (Rymaszewska and Grenda, 2008). *Anaplasma platys* infection in dogs is typically subclinical but may cause anorexia, weight loss, lymphadenomegaly, lethargy, fever, and mucopurulent nasal discharge (Bouzouraa et al., 2016; Harrus et al., 1997). Importantly, *A. platys* is often found in conjunction with other tick-borne pathogens including *Ehrlichia canis* and *Hepatozoon canis* (Andersson et al., 2013; Attipa et al., 2017; Lauzi et al., 2016), which can exacerbate the resulting illness (Gaunt et al., 2010). *Anaplasma platys* infections are highly prevalent in dog populations worldwide with seroprevalences above 20 % being reported in some regions (Matei et al., 2016; Yuasa et al., 2017). There are also reports of humans being infected with *A. platys* resulting in a mild illness with non-specific symptoms including headache, myalgia, and lethargy (Arraga-Alvarado et al., 2014; Breitschwerdt et al., 2014; Maggi et al., 2013).

Anaplasma platys is presumed to be transmitted by the brown dog tick, *Rhipicephalus sanguineus*, (Latrofa et al., 2014; Rymaszewska and Grenda, 2008; Woody and Hoskins, 1991) and *Anaplasma platys* infections in dogs are usually associated with heavy infestations by brown dog ticks (Abarca et al., 2007; Almazán et al., 2016). Currently, there are two recognized “lineages” of the brown dog tick: the temperate lineage or *R. sanguineus* sensu stricto (s.s) and the tropical lineage or *R.*

* Corresponding author at: Centers for Disease Control and Prevention, 1600 Clifton Road, MS H17-3, Atlanta, GA 30333, USA.
E-mail address: ASnellgrove@cdc.gov (A.N. Snellgrove).

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sanguineus sensu lato (s.l.) (Nava et al., 2018; Zemtsova et al., 2016). The two lineages presence in regions are presumed to correlate roughly with climatic variables such as annual mean temperature (Zemtsova et al., 2016). Additionally, there are some areas where both lineages can be found (Villarreal et al., 2018). Brown dog ticks are highly adapted to living in houses as well as kennels and are typically active year-round in tropical regions (Gray et al., 2013). The presumed involvement of the brown dog tick in vectoring *A. platys* is supported by the geographical overlap of *R. sanguineus* and *A. platys* infections (*e.g.* in Arizona, USA and regions of Taiwan); numerous field studies reporting *A. platys* DNA in both flat and engorged *R. sanguineus*; and dogs being primary hosts of both *R. sanguineus* and *A. platys* (Diniz et al., 2010; Yuasa et al., 2017). Additionally, *A. platys* infections in dogs are frequently found in conjunction with canine monocytic ehrlichiosis (CME) caused by *Ehrlichia canis,* which is known to be transmitted by *R. sanguineus* (Dagnone et al., 2009; Ramos et al., 2010; Sanogo et al., 2003; Sparagano et al., 2003). However, *R. sanguineus* ticks failed to acquire detectable levels of *A. platys* from laboratory-infected dogs in the only published study of its vector competence (Simpson et al., 1991). It is important to note that in that study, ticks were assessed for *A. platys* infection by light and electron microscopy rather than by PCR.

In vector-competent ticks, an infectious agent may be maintained transstadially – from one life stage to the next through the molt, horizontally – through a host (including cofeeding), and transovarially – from an infected female to its progeny. The transovarial transmission (TOT) route is extremely important in the natural maintenance of a variety of tick-borne pathogens, including *Rickettsia* spp., *Babesia* spp., and many viruses (Azad and Beard, 1998; Balashov, 1999; Bonnet et al., 2007; Danielová et al., 2002). It is generally accepted that TOT of *Anaplasma* spp. by tick vectors is either inefficient or nonexistent. However, published records on this topic are sparse and conflicting. For instance, *Rhipicephalus simus, Rhipicephalus microplus,* and *Ixodes ricinus* were unable to transovarially transmit *A. centrale*, *A. marginale,* and *A. phagocytophilum* respectively in laboratory studies (Esteves et al., 2015; Macleod and Gordon, 1933; Potgeiter and van Rensburg, 1987). On the other hand, *Dermacentor albipictus* has been reported to transovarially transmit *A. phagocytophilum* (Baldridge et al., 2009). In addition, *A. marginale* DNA was detected in flat field-collected larvae of *R. microplus* and *A. phagocytophilum* DNA in questing larvae of *Haemaphysalis megaspinosa* and *Haemaphysalis longicornis,* indicating the possibility for TOT (Fukui and Inokuma, 2019; Shimada et al., 2004; Yoshimoto et al., 2010). With regard to *A. platys,* naturally infected *R. sanguineus* nymphs have been reported to maintain the pathogen transstadially into the adult stage. However, the pathogen's DNA was not detected in pools of flat larvae collected from the ground in an animal shelter with an ongoing *A. platys* outbreak (Aktas and Ozubek, 2018).

Here we describe the establishment and maintenance of a laboratory colony of *R. sanguineus* s.s. persistently infected with *A. platys* over multiple generations, providing evidence of repeated efficient TOT, transstadial maintenance, and horizontal transmission.

2. Material and methods

2.1. Ticks

Sufficiently engorged females of *R. sanguineus* s.s. from a population formerly identified as belonging to the "temperate lineage" (Zemtsova et al., 2016) were collected in June 2016 off domestic dogs in eastern Arizona and brought into the laboratory for the purpose of establishing a specific pathogen free (SPF) tick colony. Ticks were held in individual vials during oviposition. Once oviposition was complete, spent females and pools of approximately 100 eggs from each clutch were tested by PCR using a broad range pan-*Rickettsia* assay targeting the 23S rRNA gene as described previously (Kato et al., 2013). All *Rickettsia*-positive ticks were discarded. Only PCR-negative egg batches produced by individual *Rickettsia*-negative females were kept for further propagation. With the presumption that TOT occurs either inefficiently or not at all in *Anaplasma* spp., neither the field-collected females (parental generation "P") nor their F1 progenies were initially tested for the presence of *Anaplasma* spp. DNA. DNA from these field collected ticks and their progeny were stored at −80 °C for later testing as needed.

The *Rickettsia*-negative F1 larval cohorts were used to establish a laboratory colony by feeding on pathogen-free and tick-naïve female New Zealand white rabbits (*Oryctolagus cuniculus*) as previously described (Levin and Schumacher, 2016). Between feedings, ticks were held in environmental incubators at 22 °C and 90–98 % relative humidity with a 16 h-day/8 h-night light cycle for the entirety of their life cycle. Feeding numbers for each life stage on rabbits are as follows: batches of larvae (up to ~5000 each) produced by three females were fed in three separate bags; 150 nymphs from individual larval progenies fed in three separate bags; 20 pairs of adults fed in separate bags (males and females from different previous cohorts). All animal procedures followed protocols for maintenance of tick colonies and were approved by the Centers for Disease Control and Prevention Institutional Animal Care and Use Committee.

2.2. Molecular evaluation of ticks

The tick species identity of this colony was confirmed by amplification and sequencing portions of the *cox1* (cytochrome c oxidase subunit 1) gene. Primers HCO2198 and LCO1490 were used to amplify a 710-bp portion of the *cox1* gene of the tick genome (Folmer et al., 1994; Hornok et al., 2018). Reactions were set up using the Taq PCR Master Mix kit (Qiagen Inc, Valencia, CA) and run on a Veriti 96 thermal cycler (Applied BioSystems, Foster City, CA). One negative control (reagent-grade water) and one positive control (known tick DNA from a different genus and species- *Dermacentor variabilis*) were included in each PCR run. Amplicons for experimental samples were visualized on 1.5 % agarose gels containing 0.1 mg/mL ethidium bromide and then extracted and purified using the Promega Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI). Sequencing reactions were set up bi-directionally using the BigDye Terminator V3.1 kit (Applied Biosystems, Carlsbad, CA) and run as recommended by the manufacturer on an Applied BioSystems 3130*xl* genetic analyser. Sequences were assembled using SeqMan Pro 14 and compared to GenBank data using BLASTn analysis.

2.3. Molecular testing for Anaplasmataceae and Anaplasma platys

When serum from a rabbit used for feeding of F1 adult ticks showed reactivity against *Anaplasma phagocytophilum* (strain Dawson) antigen using indirect immunofluorescence assay (IFA), the previously extracted tick samples from the field (P generation) and every stage in the F1 generation were retrospectively tested for the presence of Anaplasmataceae DNA as previously described (Li et al., 2002). Primers ECHSYBR-F and ECHSYBR-R were used to amplify a 147-bp product of the 16S ribosomal RNA gene. All PCR reactions were conducted in duplicate on a BioRad CFX 96 thermal cycler using the BioRad SsoFast EvaGreen Supermix kit (Life Science, Hercules, CA). Two larger DNA segments were sequenced from Anaplasmataceae-positive colony ticks: a 650-bp segment of the groEL gene and a 345-bp segment of the 16 s rRNA gene were amplified and sequenced in a subset of samples from multiple life stages and generations (Ait Lbacha et al., 2017; Inokuma et al., 2000). In all above reactions, reagent-grade water was used as a negative control and *A. phagocytophilum* genomic DNA as a positive control.

After sequence confirmation *via* these assays, a real-time *A. platys*-specific PCR assay for an 84-bp portion of the gltA gene was used on all samples to streamline further testing (da Silva et al., 2016). Table 1 presents a summary of assays used throughout this study.

Progenies of individual females (from P generation through F4) were tested for presence of *A. platys* DNA as a single pool of

Table 1
PCR primer and probe sequences.

Specificity	Target	Primer/Probe name	Primer/Probe sequence	Amplicon size	Citation
Anaplasmataceae	16s rRNA	ECHSYBR-F	AAC ACA TGC AAG TCG AAC GG	147 bp	Li et al. (2002)
		ECHSYBR-R	CCC CCG CAG GGA TTA TAC A		
A. platys	gltA	gltA84F	GAC CTA CGA TCC GGG ATT CA	84 bp	da Silva et al. (2016)
		gltA84R	TGG CGC AGT ATA CCC TTT TCT C		
		PLATYSp	FAM-TCT ACC ACC GCG GCA TGC AGC TCT G-BHQ1		
Anaplasmataceae	16s rRNA	EHR16SD	GGT ACC YAC AGA AGA AGT CC	345 bp	Inokuma et al. (2000)
		EHR16SR	TAG CAC TCA TCG TTT ACA GC		
Anaplasmataceae	groEL	AnaPlatF2	GCG TAG TCC GAT TCT CCA GT	650 bp	Ait Lbacha et al. (2017)
		AnaGro712R	CCG CGA TCA AAC TGC ATA CC		
Arthropods	*cox1*	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	710 bp	Hornok et al. (2018)
		LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G		

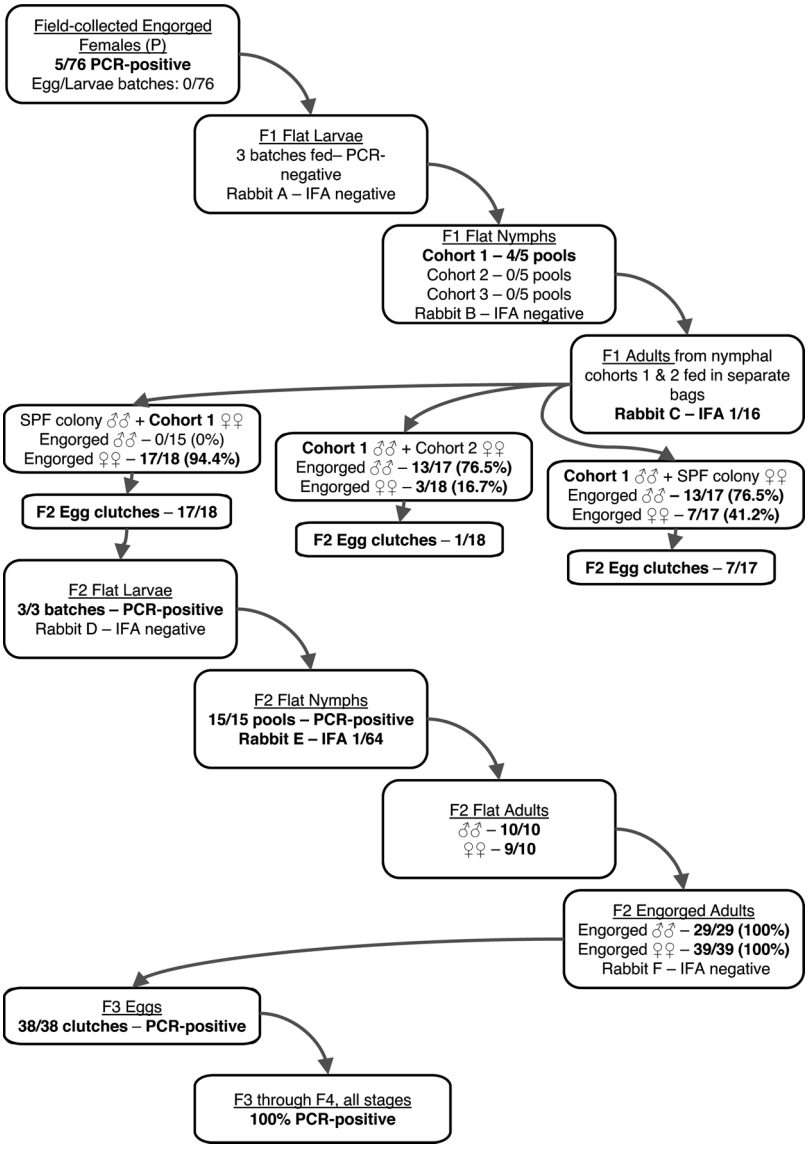


Fig. 1. Persistence of *Anaplasma platys* infection in successive generations of *Rhipicephalus sanguineus* s.s. and seroconversion in host animals. (PCR/IFA positive samples are bolded).

approximately 100 flat larvae or eggs from each egg-clutch. Flat nymphs were tested as five pools of five ticks from each cohort. Adult ticks were tested individually – five individual unfed females and five unfed males per cohort (ticks derived from nymphs fed in the same feeding bag), as well as all spent females and engorged males. Genomic DNA from each tick or pool of ticks was extracted using the Qiagen DNeasy kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol and eluted to a final volume of 100 µL.

2.4. Indirect immunofluorescence assay (IFA)

Sera from all rabbits used for tick feeding were tested by IFA for the

presence of IgG antibodies against tick-borne pathogens including *Anaplasma*, *Ehrlichia*, and *Rickettsia* spp. per the Clinical Microbiology Procedures Handbook (Leber, 2016). Serum samples were tested for antibodies against *Anaplasma* spp. using *A. phagocytophilum* (strain Dawson) antigen due to unavailability of *A. platys*-specific antigen, as this agent has yet to be isolated and grown in cell culture. For the same reason, we have not been able to assess the extent of cross-reactivity between these two *Anaplasma* species. Samples were collected at 14 days after tick placement and screened at 1/16 dilution with subsequent endpoint titration of positive samples.

2.5. Statistical analysis

Prevalence of infection in nymphal cohorts was calculated as maximum likelihood estimate (MLE) using the CDC-provided Microsoft Excel add-in for calculation of pooled infection rates (Biggerstaff, CDC, www.cdc.gov/ncidod/dvbid/westnile/software.htm). The maximum likelihood estimation takes into account the number of pools, number of positive pools, and variation in pool size thereby relaxing the assumption of the minimum infection rate that only one infected specimen exists in a positive pool, and therefore is a more accurate measure of infection rate.

3. Results

3.1. Molecular evaluation of ticks

Using the *cox1* gene for confirmation of the *R. sanguineus* species, the original P *R. sanguineus* female infected with *A. platys* and 4 individual nymphs from an F4 cohort were sequenced and matched 99.72–99.86 % to temperate lineage (*R. sanguineus* s.s.) ticks (Accession numbers: MN585197, MN593343- MN593344). Additionally, the ticks used here were collected from the same area as ticks assessed in a previous *R. sanguineus* phylogeny study that were confirmed as *R. sanguineus* “temperate lineage” (now sensu stricto) (Nava et al., 2018; Zemtsova et al., 2016).

3.2. Detection of A. platys in the tick colony

Fig. 1 visualizes pertinent PCR and IFA results throughout four generations of the tick colony from field-collected (P) females through their fourth-generation progeny. Five (~7 %) of the 76 originally collected field females tested after oviposition were *A. platys*-positive by a real-time *A. platys* specific qPCR assay, while none of the egg batches (F1) had *A. platys* DNA in detectable levels.

Three of the F1 larval batches – each the progeny of different P generation females – were fed in separate bags on Rabbit A. Cohort 1 derived from an egg clutch produced by a female that upon retrospective testing turned out to be *A. platys*-positive, while cohorts 2 and 3 were from progenies of *A. platys*-negative female ticks. Rabbit A did not develop antibodies reactive with *A. phagocytophilum* antigen after feeding these ticks.

Engorged larvae from these cohorts were kept separately and 25 molted F1 nymphs from each cohort were tested in pools of five for the presence of *A. platys* DNA by qPCR. The total number of molted nymphs was between 3000 and 4000 nymphs per cohort. In cohort 1, four of the five nymphal pools (containing 25 total ticks) tested were *A. platys* PCR-positive (estimated MEL 27.5 % ± 13.0 %). In cohorts 2 and 3 all pools were PCR-negative. Approximately 150 nymphs from each cohort were fed – again in separate bags – on rabbit B to produce F1 adult ticks. On day 14 post infestation, no antibodies reactive to *A. phagocytophilum* antigen were detected from rabbit B’s serum.

After engorged nymphs molted into F1 adult ticks, these were fed in 3 separate feeding bags on rabbit C to produce F2 progeny. The total number of adults produced by the previous nymphal feeding is not available and flat adults were not tested before feeding. In bag 1, 20 female ticks from cohort 1 were fed together with 20 males derived from a separate SPF colony of *R. sanguineus* s.s. continuously maintained in the laboratory for > 10 generations, which also originated from the same area of Arizona. In bag 2, 20 male ticks deriving from nymphal cohort 1 were combined with 20 females from nymphal cohort 2; and in bag 3, 20 cohort 1 males were combined with 20 females from the SPF laboratory colony of *R. sanguineus* s.s.

After feeding these adult ticks, rabbit C produced antibodies reactive with *A. phagocytophilum* antigen at a 1/16 dilution. This seroconversion prompted retrospective testing of all tick DNA extracts previously tested for rickettsial DNA using an *Anaplasma* spp.-specific and then an *A. platys*-specific assay as described (Table 1). From this point on, this line of *R. sanguineus* s.s. derived from field-collected females was designated as infected with *A. platys*.

All surviving engorged males and females fed on Rabbit C, as well as a sample of approximately 100 eggs from each resulting F2 egg clutch were tested for the presence of *A. platys* DNA *via* the real time PCR assay. Seventeen of 18 (94.4 %) female ticks fed in bag 1 were infected, while males originating from the SPF colony (15 tested) remained *Anaplasma*-free. In the other two bags (2 & 3), the prevalence of infection in males was 76.5 % in both bags, while 3 of 18 (16.7 %) and 7 of 17 (41.2 %) females originating from nymphal cohorts 2 and the SPF colony respectively contained *A. platys* DNA.

Overall, 17 of 18 (94.4 %), 1 of 18 (5.6 %), and 7 of 17 (41.2 %) female ticks successfully fed in bags 1, 2, and 3 respectively produced egg clutches that contained *A. platys* DNA. Contrary to the PCR-negative F1 eggs derived from the *A. platys*-infected field-collected tick, 25 of the 27 F2 egg clutches from PCR-positive females also contained *A. platys* DNA (with the exception of two females fed in bag 2). This indicates a high (92.6 % overall) frequency of TOT by *A. platys*-infected *R. sanguineus* s.s. The F2 ticks retained a high prevalence of infection (90–100 %) through the F2 nymphal and adult stages, the rabbit D used as a host for F2 larvae (3 PCR-positive batches fed) remained seronegative while rabbit E used to feed F2 nymphs (3 bags of 150 ticks each) produced IgG antibodies reactive with *A. phagocytophilum* antigen at a 1/64 dilution. Only two cohorts of adults derived from the three cohorts of F2 nymphs were tested and 10/10 (100 %) of males and 9/10 (90 %) of females were infected with *A. platys* as shown in Fig. 1. Females from Cohort 1 were crossed with males from Cohort 3 and *vice versa* on rabbit F for mating and feeding.

Of the 29 engorged male ticks and 39 females in the F2 generation that successfully fed on rabbit F, all tested PCR-positive for *A. platys* after feeding. However, rabbit F remained seronegative at 14 days after tick placement. All 38 F3 egg clutches produced by the F2 *A. platys*-infected females also contained *A. platys* DNA indicating 100 % frequency of TOT. From this point on, this colony of ticks has remained 100 % positive in every life stage and is now in the adult stage of the F4 generation. Individual testing of 50 flat nymphs from an F4 cohort as well as 30 flat females and 30 flat males from an F4 adult cohort showed that 100 % of these individual ticks were infected with *A. platys*. This is reflected in Fig. 1 by a single box indicating 100 % infection prevalence in F3 nymphs through F4 adults.

3.3. Molecular identification of Anaplasma platys

Using a 345-bp 16s rRNA assay, 5 sequenced tick samples (4 F4 individual nymphs, and one F1 engorged female) were 99.71 % identical to an *A. platys* isolate (Accession number: MN631251). Using a 710-bp portion of the groEL gene, 11 individual sequences were obtained from 4 F4 individual nymphs, 4 F1 engorged females and 3 of their respective egg batches and found to be 99.49 % identical to *A. platys*. Some sequences were 100 % identical to each other and those sequences were submitted to GenBank as one identical sequence instead of one for each individual tick (Accession numbers: MN593342, MN604393, MN604394, MN604395).

4. Discussion

Anaplasma platys is a canine pathogen with a world-wide distribution (Rymaszewska and Grenda, 2008). *R. sanguineus* has long been suspected as a vector of *A. platys* because infections in dogs are usually associated with heavy infestations by brown dog ticks (Abarca et al., 2007; Almazán et al., 2016). Yet, in the only laboratory study aimed at assessing the vector competence of *R. sanguineus* for *A. platys*, light and electron microscopy failed to visualize the pathogen in ticks feeding upon infected dogs (Simpson et al., 1991). Here we describe the ability of laboratory reared *R. sanguineus* s.s. to maintain *A. platys* infections over multiple generations *via* transstadial, cofeeding, and even transovarial routes while feeding on tick and pathogen-naïve New Zealand white rabbits, which are not known to be susceptible to this pathogen.

The detection of *A. platys* in the F1 progeny of female ticks collected from dogs in Arizona was serendipitous as TOT of *Anaplasma* spp., especially highly efficient TOT, has not been described in the available literature. If it were not for the routine serological monitoring of all animals used in the maintenance of tick colonies in our laboratory, we may have not detected the presence of this pathogen. Ticks from our continuously maintained SPF *R. sanguineus* s.s. and *R. sanguineus* s.l.-tropical colonies showed no evidence of *A. platys* infection either *via* PCR testing or from post-feeding rabbit serology. *Anaplasma platys* has also not been detected in other brown dog field ticks originating from California, Florida, or Oklahoma that we have received since. Therefore, it is logical to conclude that the adult ticks collected from dogs in Arizona were naturally infected with *A. platys*. Indeed, retrospective testing identified *A. platys* DNA in 5 out of 76 of the field-collected progenitor ticks.

Anaplasma platys DNA was not detected in samples of the F1 egg batches of the infected field-collected females, which would seem to conform with the common perception of *Anaplasma* spp. not being transovarially transmitted by their vectors. However, this perception was challenged when the pathogen was identified in flat F1 nymphs originating from one of the naturally infected field-collected ticks and in every subsequent life stage. There are at least two possible explanations for the failure to detect infection in those F1 eggs: low prevalence of infection or insufficient amount of pathogen DNA in infected eggs. An engorged *R. sanguineus* female lays approximately 1,500 − 4,000 eggs (Koch, 1982; Dantas-Torres, 2010). By selecting a sample of roughly 100 eggs, we tested between 2 and 6 % of eggs produced by individual females; and if the original prevalence of infection was < 5–6 %, no infected eggs may have been selected for testing. Alternatively, the quantity of *A. platys* DNA in F1 egg samples may have been below the limit of detection of the assays used. Regardless, the incongruence of PCR results between eggs and the subsequent life stages shows that the lack of pathogen amplification in egg samples by itself should not be interpreted as a sufficient proof of the absence of TOT.

The infected ticks maintained *A. platys* transstadially with high efficiency. By the adult stage of the F2 generation almost 100 % of ticks were infected. This is likely due to the fact that after the first generation we intentionally propagated cohorts with the highest prevalence and quantity of the pathogen. Feeding of numerous infected ticks in close proximity – within a 3″-diameter feeding bag - could have also led to enhanced proliferation of *A. platys* within this colony *via* cofeeding transmission. Notably, *A. platys* DNA was detected in engorged female ticks from previously PCR-negative cohorts after feeding together with males from an infected cohort. This suggests that *A. platys* can be transmitted between adult *R. sanguineus* s.s. *via* the cofeeding route or possibly *via* sexual transmission during mating. Conversely, uninfected males feeding with infected females remained pathogen-free, which may be due to the smaller volume of blood acquired during feeding.

Anaplasma platys has been consistently transmitted transovarially over at least 4 filial generations in this colony. Starting with the F2 generation, *A. platys* DNA was detectable in 100 % of egg clutches/larval batches produced by infected females. The enhanced proliferation of the pathogen within the infected cohorts may have resulted in increased efficiency of TOT, whereupon larger proportions of filial ticks were infected, contained larger quantities of bacteria, or both. Interestingly, some of the previously uninfected female ticks, which acquired *A. platys via* the cofeeding route during adult feeding with infected males were also able to transmit the pathogen to their progeny, albeit with a decreased frequency.

There were no observable deleterious effects in feeding/molting success, survival, or fecundity between the *A. platys*-infected and the SPF colonies of *R. sanguineus* s.s. maintained in our laboratory under identical conditions. New Zealand white rabbits are not known to be susceptible to *A. platys* infection, however, rabbits fed upon by infected ticks produced IgG antibodies detectable (albeit inconsistently) in IFA using a non-conspecific antigen. This indicates there may be some level of serological cross-reactivity between *A. platys* and *A. phagocytophilum*. Similar phenomena have been reported between other *Anaplasma* spp. For example, cross-reactivity has been reported in *Neoehrlichia mikurensis* PCR-positive patients assessed serologically with *A. phagocytophilum* antigen coated slides (Wass et al., 2018). This has also been reported in Swiss cattle and horses infected with *Anaplasma marginale* assessed by IFA using *A. phagocytophilum* antigen (Dreher et al., 2005). Due to the unavailability of quantitative data on cross-reactivity between *A. platys* and *A. phagocytophilum* serology results are interpreted only qualitatively in this study. Yet, the development of an immune response in previously naïve rabbits indicates that *R. sanguineus* s.s. is capable of inoculating *A. platys* into a host during feeding. However, the possibility of non-specific fluorescence - especially for lower titers such as 1/16 - cannot be discounted.

Altogether, the observations described here demonstrate the vector competence of *R. sanguineus* s.s. for *A. platys*, the causative agent of ICCT. Significantly, it also provides evidence that *A. platys* can be transmitted transovarially in *R. sanguineus* s.s.- at least under laboratory conditions. This discovery may have implications for our understanding of the natural cycle of *A. platys*. If TOT can be highly efficient in some populations, then it could serve as an additional route of *A. platys* maintenance alongside transstadial maintenance and cofeeding transmission. Based on the incongruities between the results in this study *versus* others reporting either no or rare events of TOT in *Anaplasma* spp., it is likely that many factors including tick species, bacterial species/strain, animal host, as well as environmental conditions may affect the probability and efficiency of TOT among *Anaplasma* species.

CRediT authorship contribution statement

Alyssa N. Snellgrove: Methodology, Investigation, Validation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Inna Krapiunaya:** Investigation, Validation. **Shelby L. Ford:** Investigation. **Hannah M. Stanley:** Investigation. **Alexandra G. Wickson:** Investigation. **Kris L. Hartzer:** Investigation. **Michael L. Levin:** Conceptualization, Methodology, Writing - review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101517.

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