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Population dynamics of multiple symbionts in the hard tick, *Dermacentor silvarum* Olenev (Acari: Ixodidae)

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ABSTRACT

Previously, we reported that *Coxiella*-like, *Rickettsia*-like and *Arsenophonus*-like symbionts could simultaneously coexist in *Dermacentor silvarum*. In this study, we examined their burdens and population dynamics in a single host during the host life cycle using quantitative PCR. Our results showed that multiple symbionts exhibited different abundances and varying trends in the tick host. *Coxiella*-like and *Rickettsia*-like symbionts were found at high densities in large quantities that fluctuated with time. This may coincide with oogenesis and mating of the host. Our findings provide insight into symbiont–tick interactions that lay the foundation for future studies.

1. Introduction

Symbioses between arthropods and bacteria are prevalent in nature, and the biological consequences of these interactions have been growing a research interest in recent years (Koropatnick et al., 2004; Dale and Moran, 2006; Duron et al., 2008). Mutually obligate symbiotic bacteria inhabit special bacteriocytes and provide the host with essential nutrients for survival (Shigenobu et al., 2000; Moran et al., 2008). Facultative symbionts are found scattered in the host and confer a broader variety of benefits, including host tolerance to biotic and abiotic stresses (Montllor et al., 2002; Oliver et al., 2003), expansion of the range of host plants (Tsuchida et al., 2004), and affects on host morphogenesis (Rakoff-Nahoum et al., 2004). In addition, facultative symbionts can alter reproductive mode of host (Stouthamer et al., 1999; Perlman et al., 2006).

Some invertebrate hosts have been reported to be co-infected or superinfected with symbionts (Distel et al., 2002; Oliver et al., 2006; Zhao et al., 2013). During superinfection, the host must balance the costs and benefits of superinfection for its survival, and thus likely developed some survival strategies to constrain the diversity and burden of symbionts. Symbionts compete with each other for limited resources and niches (Distel et al., 2002), and overcome vertical transmission bottlenecks in vertical transmission (Mira and Moran, 2002). On the other hand, symbionts may utilize some strategies to maintain superinfection, including balancing spatial and time differences during growth (Ijichi et al., 2002), which provides the host with beneficial advantages while sharing limited resources with symbiont partners (Montllor et al., 2002; Oliver et al., 2003; Tsuchida et al., 2004). For example, the aphid is a well-studied model organism for investigating symbiosis and co-infection with multiple symbionts. In addition to obligate symbiotic *Buchnera aphidicola*, the pea aphid may simultaneously harbor two facultative symbionts, including *Serratia symbiotica* and *Hamiltonella defensa*, which render heat tolerance and natural parasite enemy resistance to the host (Oliver et al., 2006). Generally, physiological processes and key life events involved in host symbiosis are accompanied by remarkable variation in the burden of symbiont. Therefore, population dynamics of symbionts are regarded as critical for host-symbiont and symbiont–symbiont interactions (Kondo et al., 2005; Rio et al., 2006).

Ticks are blood-feeding arthropods that can carry diverse symbionts in the host (Sassera et al., 2006; Dergousoff and Chilton, 2010; Ivanov et al., 2011; Zhang et al., 2011; Almeida et al., 2012; Gillespie et al., 2012; Epis et al., 2013). Moreover, *Amblyomma americanum* and *Dermacentor silvarum* have been shown to be superinfected by multiple symbionts (Clay et al., 2008; Liu et al., 2013). However, reports on the fitness granted by the tick-associated symbionts only in *A. americanum* and *Ixodes ricinus* have

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been described. *A. americanum* relies on obligate *Coxiella*-like symbiont for reproductive fitness (Jasinskas et al., 2007; Zhong et al., 2007). The facultative symbiont, *Candidatus* Midichloria mitochondrii, in *I. ricinus* corresponds with the phases of engorgement and molt (Sassera et al., 2008).

Previously, we discovered that *Coxiella*-like (CLS-Ds), *Rickettsia*-like (RLS-Ds) and *Arsenophonus*-like (ALS-Ds) symbionts coexist in *D. silvarum* (Liu et al., 2013). However, other aspects are needed to further understand symbiont–tick interactions, such as the burdens of the three different symbionts in *D. silvarum*, their population dynamics throughout the life cycle of the host, and their affects on host fitness. In this study, we estimated burdens and population dynamics of the three different symbionts in *D. silvarum* across life stages in female ticks using real-time quantitative PCR.

2. Material and methods

2.1. Sample collection

Unfed adult ticks were obtained from the Xiaowutai National Natural Reserve Area (39°50′–40°07′ N, 114°47′–115°30′ E) in China using flag dragging. Starving adults were reared on the ears of rabbits. Rabbits were maintained in a room with 50–55% relative humidity (RH) at 25–27 °C and exposed to daylight. After detachment, ticks were collected and incubated in cotton plugged glass tubes with folded filter paper in an incubator with 75 ± 5% RH and 6/18 h of L/D cycle at 26 ± 1 °C (Liu et al., 2005). All animal experiments were approved by the Institutional Animal Care and Use Committee of Hebei Normal University. Engorged adults and offspring were used for the data analysis in this study. Ticks were collected and dissected at 1, 2, 3, 4 and 5 days after blood feeding and dropping from the host to obtain different feeding and preoviposition stages.

2.2. DNA extraction

All tick samples were sterilized using 100% ethanol as described by Clay et al. (2008) prior to DNA extraction. Tissue samples were dissected and washed three times in sterile phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4{\cdot}7H_2O$, 1.4 mM KH_2PO_4, pH 7.4). DNA was extracted from 200 eggs (5 days after oviposition), 100 unfed larvae (5-day-old), 50 unfed nymphs (5-day-old) and 10 unfed adult females (5-day-old) using the DNeasy tissue kit (Qiagen, Germany) according to the manufacturer's protocol. Similarly, DNA was extracted from different pooled tissues samples (10 engorged females), including ovaries, salivary glands, Malpighian tubules, and midguts. The prevalence of CLS-Ds, RLS-Ds and ALS-Ds in females was 100%, 86% and 42%, respectively (Liu et al., 2013). All samples (larvae, nymphs, adults) investigated were obtained from a single field-collected female simultaneously infected by three symbionts (verified by detection of eggs). There were three replicates for each sample.

2.3. Quantitative PCR

Coxiella and *Rickettsia* contain a single copy of the 16S rRNA gene (Fogel et al., 1999). Symbionts densities can be determined by the ratio of symbiont-specific 16S rRNA gene copy number to the host *actin* gene copy number (Sunyakumthorn et al., 2013) while quantity is assessed by symbiont 16S rRNA copy number per tick. Quantitative PCR was performed using ABI 7500 (Applied Biosystems, Foster, USA) and SYBR Green I. The primers for amplifying specific 16S rRNA gene of CLS-Ds, RL-Ds and ALS-Ds and *actin* gene in the host are shown in Table 1. Standard curves were created with serial dilutions of plasmids containing inserts of the amplified specific 16S rRNA gene sequences from symbionts and *actin* gene sequences. The amplification efficiencies of primers CLS F/R, ALS 82F/198R, Rick 935F/1200R and Actin F/R were 94.8%, 98.9%, 90.5% and 94.4%, respectively (estimated in standards). Each of the quantitative mixtures contained 12.5 μl 2 × TansStart Green qPCR SuperMix UDG (TransGen, China), 0.5 μl 50 × Passive Reference Dye, 10 μl sterile waster, 0.5 μl primer each (10 μM), and 1 μl template DNA. The cycling conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Sterile water was used as a negative control in the qPCR assays.

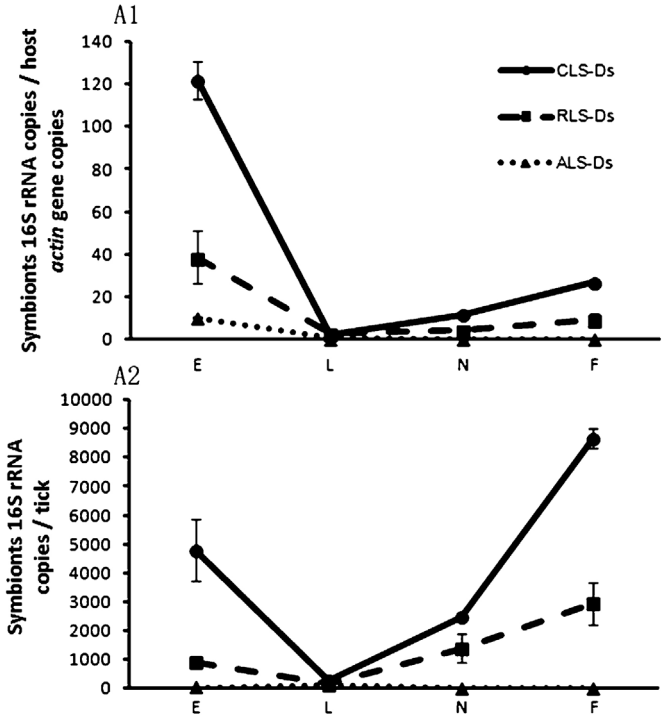

Fig. 1. Population dynamics of CLS-Ds, ALS-Ds and RLS-Ds in *D. silvarum* in different life stages. E, eggs; L, unfed larvae; N, unfed nymphs; F, unfed females. (A1) CLS-Ds, ALS-Ds and RLS-Ds densities in terms of the number of 16S rRNA gene copies per *actin* gene copy; (A2) CLS-Ds, ALS-Ds and RLS-Ds quantities in terms of the number of 16S rRNA gene copies per tick. Means and standard errors of the means are shown.

3. Results

The burden of the three symbionts in the host differed during the host life cycle. During development, the densities and quantities of CLS-Ds were found higher than the others, while ALS-Ds' were the lowest (Fig. 1). Interestingly, the variation trends of CLS-Ds and RLS-Ds were nearly identical in both densities and quantities. Their densities and quantities in the egg dropped remarkably to the lowest point after egg hatch. Then the amount and densities of symbionts increased progressively from larvae to adults. Moreover, the densities of CLS-Ds and RLS-Ds in eggs were higher than that of females, while their quantities were lower than that of females. Unlike CLS-Ds and RLS-Ds, there was no apparent difference in densities and quantities of ALS-Ds during host development.

During the reproductive stage, including the feeding and the preoviposition stages, burdens and population dynamics of the dominant symbionts, CLS-Ds and RLS-Ds, were detected in females. Our previous studies showed that CLS-Ds inhabited the ovaries and Malpighian tubules, while RLS-Ds and ALS-Ds infected ovaries, Malpighian tubules, midguts and salivary glands. Therefore, in this study, the densities and quantities of CLS-Ds and RLS-Ds were tested in infected tissues. High densities and large quantities were observed with CLS-Ds and RLS-Ds with markedly varied trends (Fig. 2). In ovaries, the changes in densities and quantities of

Table 1
Oligonucleotide primers used for RT-qPCR amplification.

Primer name	Genera	Target gene	Nucleotide sequence (5′-3′)	Reference
CLS F	*Coxiella*	16S rRNA	CACGTAGGAATCTACCTTGTAG	18
CLS R			CGTTTTGTTCCGAAGAAATTAT	
ALS F	*Arsenophonus*	16S rRNA	AGGGAGCTTGCTTCCTGGCCGG	18
ALS R			CGAAGGTGTGAGGCCTAATGG	
RLS F	*Rickettsia*	16S rRNA	TGCGGATCGCAGAGATGCTT	This study
RLS R			GTCTTGCTTCCCTCTGTAAAC	
Actin F	*Dermacentor*	*actin*	TTCCAGCCCTCGTTCCTGGGTAT	This study
Actin R			AATGATCTTGATCTTCATGGT	

CLS-Ds and RLS-Ds both displayed M-shaped wavy curves with three distinctive density peaks. The first density peak appeared on the third day of the feeding stage, the second was in the middle of preoviposition, and the third was on the last day before oviposition.

CLS-Ds were also found in Malpighian tubules at lower densities and quantities compared to those in the ovaries. Densities and quantities decreased continuously from the start of feeding to oviposition (Fig. 2). The population of RLS-Ds exhibited almost identical infection dynamics in the midguts, salivary glands, and Malpighian tubules, wherein two density peaks were observed (Fig. 2).

4. Discussion

During the developmental stage, the symbionts CLS-Ds and RLS-Ds were found at high density and large quantity that were closely related to the host biological events. In this stage, there was a notable decline in the density and quantity after hatching from eggs to newly hatched larvae. More specifically, genetic copies of 16S rRNA decreased dramatically, while the copies of the host *actin* gene remained constant during this stage (data not shown). These results were consistent with previous reports that the number of *Ca.* Midichloria mitochondrii in *I. ricinus* dropped remarkably from eggs to larvae (Sassera et al., 2008). This notable decrease may be associated with "transmission bottleneck," where symbionts are excluded during embryonic development. The vertical transmission of symbionts also endured "transmission bottleneck" from mother to eggs or embryos in other arthropod hosts (Mira and Moran, 2002; Weeks et al., 2002; Oliver et al., 2013).

From larvae to adults, the densities and quantities of CLS-Ds and RLS-Ds relatively increased over time (Fig. 1). During these developmental stages, CLS-Ds and RLS-Ds recovered from quiescence and appeared to multiply in parallel with host cell growth. In the immature stage, a relatively low symbiont burden helps to maintain low level of metabolic cost in the host. High densities and quantities of symbiont in mature females may confer some advantages to hosts' fitness and benefit vertical transmission. This phenomenon that the burden of symbionts increases with host growth and maturity has been shown in other arthropod hosts (Koga et al., 2003; Sakurai et al., 2005; Kono et al., 2008). Moreover, ALS-Ds was rarely transmitted vertically to the next generation with very low burdens in females that were removed over time. This illustrated that they may not play a key role in the physiological functions of their host (Weeks et al., 2002; Lo et al., 2006; Bansal et al., 2013).

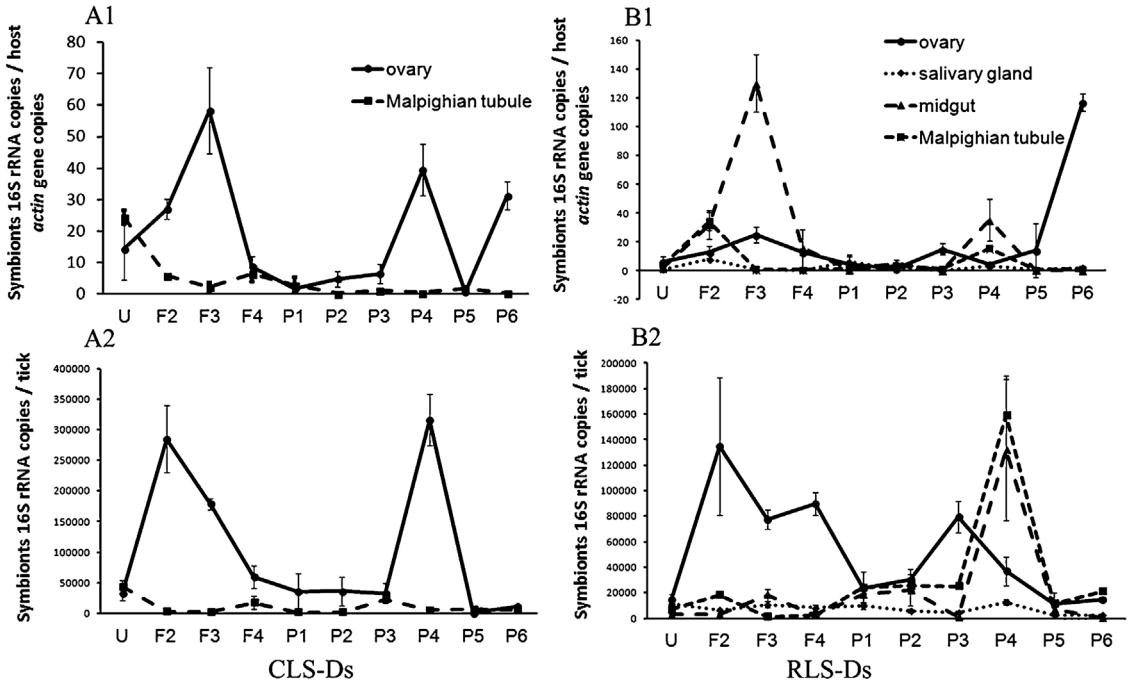

Fig. 2. Population dynamics of CLS-Ds and RLS-Ds in different tissues of adult *D. silvarum*. U, unfed stage; F2, the 2nd day in the feeding stage; F3, the 3rd day in the feeding stage; F4, the 4th day in the feeding stage; P1, the 1st day in the preoviposition stage; P2, the 2nd day in the preoviposition stage; P3, the 3rd day in the preoviposition stage; P4, the 4th day in the preoviposition stage; P5, the 5th day in the preoviposition stage; P6, the 6th day in the preoviposition stage. (A1) CLS-Ds densities in terms of the number of 16S rRNA gene copies per *actin* gene copy. (A2) CLS-Ds quantities in terms of the number of 16S rRNA gene copies per tick, (B1) RLS-Ds densities in terms of the number of 16S rRNA gene copies per *actin* gene copy. Means and standard errors of the means are shown; (B2) RLS-Ds quantities in terms of the number of 16S rRNA gene copies per tick. Means and standard errors of the means are shown.

In the first stage of reproduction, there was a remarkable increase in the densities and quantities of both CLS-Ds and RLS-Ds during the feeding stage, which was sustained up until the third day after the host sucked blood. Coincidentally, the third day is usually the time of mating. During this process, the most important biological event is oogenesis. Therefore, CLS-Ds and RLS-Ds could potentially be intimately associated with the reproduction fitness of the host, specifically during oogenesis and mating. It was demonstrated that laboratory-reared *A. americanum* cannot reproduce normally after antibiotic treatment to remove the *Coxiella*-like symbiont, illustrating the association between reproduction and symbiont (Zhong et al., 2007). Effective removal of symbiont by antibiotic treatment depends on the time and duration of antibiotic treatment (Ninio et al., 2015). Our results provide insight into the optimal time for antibiotic sensitivity, which usually coincides with rapid proliferation. Interestingly, on the last day before oviposition, the host accumulated a large amount of symbionts, which appears to counteract the "replication bottleneck" from eggs to larvae. Our findings also demonstrated that CLS-Ds and RLS-Ds could be transmitted transovarially.

CLS-Ds also was detected in Malpighian tubules at lower densities and quantities than in ovaries. From the beginning of the feeding stage to oviposition, its densities and quantities continuously decreased. The population of RLS-Ds exhibited similar infection dynamics in the midguts, salivary glands and Malpighian tubules, wherein two density peaks were observed. Similar variation trends in different tissues could possibly occur and is due to their circulation in hemolymph in the body cavity, which also demonstrated that RLS-Ds might be facultative. Additionally, except for the ovaries, the densities and quantities of RLS-Ds and CLS-Ds in other infected tissues did not expand prior to oviposition, suggesting that symbionts were transmitted via ovarian tissues.

Our study illustrated that the population of the three different symbionts was active and varied spatially and temporally throughout the life cycle of *D. silvarum*. Our results provide insight into symbiont–tick interactions. The three symbionts in a single host exhibited different abundance and varying trends. CLS-Ds and RLS-Ds were dominant and may be associated with oogenesis and mating of the host tick. In addition, our results corroborated that symbionts in ticks endure "replication bottlenecks" during development from the egg to the larval stage.

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