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Conspecific hyperparasitism: an alternative route for *Borrelia hermsii* transmission by the tick *Ornithodoros hermsi*

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Abstract

Ixodid and argasid ticks may hyperparasitize other individuals of their own species to acquire a blood meal, however most accounts are based on single observations and the behavior has rarely been studied. While maintaining laboratory colonies of *Ornithodoros* species, we noticed that unfed ticks occasionally fed on other ticks that were feeding on mice, and unfed ticks parasitized engorged ticks when confined together in tubes. Therefore, we investigated hyperparasitism by *Ornithodoros hermsi* and the ability of these ticks to acquire and transmit the relapsing fever spirochete *Borrelia hermsii* when feeding on other ticks. Various combinations of unfed and recently engorged male, female and nymphal ticks were confined for 1–2 hr as individual pairs or in groups, then examined to determine the number of ticks that acquired blood by feeding on others. Unfed *O. hermsi* males were far more likely to hyperparasitize other ticks than were females and nymphs, as 78.6% of males (114 of 145 ticks) fed when confined with recently engorged ticks. Unfed females and nymphs also hyperparasitized other ticks but far less frequently (only 6.7% combined; 17 of 254 ticks). Infection experiments demonstrated that unfed males acquired *B. hermsii* when parasitizing nymphs that had recently engorged on a spirochetemic mouse, and unfed infected males transmitted spirochetes to recently engorged nymphs. Some ticks infected via hyperparasitism subsequently transmitted *B. hermsii* to mice. Hyperparasitism by *O. hermsi* occurred more frequently than expected, although possibly influenced by our experimental design. The significance of this behavior as it may influence the horizontal transfer of *B. hermsii* in nature is not known but worthy of future consideration.

Keywords

Relapsing fever; spirochete; Argasidae; hematophagy

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1. Introduction

Hyperparasitism, the act of one parasite parasitizing another, is commonly found in three orders of the Class Insecta, including the Hymenoptera, Diptera, and Coleoptera (Sullivan and Völkl, 1999). In these groups, hyperparasitism is most often interspecific, with one species parasitizing a different parasitic species. Hyperparasitism is less common in the Order Ixodida, Class Arachnida. In ticks, hyperparasitism is conspecific, with ticks feeding upon other ticks of the same species. Cases of conspecific hyperparasitism (herein referred to as hyperparasitism) have been documented for many species of argasid (soft) ticks, including *Ornithodoros puertoricensis*, *Ornithodoros erraticus*, *Ornithodoros tartakovskyi*, *Ornithodoros tholozani*, *Ornithodoros turicata*, *Ornithodoros parkeri*, a few *Argas* species, as well as the ixodid (hard) ticks *Amblyomma rotundatum*, *Rhipicephalus (Boophilus) annulatus*, *Hyalomma detritum* and several species of *Ixodes* (Nuttall and Warburton, 1911; Klyushkina, 1956; Uzakov, 1961; Moorhouse, 1966; Bhat, 1969; Moorhouse and Heath, 1975; Londono, 1976; Helmy et al., 1983; Endris et al., 1991; Labruna et al., 2007). In most cases, the hyperparasitism was a single documented case. However, one study focused specifically on hyperparasitism in a laboratory colony of *O. erraticus* (Helmy et al., 1983). The authors observed a high frequency of hyperparasitism in specific tick stages, and the spirochete *Borrelia crocidurae* was acquired and transmitted between ticks when feeding on each other.

The bite of another argasid tick, *Ornithodoros hermsi*, has the potential to transmit the spirochete *Borrelia hermsii*, the primary cause of tick-borne relapsing fever in the United States (Dworkin et al., 2008; CDC, 2015). These ticks reside in and near rodent and bird nests, where they emerge from cracks and crevices to feed on their vertebrate hosts. These soft ticks feed rapidly at night, usually within an hour or less, and can survive long periods of fasting, as some females have lived in the laboratory for over four years without a blood meal (Wheeler, 1943). The *O. hermsi* lifecycle includes 2–4 nymphal stages, which require a blood meal before each molt, although later-stage nymphs may feed more than once during the winter before developing into adults (Davis and Walker, 1940). Ticks may become infected for life while feeding on a spirochetemic vertebrate host, or when larvae are infected transovarially from an infected female (Wheeler, 1938a). Upon acquisition by tick feeding, the spirochetes disseminate from the midgut, enter the hemocoel, and ultimately reach the salivary glands where they establish a persistent infection. Once in the salivary glands, spirochetes are quickly transmitted to the host during the tick's short feeding (Schwan and Hinnebusch, 1998; Boyle et al., 2014).

While maintaining several species of soft ticks at the Rocky Mountain Laboratories (RML), we observed a few cases of hyperparasitism both on and off their host (Figure 1). Therefore, we investigated the likelihood that *O. hermsi* would parasitize each other, and examined the ability of *B. hermsii* to be horizontally transferred between ticks via this route. Here we show that hyperparasitism was a common event in the *O. hermsi* laboratory colony, that ticks acquired and transmitted *B. hermsii* when feeding on each other, and that ticks infected by hyperparasitism could subsequently transmit spirochetes to mice. Our findings also suggest that this phenomenon may play an under-appreciated role in the enzootic cycle of these spirochetes and ticks in the wild.

2. Methods

2.1. Ethics statement

The Rocky Mountain Laboratories, NIAID, NIH, Animal Care and Use Committee approved study protocols #2009-32, #2009-87, #2012-29 and #2012-70 for the feeding of ticks on mice, infecting mice with spirochetes, and for collecting blood samples from mice. All work in our study was conducted adhering to the institution's guidelines for animal husbandry, and followed the guidelines and basic principals in the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals, United States Institute of Laboratory Animal Resources, National Research Council.

2.2. Ticks and spirochetes

Ornithodoros hermsi ticks used in this study were from a colony maintained at the RML that was established with a single, uninfected, fertilized female from Siskiyou County, California (collected October 29, 1997). Ticks were kept either in groups in 15 or 50 mL plastic tubes (Corning Inc., Corning, NY) or individually in 1.5 mL snap-cap micro-centrifuge tubes. The tubes were ventilated with holes in the cap and filter paper was placed inside to absorb fluids. Tick tubes were kept in closed glass desiccator jars at 22–25°C, 85% relative humidity (Winston and Bates, 1960) with ambient light. *B. hermsii* DAH was used for the tick acquisition and transmission experiments (see below). Spirochetes were grown in mBSK-c medium (Barbour, 1984; Battisti et al., 2008), and 5×10^5 cells were injected intraperitoneally into a mouse (*Mus musculus*) (RML strain; a closed colony used at RML since 1937 that originated from outbred Swiss-Webster mice). After 2 – 4 days of infection, the spirochetemia in the mouse became $\sim 10^6$ cells per ml or greater, and ticks were fed to repletion. These infected ticks were used either immediately (as hosts) for the acquisition experiments, or were allowed to molt and then feed (as parasites) in the transmission experiments (see below).

2.3. Hyperparasitism experiments

Ticks were fed to repletion on an uninfected mouse, divided into groups by stage (nymphs, males, females), and then combined with unfed ticks of one of the three stages either in individual pairing or in groups (Table 1). These combinations of ticks were kept in 15 mL tubes (Corning Inc.) with filter paper, placed in the dark at 22–25°C for 1–2 hr, and then examined. The ticks were separated by stage, engorgement status, and checked for possible wounds resulting from being parasitized by another tick, all viewed with a stereoscopic microscope. The number of engorged ticks at the start and end of each experiment allowed us to determine the number of ticks that parasitized other ticks. The ticks were maintained for two to eight months to observe mortality and molting of the nymphs.

2.4. Spirochete acquisition and transmission during hyperparasitism

To study spirochete acquisition by the parasite from the host tick, twenty late-stage, uninfected nymphs were fed to repletion on a spirochetemic mouse, and each engorged nymph (host) was paired individually with an unfed, uninfected male (parasite). To study

spirochete transmission from the parasite to host, eighteen third and fourth stage, uninfected nymphs were fed to repletion on an uninfected mouse and paired individually with unfed males (parasites) previously infected with *B. hermsii*. All tick pairs were kept in the dark for 4 hrs at 22–25°C, then separated by stage (nymphs and males), and assessed for wounds and blood meal status. Three months later, ticks were pooled into two groups of nymphs and five groups of males for each experiment, and each group was fed on an uninfected mouse. Blood samples were collected daily from the tail vein of the fourteen mice and examined for spirochetes by dark field microscopy.

2.5. Indirect fluorescent antibody (IFA) assays of ticks

Six ticks used in the acquisition and transmission experiments, which fed on mice that did not become infected, were examined by IFA for spirochete infection. The midgut from all six ticks and salivary glands of four ticks were placed in PBS. Midgut tissues were teased apart in PBS on glass microscope slides, while the salivary glands were squashed in PBS onto 22×22 mm glass coverslips. The samples were dried at room temperature, fixed in acetone, incubated in a 1:50 dilution of a rabbit anti-*B. hermsii* DAH hyperimmune serum (produced at RML), washed with PBS, incubated in a 1:100 dilution of FITC-labeled goat anti-rabbit antibody (Kirkegaard & Perry, Gaithersburg, MD), rinsed again in PBS and distilled H₂O. These samples were examined with a Nikon Eclipse E800 epifluorescence microscope.

2.6. Serology

Convalescent serum samples from mice fed upon by potentially infected ticks were collected two months later and tested by immunoblot for antibodies to *B. hermsii*. Whole-cell lysates and purified recombinant glycerophosphodiester phosphodiesterase (rGlpQ) from *B. hermsii* DAH were prepared as described (Schwan et al., 1996) and electrophoresed in adjacent lanes by SDS-PAGE using Novex 12% Tris-Glycine Mini Gels-1mm (Thermo Fisher Sci, Waltham, MA). Proteins were transferred onto nitrocellulose membranes, blocked, and cut into two-lane strips for testing. Each mouse serum sample was tested at a 1:100 dilution, and bound antibodies were detected with HRP-rec Protein A (Thermo Fisher Sci, Waltham, MA), and the Amersham ECL Western Blotting Detection Reagents (GE Healthcare Ltd., Buckinghamshire, UK). A sample was considered positive if it contained antibodies reactive to five or more proteins of the whole-cell lysate and to the rGlpQ protein.

2.7. Statistical analysis

The Fisher's exact test was performed on the molting and tick feeding data. No significant difference was found between the nymph and female percent hyperparasitism, therefore the data were combined and nymphs and female hyperparasitism was compared to the male hyperparasitism. The one-way ANOVA with Dunnett's multiple comparisons test was applied to the mortality data which included seven groups: host females, host males, host nymphs, parasite females, parasite males, parasite nymphs and the controls. The GraphPad Prism 7.01 program (La Jolla, CA) was used for all analysis.

3. Results

3.1. Hyperparasitism

In the first experiments with only uninfected ticks, more unfed males parasitized fully engorged ticks than did unfed nymphs and females (Table 1). In the group experiments, 83% of the males parasitized other ticks, while in single pairings, 70.6% of the males fed upon the other tick (Table 1). When combined, 78.6% ($n = 114$ of 145) of the males parasitized other ticks. In contrast, neither unfed females nor late-stage nymphs fed on engorged males, and unfed females only occasionally fed on engorged nymphs (5.3–12.5%) and rarely on other engorged females (0–5%) (Table 1). Unfed nymphs occasionally fed on engorged nymphs (15–25%) but less often on females (0–10%) (Table 1). When combined, only 6.7% of the unfed nymphs and females ($n = 17$ of 254) parasitized other ticks. In the single pooled experiment combining unfed larvae with engorged 1st nymphs, no hyperparasitism occurred (Table 1).

All engorged ticks serving as hosts were examined after being confined with unfed ticks. Wounds were visible on 10% of the males, 25.5% of the females and 42.9% of the nymphs. Often the midgut was retracted from the cuticle wall, which created a clear space within the body cavity (Figure 2A–C).

3.2. Mortality and molting

Mortality for parasite and host ticks, grouped by stage, was not statistically different than mortality in the control group fed on a normal mouse at the same time ($n = 24$, mortality 8.7%). The mortality in host ticks ranged from 0 - 6.5% with the highest percentage in the nymphs (Table 2). Mortality in the parasite group was less with the highest mortality of only 0–1.1% in the males (Table 2).

Molting was monitored for engorged nymphs that were or were not parasitized by another tick. Fifteen of 21 nymphs (71.4%) that were parasitized, after having engorged upon a mouse, successfully molted, while 17 of 31 nymphs (54.8%) that were not parasitized molted during the observation period. These data suggest that molting was not hindered in those ticks that were parasitized by another tick, however, the numbers are small and the difference between groups was not statistically significant.

3.3. Spirochete acquisition and transmission between ticks

Given our previous results that males were much more likely to parasitize other ticks, the *B. hermsii* infection experiments used unfed males as the parasite and engorged nymphs as the host in the tick pairings. The ability of ticks to acquire or transmit spirochetes to other ticks was assayed primarily by subsequently feeding ticks on mice and following the animals for infection and antibody responses. Six ticks were also examined directly for infection.

In the acquisition experiment, 20 unfed, uninfected males were paired individually with nymphs immediately after they had engorged on a spirochetemic mouse. All of the males parasitized the nymph they were paired with, and three months later the males were pooled into five groups and fed on naïve mice (Table 3). Not all the males fed, which resulted in

mice being fed upon by 2 – 5 ticks, and only one of the five mice became infected. However, three of five males that fed on two mice that did not get infected (M8 & M9) did have midgut infections visualized by IFA microscopy, showing that these males also acquired spirochetes when they parasitized the nymphs. No spirochetes were found in the salivary gland IFAs from these ticks (n=3), though the preparations were poor and it is unknown if the negative result is due to a true lack of spirochetes in the salivary glands or the poor prep. Two groups of nymphs (original hosts) were also fed on mice (1 and 4 ticks fed), and both animals became infected and seroconverted, showing that these ticks also acquired spirochetes previously while feeding on the spirochetemic mouse.

In the transmission experiment, 18 late-stage, uninfected nymphs were fed to repletion on an uninfected mouse and immediately paired individually with an unfed male infected previously with *B. hermsii*. All males parasitized the nymphs, and three months later the nymphs were pooled into five groups and placed on mice. Fourteen of the original 18 nymphs fed (1, 3, 3, 3, and 4 ticks fed per mouse), and four of the five mice became infected and seroconverted (Table 4). However, a single tick that fed on the mouse that did not become infected or seroconvert had spirochetes in its salivary glands and midgut, demonstrating that this tick too had become infected when parasitized by the infected male. The infection status of males was verified by successfully feeding one and three males on two mice, both of which became infected and seroconverted.

4. Discussion

Hyperparasitism is a unique and, thought to be, unusual method of feeding among ticks, although this behavior may be rather common in some species of *Ixodes* with males heavily parasitizing females (Moorhouse, 1966; Moorhouse and Heath, 1975). Bhat (1969) observed that male *O. tholozani* would not feed on rabbits but readily fed on engorged nymphs and females, however numbers of observations were not given. Helmy et al. (1983) subsequently studied this behavior in *O. erraticus*, and as we observed for *O. hermsi*, *O. erraticus* males hyperparasitized other ticks far more frequently than did females and nymphs. Additionally, these investigators followed individual nymphs through the molt to determine whether they developed into males (designated N-♂) or females (designated N-♀). In doing so, they observed preferential feeding differences by adult ticks on the two nymphal groups, and how the two groups of nymphs fed prior to becoming adults. The N-♂ ticks fed on any stage (males, females, N-♂ or N-♀), but preferred females and N-♀ over males and N-♂; N-♀ never fed on males or nymphs that became males (N-♂). By combining all nymphal data for *O. erraticus*, the frequencies of hyperparasitism were similar to *O. hermsi*, except by males feeding on engorged nymphs, which occurred more readily by *O. hermsi* (85%) than by *O. erraticus* (25%). Overall, nymphal feedings by *O. erraticus* were more frequent (24.1%) (Helmy et al., 1983) compared to *O. hermsi* (only 7.7%) (this study).

Nearly 30% of the female *O. erraticus* parasitized by males, and 80% of the females fed upon by other females, died within three months compared to only 6.7% mortality in their control group (Helmy et al., 1983). In contrast, *O. hermsi* ticks, either as the parasite or host, had no increase in mortality over our control ticks. This could be due to a greater proportion of blood, in relation to body weight, consumed by *O. hermsi* females compared to *O.*

erraticus females when feeding on mammals (McCoy et al., 2010). While both species are relatively small ticks, *O. hermsi* females take in 8.2 times their unfed weight in blood compared to 2.9 to 5.2 times for *O. erraticus* females (Khalil et al., 1986; McCoy et al., 2010). The larger blood meal in proportion to body size could allow for *O. hermsi* to yield a larger amount of blood when being parasitized without impacting survival. *O. hermsi* may also take less blood when feeding on other ticks compared to *O. erraticus*, however, we did not measure the amount of blood ingested during hyperparasitism. Additionally, *O. hermsi* may cause less trauma during feeding, or these ticks may be better equipped to handle cuticular damage. Possible differences in the mechanics of feeding between *Ornithodoros* species are not known, but observations on host skin reactions to the bite of *O. hermsi* and *O. erraticus* may hint at possible effects of feeding. Wheeler (1938b; 1943) observed a mild reaction on man, mouse and monkey when bitten by *O. hermsi*, while Lavoipierre and Riek (1955) observed extensive bruising and blood pooling from the bite of *O. erraticus* on the ear of rabbits. However, these observations are complicated by many factors including different host species, physical damage to the skin, vascularization of species tissues, and the chemical complexity of tick saliva.

An intriguing finding from our study was the higher molting success for the engorged *O. hermsi* nymphs fed upon by other ticks (71.4%) compared to ticks not fed upon (54.8%). In comparison, Davis and Walker (1940) observed molting successes of 93% for *O. hermsi* second-stage nymphs, 21% for third-stage nymphs, and 60% for fourth-stage nymphs, all following one replete blood meal. Blood meal size affects molting of soft ticks and most species probably have a minimum volume of blood needed to molt. For example, *O. papillipes* [*O. tholozani* (Laboulbène and Méglin)] nymphs require at least 30-35% of their average blood meal volume to molt (Sonenshine, 1991), while *O. tartakovskyi* requires only 20-25% of their average blood meal volume to molt (Balashov, 1972; Sonenshine, 1991). Thus, it may not be surprising that *O. hermsi* nymphs fed upon by other ticks still molted successfully. Additionally, the molting process may be triggered by neural stimulation of the distended midgut tissues, setting the hormonal initiation in progress in spite of the subsequent reduction in blood volume when parasitized.

We expected that a tick, feeding upon another tick with a fresh spirochetemic blood meal, would imbibe spirochetes along with blood and become infected. Indeed, some unfed *O. hermsi* ticks acquired and later transmitted *B. hermsii* when feeding on recently engorged ticks. What was unanticipated was the ability of infected ticks to transmit spirochetes to another tick, leading to the establishment of a spirochete infection in the host tick. In the former case, ticks infected by hyperparasitism subsequently transmitted spirochetes to only one of five mice, although several ticks that did not transmit *B. hermsii* to the mice were infected. *O. hermsi* ticks infected with *B. hermsii* may transmit spirochetes during some but not all feedings (Davis and Walker, 1940; Wheeler, 1943; Schwan and Hinnebusch, 1998). Therefore, if fed again, some of our ticks might have eventually transmitted spirochetes. The implications of spirochete transmission through hyperparasitism remain undetermined. Theoretically, an increased number of infected ticks could spread *B. hermsii* infections to other ticks in the population.

Ticks would need to aggregate in or near the nest of a vertebrate host for hyperparasitism to occur. Both hard and soft ticks have a well-documented propensity to do this, which is driven by the production and sensing of pheromones (Leahy et al., 1973; Leahy et al., 1975; Leahy, 1979; Allan and Sonenshine, 2002; Yoder et al., 2013), with individuals of multiple tick species attracting other ticks up to a meter or more away (Sonenshine, 2006). Unfed ticks show a greater tendency to aggregate than fed ticks, suggesting aggregation pheromones assist in finding food (Leahy, 1979). The role of pheromones in the movement and aggregation of *O. hermsi* ticks is unknown. However, one could perceive a scenario where ticks in an abandoned nest, which have not fed for many months, are hungry when a new vertebrate host inhabits the nest. A subset of ticks feed on the squirrel or bird, return to the cracks and crevices from where they came, and are then hyperparasitized by those ticks having not yet fed.

Hyperparasitism in our *O. hermsi* experiments occurred frequently although our protocol, which kept unfed ticks confined closely with others recently engorged, may have increased the likelihood of such feeding. This behavior has the potential to increase the survival of those ticks not feeding on vertebrates, therefore increasing sustainability of ticks in laboratory or natural colonies, as suggested by Wheeler (1938b) many years ago. Additionally, hyperparasitism in spirochete-infected laboratory colonies could affect the prevalence of infection, and influence the outcome of experiments tracking spirochete-tick interactions. Future studies of the potential significance of hyperparasitism and how it affects the horizontal transmission of *B. hermsii* in nature are warranted.

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Figure 1.
Hyperparasitism by an unfed *Ornithodoros hermsi* nymph on a recently engorged nymph.

Figure 2.
A–C. *Ornithodoros hermsi* female ticks with visible wounds (white rectangle) from hyperparasitism by other *O. hermsi* ticks. The blood-filled midgut is visibly retracted from the body wall where the wound is located.

Table 1

Hyperparasitism during pooled and individual tick pairings.

Parasite	Host	Pooled		Individually tracked	
		No. fed/total	% Parasitized	No. fed/total	% Parasitized
♀	♀	1/20	5	0/5	0
	N _L	5/40	12.5	1/19	5.3
	♂	0/20	0	0/20	0
Total		6/80	7.5	1/44	2.3
♂	♀	44/54	81.5	13/21	61.9
	N _L	34/40	85	16/20	80
	♂	–	–	7/10	70
Total		78/94	83*	36/51	70.6**
N _L	♀	2/20	10	0/20	0
	N _L	3/20	15	5/20	25
	♂	0/30	0	0/20	0
Total		5/70	7.1	5/60	8.3
L	N _I	0/56	0	–	–

* $p < 0.0001$;

** $p < 0.0001$ (compared to female and N data combined)

N_I=first-stage nymphs; N_L=late-stage nymphs (third and fourth stage); L=larvae

Table 2

Mortality rate two months after pairing for individually tracked ticks, 1–2 months for pooled ticks.

		All ticks	
	Stage	No. died	% Mortality
Host Ticks	♀	0/100	0
	♂	5/89	5.6
	N _L	8/123	6.5
Parasite Ticks	♀	1/124	0.8
	♂	2/175	1.1
	N _L	0/130	0

N_L=late-stage nymphs (third and fourth stage)

Table 3

Spirochete transmission to mice by male *Ornithodoros hermsi* ticks fed previously by hyperparasitizing infected nymphal ticks, and nymphs fed previously on infected mice. Mouse blood was examined by light microscopy for spirochetemia and western blot for antibodies.

Mouse	No. ticks fed on mouse	Mouse spirochetemia	Mouse serology
M8	2 ♂♂ ^{a,b}	–	–
M9	3 ♂♂ ^c	–	–
M10	4 ♂♂	–	–
M11	5 ♂♂	+	ND
M12	4 ♂♂	–	–
M13	4 NN ^d	+	ND
M14	1 N	+	ND

^a All ♂♂ fed previously by hyperparasitizing recently engorged, infected nymphs.

^b Both ticks with midgut infections demonstrated by IFA (400X).

^c 1 of 3 ticks with midgut infection demonstrated by IFA (400X).

^d NN = nymphs, fed previously on infected mouse, part of group hyperparasitized by ♂♂.

Table 4

Spirochete transmission to mice by nymphal *Ornithodoros hermsi* ticks hyperparasitized previously by infected males, infected males fed previously on nymphs. Mouse blood was examined by light microscopy for spirochetemia and western blot for antibodies.

Mouse #	No. ticks fed on mouse	Mouse spirochetemia	Mouse serology
M1	4 NN ^a	+	+
M2	1 N ^b	–	–
M3	3 NN	+	+
M4	3 NN	+	+
M5	3 NN	+	+
M6	1 ♂ ^c	+	+
M7	3 ♂♂	+	+

^a All nymphs (NN), fed previously on uninfected mouse and hyperparasitized by infected ♂♂.

^b Tick with midgut and salivary gland infections demonstrated by IFA (400X).

^c All ♂♂ fed previously on infected mice and then by hyperparasitizing uninfected nymphs.